**Natural history and trophic ecology of three populations of the Mexican cavefish, *Astyanax mexicanus***

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**Abstract**

The Mexican cavefish, *Astyanax mexicanus* (Characidae), has become an important model in evolutionary physiology and developmental biology, providing insights into the evolution of sensory systems, pigmentation, and metabolism. In contrast, comparatively little is known about the natural history and trophic ecology of this elusive cave inhabitant. We investigated cavefish from three independently colonized cave systems (Pachón, Tinaja, and Sabinos), which are located in the Sierra de El Abra of Northeastern Mexico. Samples were collected multiple times throughout the year to investigate variation in body size, sex ratios, proportions of individuals with empty guts, and diet composition. We found consistent differences in body size among caves, and sex ratios were generally female biased, although to varying degrees. Gut content analyses indicated that cavefish consume food throughout the year, and diets are dominated by detritus, plant materials, and aquatic invertebrates. Especially in the Pachón cave, where we had the densest sampling, there was evidence for seasonal changes in diet composition that coincided with the rainy and dry seasons. Our findings potentially suggest that the cave environments in this system are characterized by continual nutrient limitation, rather than intermittent periods of starvation.

Keywords: Caves, Characidae, Gut content analysis, Trophic ecology

**Introduction**

The Mexican cavefish, *Astyanax mexicanus*, is a well-established model organism for addressing evolutionary questions that span a variety of biological fields, including genomics, developmental biology, metabolic physiology, and behavior (Jeffery 2009; Frøland Steindal et al. 2018; Kowalko 2020). Several populations of *A. mexicanus* independently colonized caves from adjacent surface streams, adapting to the unique challenges that cave environments impose on life (Gross 2012; Pérez-Rodríguez et al. 2021). Caves are often characterized by complete darkness, low food availability, and low temperature variability, driving the convergent evolution of several traits among subterranean fauna (Porter and Crandall 2003; Kowalko et al. 2013; Rétaux and Casane 2013). Comparisons between cave and surface populations of *A. mexicanus*, as well as experiments involving surface-cave hybrids, have provided insight into the molecular mechanisms underlying the evolution of cave-specific traits such as loss of pigmentation, eye regression, and modification of other sensory systems (Gross 2012; Aspiras et al. 2015; Kowalko 2020). The evolution of many traits in *A. mexicanus*, particularly sensory system modifications and metabolic adaptations, are driven by the extreme nutrient limitations brought about by darkness and food scarcity (Moran et al. 2014; Riddle et al. 2018).

To meet nutritional demands while living in darkness, cavefish have evolved ways to discover and capture food non-visually and exhibit extraordinary starvation resistance. Feeding experiments have shown that cave *A. mexicanus* outcompete surface fish for food in darkness (Hüppop 1987; Yoshizawa et al. 2010; Espinasa et al. 2014). Their success in locating prey in darkness is attributed to vibration attraction behavior (VAB), which allows cavefish to move toward a disturbance in the water (Yoshizawa et al. 2010). VAB is largely dependent on the lateral line system, which can detect vibration frequencies at which copepods and other invertebrates move (Yoshizawa et al. 2010). In addition to the sensory enhancements associated with VAB, cavefish have more taste buds than surface fish, particularly on the ventral side of the head, which aid in feeding along the bottom of the cave (Franz-Odendaal and Hall 2006). There is evidence that cavefish have heightened olfaction as well, further contributing to their foraging success in darkness (Kasumyan and Marusov 2015; Hinaux et al. 2016). Multiple cavefish populations have independently evolved a lower feeding angle relative to their surface conspecifics, and while it is yet unclear how this posture affects bottom feeding, convergence in this trait suggests that it could be adaptive (Kowalko et al. 2013).

While it is important to maximize feeding efficiency in caves, cavefish also must tolerate periods of starvation. This is due to the lack of photosynthetic primary producers in caves, as well as inconsistent input of food items from external sources (Xiong et al. 2018). Inconsistencies in food availability are likely a result of seasonality; caves in subtemperate areas have relatively stable temperatures year-round, but the amount of rainfall varies between rainy and dry season (Simon et al. 2017; Xiong et al. 2018). During the rainy season, flash flooding can wash in organic matter from the surface, interrupting periods of limited food in the dry season (Simon et al. 2017). Cavefish have been shown to have lower metabolic rates, a loss of circadian rhythm in metabolism, elevated appetites, and increased fat storage, allowing them to withstand food shortage for extended periods of time (Hüppop 1987; Moran et al. 2014; Aspiras et al. 2015). The greater body mass of *A. mexicanus* cavefish compared to their surface ancestors has been linked to a mutation that causes insulin resistance, which hinders the body’s ability to regulate glucose (Riddle et al. 2018). In humans, the same phenomenon causes health issues that are associated with diabetes (Riddle et al. 2018). Cavefish, however, do not suffer from the same pathological manifestations of disrupted glucose regulation (Riddle et al. 2018). Due to their adaptive outcomes regarding insulin resistance and elevated body fat, cave-adapted *A. mexicanus* have been studied for their relevance to human metabolic diseases as well (Riddle et al. 2018).

