Evolution in caves: selection from darkness causes spinal deformities in teleost fishes

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**Only few fish species have successfully colonized subterranean habitats, but the underlying biological constraints associated with this are still poorly understood. Here we investigated the influence of permanent darkness on spinal-column development in one species (Midas cichlid, *Amphilophus citrinellus*) with no known cave form, and one (Atlantic molly, *Poecilia mexicana*) with two phylogenetically young cave forms. Specifically, fish were reared under a normal light:dark cycle or in permanent darkness (both species). We also surveyed wild-caught cave and surface ecotypes of *P. mexicana*. In both species, permanent darkness was associated with significantly higher rates of spinal deformities (especially in *A. citrinellus*). This suggests strong developmental (intrinsic) constraints on the successful colonization of subterranean environments in teleost fishes and might help explain the relative paucity of cave-adapted lineages. Our results add depth to our understanding of the aspects of selection driving trait divergence and maintaining reproductive isolation in cave faunas.**

**Keywords:** *Amphilophus citrinellus*, cave fauna, ecological speciation, *Poecilia mexicana*, scoliosis

**1. Introduction**

Fishes have repeatedly invaded subterranean habitats around the globe; however, only 165 species have been described as living exclusively in subterranean environments [1]. Thirty three and 26 species of cavefishes are known from South and North America, respectively [1,2]. While four families dominate teleost fish diversity across American freshwater habitats (i.e., Characidae, Cichlidae, Loricariidae, and Poeciliidae; e.g., [3,4]), only three of these have evolved cave forms (Loricariidae with three, Characidae with two, and Poeciliidae with one species; [1]). This begs the question of why these widespread families are not more common amongst the 59 cave-dwelling fish species of the Americas.

Animals that live in permanent darkness have to cope with a multitude of unique challenges. For example, absence of light renders visual senses useless, and subterranean taxa have to rely on other sensory systems to navigate, find and exploit resources, detect and evade predators, find mates, or communicate with conspecifics [5]. While we have made substantial advances in our understanding of certain evolutionary processes associated with adaptation to cave environments (e.g., enhanced mechanosensation [5]; vibration-attraction behaviour [6]; loss of pigmentation: [7]; eye reduction: [8]), we still only have a limited understanding of the evolutionary/developmental constraints associated with the initial colonization of cave environments by surface-dwelling founder populations (but see [9,10]).

We investigated this issue while focusing on two American species, *Amphilophus citrinellus* (Cichlidae) and *Poecilia mexicana* (Poeciliidae). In cichlids, cave populations of uncertain status were occasionally reported from sinkholes (cenotes) on Yucatan (e.g., [11]), while *P. mexicana* has evolved two phylogenetically young cave forms in southern Mexico [12]. Here, we raised *A. citrinellus* offspring in a common-garden experiment—half under a light:dark cycle and half in permanent darkness. Based on previous experimentation on *P. mexicana* [9,10], we predicted to find decreased viability in fish reared in permanent darkness, but instead uncovered a high incidence of spinal deformities. Leveraging our extensive database of wild-caught and lab-reared *P. mexicana*, we confirmed a general pattern of an association between darkness and spinal deformities.

**2. Material and Methods**

**(a) *Mortality and spinal deformities in* Amphilophus citrinellus**

Laboratory-reared Midas cichlids originated from a mixed-sex stock tank comprising wild-caught individuals collected in Lake Nicaragua in 2010 and 2013. For the experiment, conducted in 2017, embryos from an *A. citrinellus* brood were divided into twelve groups two days post fertilization (*n=*10 each); six were placed in a 