While *A. mexicanus* has been an exceptional model for studies in evolutionary developmental biology and metabolic physiology, there is limited research regarding their natural history, including trophic ecology. Espinasa et al. (2017) found that juvenile fish from the Pachón cave in Mexico are well-fed, and their diets are comprised mostly of invertebrates that were consumed as live prey. In contrast, their analysis of five individuals indicated that adult cavefish primarily feed on detritus during both the rainy and dry seasons (Espinasa et al. 2017). An additional study investigating the diet of *A. mexicanus* from another cave, Micos, similarly found that a mixture of a brown substance (guano or detritus), fish parts, and insect exoskeleton parts comprises the majority of the adult cavefish diet (Wilkens and Burns 1972). Consequently, *A. mexicanus* have generally been described generalists with opportunistic feeding habits at maturity (Wilkens and Burns 1972; Trajano 2001; Espinasa et al. 2017). To our knowledge, there has been no other research on temporal change in diet or comparison of gut item composition in *A. mexicanus* between the rainy (June to November) and dry (December to May) seasons (Espino del Castillo et al. 2009).

The objective of this study was to broaden our knowledge of the natural history of cave-adapted *A. mexicanus*, with a particular emphasis on diet composition and diet variation across time and populations. Diet, as well as starvation, influences the well-studied metabolic physiology of cavefish, yet there is limited research on what cavefish actually eat in nature. We assessed body size and sex ratio variation across populations from three caves (Pachón, Sabinos, and Tinaja), and examined the gut contents of populations from the Pachón and Sabinos caves at several time points. Based on previous research, we expected fish from Pachón to be smaller than those from Tinaja (Simon et al. 2017), but we did not have any prior data to inform predictions about how Sabinos cavefish sizes would compare to these populations. Subterranean species have been shown to have female-biased sex ratios, presumably due to limited dispersal and inbreeding causing reductions in genetic variation (Culver and Holsinger 1969; Frank 1990; Premate et al. 2021), and *A. mexicanus* cavefish populations do have less genetic variation than their surface conspecifics (Bradic et al. 2012). We therefore expected to find female-biased sex ratios in all populations. Consistent with an opportunistic feeding style, we also predicted that the cavefish would have a variety of food items in their diet, including detritus. Furthermore, we predicted that all three populations would have a greater proportion of individuals with food in their guts during the rainy season when flooding can wash in organic debris from the surface. Understanding what cave *A. mexicanus* eat in the wild and how their diets change over time will help inform research addressing many aspects of physiology that are heavily impacted by nutrition.

**Methods**

*Study area*

The Sierra de El Abra is a limestone ridge in Northeastern Mexico that contains at least 30 caves inhabited by *A. mexicanus* (Fig. 1) (Mitchell et al. 1977; Espinasa et al. 2018). Throughout the region, surface populations of *A. mexicanus* are also widespread (Miller et al. 2005). The Pachón cave is the northernmost of the three sampled caves, while the Sabinos cave is about 60 km south of Pachón and in close proximity to the Tinaja cave (Fig. 1) (Avise and Selander 1972; Espinasa and Borowsky 2001). Pachón is also located at the highest elevation in the valley (Avise and Selander 1972). All three caves are comprised of highly branching subterranean tunnels as well as pools that open up into cave chambers (Avise and Selander 1972). The rainy season in this region occurs from June to November, while the dry season is approximately during December to May (Espino del Castillo et al. 2009).

*Biometrics*

To compare body size variation and sex ratios (quantified as the proportion of males in the population) among the caves, we sampled *A. mexicanus* from the Pachón (*N* = 611), Sabinos (*N* = 197), Tinaja (*N* = 73) caves at several time points between February 2001 and March 2002 (Table 1). We sampled the first pools that were accessible upon entering the caves, and we returned to the same pools for each subsequent sampling time. Fish were caught using a seine (2 meters long by 1.5 meters tall) and dip nets. Standard length was measured with calipers to the nearest millimeter, and sex was determined based on the presence of denticles on the anterior rays of the anal fin in males (Borowsky 2008). The fish were then released at the collection location.

All statistical analyses were conducted in R version 4.0.3 (R Core Team 2020). To analyze variation in standard length, we ran generalized linear mixed models (GLMM) with a Gaussian distribution using R’s lme4 package (Bates et al. 2014). We excluded 89 juveniles from Pachón, 15 from Sabinos, and 1 from Tinaja from analyses because their sex could not be determined with confidence. We explored multiple GLMMs to better understand the effects of different predictor variables: (1) a null model, including only the date of collection as a random factor; (2) a model adding sex as a fixed factor; (3) a model adding cave of origin as a fixed factor; (4) an additive model adding sex and cave of origin as fixed factors; and (5) an interaction model adding sex and cave of origin as fixed factors. The small sample unbiased Akaike information criterion (AICC) approach was used for model selection (Johnson and Omland 2004), and models with ΔAICC < 2 were considered to be equally well-supported (Burnham and Anderson 2001). Similarly, binomial GLMMs were used to test for differences in sex ratios across caves. The null model included the date of collection as a random factor, and it was compared to a model adding cave of origin using AICC.

*Gut content analysis*

Fish were sampled from the Pachón (*N* = 146), Sabinos (*N* = 58), and Tinaja (*N* = 13) caves for gut content analysis (Table 1). These individuals were also collected across time points between February 2001 and March 2002 (6 sampling times for Pachón, 3 for Sabinos, and 3 for Tinaja; Table 1) using the same capturing methods as previously described. The fish that were collected for gut content analysis were haphazardly selected. We aimed to collect at least 25 fish per sampling time for gut content analysis, unless the population size was small, in which case we aimed for at least 10 fish. During some sampling times, fewer than 10 individuals were collected to avoid depleting the populations. The fish were immediately euthanized in the field, preserved in ethanol, and transported back to the laboratory for dissection and measurements. The digestive tracts were dissected, and the contents were examined under a dissecting microscope. The presence or absence of the following diet categories was recorded for each individual: fish parts, invertebrates or invertebrate parts, algae, seeds, other plant material, detritus, and inorganic materials. Note that the detritus category included all unidentifiable organic matter, and the inorganic materials category included rock granules and sandy sediment.

Due to the small sample size of Tinaja cavefish for gut content analyses, individuals from that cave were excluded from quantitative analyses. We first compared the frequency of empty guts between caves using binomial generalized linear models (GLMs) as implemented in the lme4 package in R (Bates et al. 2014). We ran five alternative models that were contrasted using AICC: (1) a null model, including only the intercept; (2) a model adding collection date as a fixed factor; (3) a model adding cave of origin as a fixed factor; (4) an additive model adding collection date and cave of origin as fixed factors; and (5) an interaction model adding collection date and cave of origin as fixed factors. Additionally, we compared variation in diet composition over time and across caves. We first removed individuals with empty guts from the dataset and analyzed the remaining data using permutational multivariate analysis of variance (PERMANOVA) with Jaccard distances and 999 permutations using the adonis2 function in the R package vegan (McArdle and Anderson 2001; Oksanen et al. 2020). Sampling date, cave of origin, and their interaction term were included as predictor variables. For data visualization, we used non-metric multidimensional scaling via the metaMDS function in R’s vegan package (Oksanen et al. 2020).

**Results**

*Biometrics*

After excluding juveniles that could not be sexed, the standard lengths of fish used in the analyses ranged from 1.9-6.2 cm in Pachón (*N* = 522 adults), 1.9-6.4 cm in Sabinos (*N* = 182), and 2.1-6.4 cm in Tinaja (*N* = 72). Analyzing variation in standard length using mixed-effects models yielded two models that were equally supported (ΔAICc < 2), including both the additive and interactive models with all variables (Table 2A). Hence, variation in standard length was affected by both sex and cave of origin. Visualizing the marginal effects of the interaction model indicated that Tinaja cavefish were larger than both Pachón and Sabinos cavefish (Fig. 2). This trend was consistent for both males and females. In addition, males were larger than females in the Pachón and Sabinos caves, while there was no difference in body size between the sexes in the Tinaja cave (Fig. 2).

There were significant differences in sex ratios between the caves and across sampling times, as indicated by the mixed model analysis of the frequency of males and females (Table 2B). For all the cave populations and across all sampling times between February 2001 and March 2002, females outnumbered males (Table 1). The sex ratio in Pachón ranged from 0.01 (11/4/2001) to 0.38 (2/3/2001) (Table 1). In the Sabinos population, it ranged from 0.21 (11/2/2001) to 0.42 (6/23/2001) (Table 1). Lastly, the sex ratio in the Tinaja population ranged from 0.27 (4/17/2001) to 0.58 (6/23/2001) (Table 1). The average sex ratios (with all sampling times included) were 0.17 for Pachón, 0.29 for Sabinos, and 0.39 for Tinaja, indicating that all three populations have a female-biased sex ratio.

*Gut content analysis*

At any given sampling time, the percentage of individuals with empty guts ranged from 44.4% to 66.7% in the Pachón population and from 0.0% to 66.7% in the Sabinos population (Fig. 3A). Although they were not included in the gut content analysis due to small sample sizes, the percentage of Tinaja individuals with empty guts ranged from 25.0% to 66.7% (Table 1). There was no evidence that the frequency of individuals with empty guts significantly varied between caves or sampling periods (Table 2C). Among the guts that contained food items, there were a variety of diet categories present overall. The Pachón population had all diet item categories (fish, invertebrates, algae, plant material, seeds, detritus, and inorganic materials) represented, and the Sabinos population had all categories represented except for seeds. The median number of diet categories present in fish from both caves was 2, with a range of 1-6 in Pachón and 1-4 in Sabinos (Fig. 3B). Tinaja cavefish had a median of 1 diet item category, ranging from 1-4 categories (Table S1).

Examining diet composition among caves and sampling periodsusing a PERMANOVA, we found significant effects for cave (PERMANOVA: *R*2 = 4.6%, *P* = 0.002) and time of sampling (PERMANOVA: *R*2 = 3.7%, *P* = 0.002), as well as for the interaction of the two factors (PERMANOVA: *R*2 = 2.1%, *P* = 0.046; Table 3). The ordination plot based on NMDS (Fig. 4A) indicated that the Pachón population exhibited greater variation in diet item composition across sampling times than the Sabinos population. While not all diet item compositions changed significantly over shorter time scales (such as 1-3 months), there were significant gut content changes between sampling times that were 4 or more months apart. There is some evidence that sampling times toward the end of the dry season cluster together, as the points for February 2001 (P1), April 2001 (P2), and March 2002 (P6) group near each other. This grouping is driven largely by the presence of seeds and organic detritus. The greatest sequential shifts in gut content composition occurred across three time points, from 4/19/2001 (P2) to 6/24/2001 (P3) to 11/3/2001 (P4). These dates represent a period from the end of the dry season (April 2001) to the early part of the rainy season (June 2001) and then finally to the end of the rainy season (November 2001) (Fig. 4B). Time point P3, the June 2001 sampling date, particularly stands out in the middle of this shift, as it does not cluster with any other sampling time and is uniquely characterized by the presence of plant material. Following the end of the rainy season in November 2001 (P4), the next sampling point in February 2002 (P5, during the dry season) groups closely with the November 2001 time point (Fig. 4). The diets at these two sampling times cluster together due to the presence of inorganic materials. As previously mentioned, the final sampling time for Pachón (March 2002, point P6) is more similar to the 2001 sampling times for February and April, so there is a considerable difference in gut content composition over the course of a month between February 2002 (P5) and March 2002 (P6).

In the Sabinos population, there were no significant changes in diet from the rainy to dry season. All 3 sampling times cluster together, and the separation of this grouping from the Pachón samples is largely driven by the abundance of invertebrates in the diet. All the time points for Sabinos most closely reflect points P4 and P5 from Pachón, which are the gut content samples collected in November 2001 and February 2002. November 2001 marks the transition from the rainy season to the dry season, while February 2002 is within the dry season (Fig. 4B). Invertebrates and inorganic materials drive the clustering of these two Pachón time points with the Sabinos time points.

In the Tinaja population, gut contents were described for individuals collected at three time points—2/4/2001 (n=6), 6/23/2001 (n=3), and 11/2/2001 (n=4) (Table 1). While the majority of individuals had empty guts, the gut contents from cavefish sampled in February 2001 included inorganic materials and fish parts (Table S1). In June and November of that same year (during the rainy season), plant material was more frequently found in cavefish guts (Table S1). Invertebrates were only identified in cavefish guts during the November sampling time, and one individual from November had seeds and detritus in addition to invertebrates and plant material (Table S1).

**Discussion**

The cavefish *Astyanax mexicanus* has been well-studied for its adaptations to a subterranean lifestyle, but the natural history and diet of cavefish in the wild are less well known. Our analyses found variation in standard length, sex ratios, and diet between cave populations of *A. mexicanus*, as well as variation in sex ratios and diet through time. Across three cave populations, Tinaja cavefish were larger than Pachón and Sabinos cavefish. Males were larger than females in Pachón and Sabinos, while there was no difference in size between the sexes in Tinaja. Additionally, all three cave populations had a female-biased sex ratio. Most importantly, our gut content analyses indicated that the majority of cavefish consumed food in similar proportion throughout the year. Pachón and Sabinos populations differed in their diet, which was primarily driven by the frequency of detritus, invertebrates, seeds, other plant material, and inorganic materials. Furthermore, in Pachón, for which we had the most rigorous sampling, there was also evidence for dietary shifts occurring across the rainy and dry seasons.

*Sex ratios, body size, and energetics in subterranean environments*

Animals living in subterranean environments experience space limitations and isolation from the surface, often driving changes in population structure (Premate et al. 2021). Reductions in heterozygosity and female-biased sex ratios have been described for many subterranean species and are attributed to inbreeding due to constraints in physical space (Konec et al. 2015; Behrmann-Godel et al. 2017; Premate et al. 2021). *Astyanax mexicanus* cavefish populations also have reduced genetic variation when compared to their surface ancestors (Bradic et al. 2012), and we found female-biased sex ratios in all three cave populations sampled. As has been suggested for other subterranean species, increased mate competition in a confined space and more frequent mating between siblings, further reinforced by kin selection, can result in female-biased sex ratios because competition between brothers favors a decline in investment in males (Frank 1990; Premate et al. 2021). It is possible that these interacting forces are similarly contributing to the female-biased sex ratios in the Pachón, Sabinos, and Tinaja caves. Differential sex determination between males and females can also drive variation in sex ratios. Interestingly, Pachón cavefish carry B chromosomes that are predominantly found in males and contain two copies of a sex determination gene that is only expressed in male gonads (Imarazene et al. 2021). When this gene was knocked out in a study by Imarazene and colleagues (2021), males were sex-reversed, indicating a role for the gene in driving sex determination in Pachón cavefish. Populations that are biased toward one sex have been shown to carry B chromosomes that are predominant in the more abundant sex, supporting a role for B chromosomes in sex determination (Beladjal et al. 2002; Yoshida et al. 2011; Clark and Kocher 2019). Accordingly, although we might expect male-biased sex ratios from these patterns, skewed sex ratios can emerge from a variety of environmental, genetic, and demographic factors, and variation in sex determination is one of many potential contributors to sex ratio bias (Beladjal et al. 2002; Yoshida et al. 2011; Cornelio et al. 2017; Clark and Kocher 2019). Furthermore, in a study of 200 Pachón cavefish that were born in captivity from laboratory stocks, the sex ratio was found to be 1:1, suggesting that mechanisms of sex determination alone are not the only factors responsible for sex ratio bias, but there are likely environmental effects influencing sex ratios in wild population (Imarazene et al. 2020). Finally, if female-biased sex ratios are a result of selection against males for any number of reasons, then the presence of a male sex determination gene may be selectively maintained in the population (Natri et al. 2019). Regardless of the selective pressures associated with cave environments, female-biased sex ratios are common in other fish species due to greater survival or longevity of females (Reichard et al. 2014; Fryxell et al. 2015). Further investigations into the genetic, demographic, and environmental interactions in wild cavefish populations, as well as comparisons between cavefish and surface fish life histories and their sex-specific differences, will provide insight into the mechanisms underlying female-biased sex ratios in *A. mexicanus* cavefish.

In addition to limitations on population size and genetic diversity, subterranean environments with their lack of sunlight and nutrients impose significant energetic constraints on the organisms that inhabit them. Cave-adapted animals cope with these challenges by conserving energy in various ways, such as optimizing body stores and lowering metabolic rate relative to ancestral surface populations (Hüppop 1986; Hervant and Renault 2002; Hervant 2012; Xiong et al. 2018). Additionally, body size reduction in cave animals can help lower energetic demands and may occur independently or in conjunction with a decrease in metabolic rate, potentially changing the allometric relationship between body size and metabolic rate (Passow et al. 2015). Like many subterranean fauna, cavefish species are generally smaller than their surface relatives, but differences in nutrient availability between cave populations may drive variation in strategies for conserving energy, including body size modifications (Hüppop 1985; Trajano 2001; Simon et al. 2017). We did find differences in standard length between the Pachón, Sabinos, and Tinaja cave populations, and Tinaja cavefish were larger than Pachón and Sabinos individuals. In support of this finding, there is evidence for a genetic limitation on size for some cave populations, as Pachón cavefish have been shown to reach smaller sizes than Tinaja cavefish and another population, Subterráneo, in the wild and when reared under identical treatments in the laboratory (Simon et al. 2017). If cave-specific body size variability is dependent on food limitation, it is possible that the Pachón and Sabinos caves are more nutrient-poor than Tinaja and that this drives a more substantial body size reduction in these cave populations. This matches the observation that Tinaja’s mud is richer in carbon content than Pachón’s (Simon et al. 2017). Alternatively, Tinaja cavefish might indeed face similar nutritional challenges as Pachón and Sabinos cavefish but utilize other strategies for conserving energy or optimizing nutrient uptake and consequently do not rely as much on body size reduction. For example, Pachón cavefish accumulate fat stores earlier in development than Tinaja cavefish, providing them starvation resistance sooner in life, but Tinaja cavefish exhibit hyperphagia when food is available, unlike those from Pachón (Aspiras et al. 2015; Xiong et al. 2018). Accordingly, any combination of physiological or behavioral traits could provide a means of resisting starvation and maintaining energy homeostasis. To understand how body size reduction interacts with other energy conservation strategies in response to nutrient variation across cave environments, it is crucial to better understand the trophic ecology of cavefish in their natural habitats.

*Nutrient limitation and starvation*

Because consistent or periodic starvation forces cave-dwelling animals to reduce their energy expenditure, understanding to what extent individual populations experience starvation can provide insight into the drivers of behavioral, morphological, and physiological traits of subterranean fauna. Caves are expected to limit food access and cause periods of starvation in cave-adapted *A. mexicanus*. We predicted that during the rainy season, more cavefish would have food in their guts due to flooding washing in debris. However, we found that Pachón and Sabinos cavefish seem to eat throughout the year, without significant seasonal variation in the frquency of empty guts. There were consistently large portions of the population that had empty guts (44%-67% of the population for both Pachón and Sabinos at all sampling times, with the exception of one collection of 3 Sabinos cavefish that all had full guts) (Fig. 3A). This indicates that the caves might not experience substantial changes in food availability between the rainy and dry seasons or that shifts in food accessibility are short-lived. For example, the way that water from the surface moves into the caves could play a role in how effectively nutrients reach the cave pools. There is evidence that water influx from flash flooding in Pachón comes more from sump inflow than stream inflow because the Pachón cave is at a higher elevation than the nearby Río Mante, potentially reducing the quantity and frequency of debris entering the cave (Keene et al. 2015; Espinasa et al. 2017; Simon et al. 2017). This would diminish seasonal impacts on the percentage of individuals with empty guts, which was corroborated by our findings in both caves. Flooding in Sabinos, however, may be more likely to push debris in, as it does not have that elevational effect and fragments of organic material have been documented in the cave (Keene et al. 2015). Although we found no evidence that there is enough influx of organic debris to impact the gut contents of cavefish during the rainy season, seasonal trends for Sabinos could potentially emerge with more extensive temporal sampling. In all the populations, sampling at additional time points in the rainy season (particularly in August-October) could reveal greater influxes of food, even if a significant proportion of the populations do have food in their guts during the drier parts of the year.

Alternatively, there could be bursts of nutrient availability after heavy rains in the caves, but the effects of these flash floods might be brief, and thus not detected at our sampling times. If cavefish do experience consistent low food availability, then that may explain why cave-adapted *A. mexicanus* are smaller than surface *A. mexicanus*. Intermittent starvation is predicted to select for larger individuals because of the greater energy stores associated with larger size, and the periods of food abundance would provide opportunities for larger individuals to gain the energy required to maintain homeostasis (Culver et al. 1995; Christiansen 2012). If food availability is instead consistently low throughout the year, the benefit of using less energy to power a smaller body may outweigh the cost of having reduced energy stores, particularly if buffered by other adaptations for energy storage (such as increased fat accumulation in cave-adapted *A. mexicanus*).

Finally, it is also important to consider that reduced rates of energy acquisition may not only be driven by low resource availability, but by the cost-to-benefit ratio of foraging. In cave populations of *Poecilia mexicana*, 35% of guts are consistently empty, even though these fish occur in a chemoautotrophic cave with high resource availability (Tobler 2008; Roach et al. 2011). In these populations, the high frequency of empty guts arises from intrinsic limitations associated with a trade-off between benthic foraging and surface breathing in hypoxic water (Tobler et al. 2009). Other intrinsic limitations may be associated with the cost of food finding and processing, digestion, and nutrient assimilation (Whelan and Brown 2005; Secor 2009). Hence, intermittent starvation in nutrient poor environments could also be an adaptive strategy to balance costs associated with energy acquisition.

*Trophic ecology and seasonal variation in diet*

In most subterranean environments, energy sources that are carried in from surface habitats can be in the form of suspended organic particles, invertebrates, plant pieces, microorganisms, bat guano, and carcasses (Hervant 2012). Consequently, hypogean animals are expected to be generalist feeders (Trajano 2001). Several cavefish species show similar feeding preferences to their epigean relatives (as omnivores or generalist carnivores), suggesting that generalist feeding could be a preadaptation for cave colonization (Trajano 2001). Despite dietary overlap between related populations of surface fish and cavefish, behavioral and sensory adaptations for finding food often arise with cave colonization (Soares and Niemiller 2013). We found a variety of food items present in the diet of cave-adapted *A. mexicanus* from the Pachón and Sabinos caves, including plant material and invertebrates, which comprise a large part of the diet of surface *A. mexicanus* as well (Riddle et al. 2019). Unlike surface *A. mexicanus*, however, the enhanced sensory systems for food detection and lower feeding angle of cavefish suggest that they feed at the bottom as well as consume prey moving in the water column. We commonly found detritus and inorganic materials in the cavefish guts, supporting the hypothesis that adult cavefish feed along the cave floor. Detritus is comprised of a mixture of organic materials and can be an energy-rich food source for hypogean fauna (Fenolio et al. 2006; Tobler et al. 2015). Guano has been identified as a major component of detritus in other caves, and stable isotope analyses of cave-adapted salamander gut contents have revealed that guano could have a similar nutritional value to invertebrate prey (Fenolio et al. 2006). Detritus is a heterogeneous mixture (Moore et al. 2004), and guano itself contains protein-rich exoskeleton fragments (Wilkens and Burns 1972; Fenolio et al. 2006), so further isotope analyses and nutrient assimilation studies will be necessary for understanding whether cavefish selectively uptake certain components of detritus based on their nutritional value (Lin et al. 2007; Enyidi et al. 2013). Our finding of inorganic materials, invertebrates, seeds, and other plant material in addition to detritus is in line with Espinasa et al.’s (2017) finding of a mixture of organic materials and arthropods in adult Pachón cavefish and supports that *A. mexicanus* cavefish are opportunistic feeders.

While it is true that cave *A. mexicanus* seem to eat whatever they have available, we detected trends in what food items are present in their guts based on cave of origin and seasonality. Diet varied between the rainy (June to November) and dry (December to May) seasons for the Pachón population, but this seasonal variation was not as prevalent for Sabinos. In Pachón, there is a very clear pattern in diet change over the course of the year, which is completed with the last sampling time in March 2002 falling into place with collection time points from almost exactly a year prior. Dry season diets were dominated by the presence of seeds, detritus, and inorganic materials at different sampling times. The time point immediately following the April 2001 collection date, in June 2001, represents the wettest part of the year out of all the sampling times, and the diet composition at this time point is categorized by the presence of plant material. This suggests that during the rainy season, macroscopic organic material from the surface can make its way into Pachón, indicating that surface debris can enter the cave and is consumed by cavefish. As the rainy season transitioned into the dry season, the following November 2001 and February 2002 sampling times (points P4 and P5 in Fig. 4) clustered together based on the presence of inorganic materials. Periods of time during the dry season could make the caves very nutrient poor without rainfall, possibly causing the cavefish to pick up more inorganic materials than anything of nutritional value. The November 2001 and February 2002 sampling times for Pachón are also the most similar to all three Sabinos time points, but the Sabinos collection times are shaped by the presence of invertebrates. It should be noted as well that six sampling times were included for Pachón (n=146), and three were included for Sabinos (n=64), so this difference in resolution could play a role in the lack of seasonal change detected for Sabinos in comparison to Pachón. Future research including multiple sampling points throughout the year, particularly with additional times during the rainy season, and further gut content analysis to include food item measurements by mass will be necessary for understanding how and what cave *A. mexicanus* eat in nature.

*Conclusions*

Our findings show that while cave-adapted *A. mexicanus* are known to be smaller than closely-related surface populations, there is variation in body size of adult cavefish between caves. Cave-specific differences in body size could be driven by variation in food availability between cave environments, and body size modifications are likely accompanied by other behavioral and metabolic adaptations for reducing energetic costs. Strategies for maximizing energy use efficiency such as metabolic rate suppression, accumulation of fat stores, and hyperphagia may accompany body size reduction and co-occur to varying degrees. Additionally, *A. mexicanus* cavefish were found to eat during both the rainy and dry seasons, suggesting that the cave environments in this system are potentially characterized by continual nutrient limitation, rather than intermittent periods of starvation. These conditions are expected to select for smaller body size and an opportunistic feeding style, and our diet analyses revealed a variety of food items that provide support for opportunistic feeding. Detritus, inorganic materials, invertebrates, seeds, and other plant material drove differences in diet over time and between two cave populations. Open questions regarding the diet of cave-adapted *A. mexicanus* are centered on understanding how the quantity, quality, and relative proportions of food items in their guts seasonally vary and identifying what nutrients most contribute to their metabolic physiology.

**Data Accessibility**

All data associated with this manuscript will be made available in a public repository (Dryad) upon acceptance of the manuscript or can be obtained directly from the corresponding authors.

**Ethical Approval and Conflict of Interest**

Our study was conducted at the Technological Institute of Ciudad Victoria (ITCV) in Tamaulipas, México, where despite the lack of any animal care protocol, the animals were all collected under standard care procedures. Authors are responsible for correctness of the statements provided in the manuscript. The authors declare no conflicts of interests.

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**Tables and Figures**

Table 1: Individuals collected for biometrics and gut content analysis at time points between February 2001 and March 2002. *N*biometrics represents all the individuals included in the biometrics analyses, and the sex ratios are reported as the proportion of the population that is male. *N*gut is the subset of individuals that were then investigated for their gut contents. Finally, *N*empty is the number of individuals with empty guts, which is also represented as a percentage (100\* *N*empty/*N*gut).

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Cave** | **Date** | ***N*biometrics** | **Sex ratio** | ***N*gut** | ***N*empty** | **Percent empty** |
| Pachón | 2/3/2001 | 50 | 0.38 | 34 | 18 | 52.9% |
|  | 2/21/2001 | 67 | 0.21 |  |  |  |
|  | 4/19/2001 | 39 | 0.13 | 34 | 18 | 52.9 % |
|  | 6/24/2001 | 100 | 0.16 | 27 | 12 | 44.4 % |
|  | 11/3/2001 | 70 | 0.11 | 29 | 15 | 51.7 % |
|  | 11/4/2001 | 31 | 0.01 |  |  |  |
|  | 2/21/2002 |  |  | 12 | 8 | 66.7 % |
|  | 2/22/2002 | 21 | 0.24 |  |  |  |
|  | 2/23/2002 | 19 | 0.21 |  |  |  |
|  | 3/22/2002 | 65 | 0.03 | 10 | 5 | 50.0 % |
|  | 3/23/2002 | 38 | 0.08 |  |  |  |
|  | 3/24/2002 | 22 | 0.23 |  |  |  |
| Sabinos | 4/18/2001 | 35 | 0.23 | 25 | 11 | 44.0 % |
|  | 6/23/2001 | 76 | 0.42 | 30 | 20 | 66.7 % |
|  | 11/2/2001 | 71 | 0.21 | 3 | 0 | 0.0 % |
| Tinaja | 2/4/2001 | 26 | 0.35 | 6 | 4 | 66.7 % |
|  | 4/17/2001 | 11 | 0.27 |  |  |  |
|  | 6/23/2001 | 24 | 0.58 | 3 | 1 | 33.3 % |
|  | 11/2/2001 | 11 | 0.36 | 4 | 1 | 25.0 % |

Table 2: Results of GLMM analysis for the response variables (A) body size variation (measured as standard length) and (B) sex distributions, and results of GLM analysis for the response variable (C) percentage of the population with empty guts. Note that collection date is designated in as a random effect (1|Date) in analyses of standard length and sex ratios.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **AICc** | **ΔAICc** | **df** | **AICc Weight** |
| (A) Standard length |  |  |  |  |
| (1|Date) (null model) | 1950.4 | 61.9 | 3 | <0.001 |
| Sex + (1|Date) | 1931.8 | 43.3 | 4 | <0.001 |
| Cave + (1|Date) | 1902.6 | 14.1 | 5 | <0.001 |
| Sex \* Cave + (1|Date) | 1888.8 | 0.3 | 8 | 0.46 |
| Sex + Cave + (1|Date) | 1888.5 | 0.0 | 6 | 0.54 |
|  |  |  |  |  |
| (B) Sex ratios |  |  |  |  |
| (1|Date) (null model) | 778.7 | 4.4 | 2 | 0.1 |
| Cave + (1|Date) | 774.3 | 0.0 | 4 | 0.9 |
|  |  |  |  |  |
| (C) Empty guts |  |  |  |  |
| Intercept (null model) | 284.3 | 0.0 | 1 | 0.679 |
| Intercept + Cave | 286.3 | 2.0 | 2 | 0.249 |
| Intercept + Date | 290.3 | 6.0 | 6 | 0.034 |
| Intercept + Cave + Date | 292.5 | 8.1 | 7 | 0.012 |
| Intercept + Cave \* Date | 291.9 | 7.5 | 9 | 0.016 |

Table 3: PERMANOVA results of gut content composition relative to cave of origin (Pachón and Sabinos), sampling time (February 2001-March 2002), and their interactions

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | ***df*** | **Sum of squares** | ***R*2** | ***F*** | ***P*** |
| Cave | 1 | 1.325 | 0.046 | 4.750 | 0.002 |
| Time | 1 | 1.070 | 0.037 | 3.836 | 0.002 |
| Cave × Time | 1 | 0.618 | 0.021 | 2.215 | 0.046 |
| Residual | 93 | 25.944 | 0.896 |  |  |
| Total | 96 | 28.958 | 1.00000 |  |  |

Fig. 1: A. The location of the study region in Northeastern Mexico is indicated by the gray square. B. Caves studied here are indicated by green circles. Black circles indicate the location of other caves inhabited by *A. mexicanus*. Maps were created in R using data associated with the rnaturalearth (South 2017) and elevatr packages (Hollister 2021).

Fig. 2: Large circles with bars represent marginal means and standard errors of standard length across the Pachón, Sabinos, and Tinaja caves, as inferred by the interaction model: Standard length ~ Sex \* Cave + (1|Date). Small circles represent the raw data used in the model. Males are indicated in orange, females in blue.

Fig. 3: A. Frequency of individuals with empty guts in the Pachón and Sabinos populations across multiple sampling times. B. The average number (and standard error) of diet categories per individual for the Pachón and Sabinos populations across multiple sampling times.

Fig. 4: A. NMDS plot showing the gut composition (means and standard deviations) of caves and sampling times for the Pachón and Sabinos populations. Ordination ranking was conducted using Jaccard distance, and the stress with 3 dimensions is 0.079. The plot points for Pachón and Sabinos are numbered in order corresponding to their sampling times as follows: For Pachón, P1 = 2/3/2001, P2 = 4/19/2001, P3 = 6/24/2001, P4 = 11/3/2001, P5 = 2/21/2002, P6 = 3/22/2002; for Sabinos, S1 = 4/18/2001, S2 = 6/23/2001, S3 = 11/2/2001. B. Precipitation (in mm) during a typical year near the Sierra de El Abra region (monthly average rainfall data from Tampico, Mexico; *World Weather Online* 2021). Points P1-P6 and S1-S3 correspond to the sampling times in Fig. 4A.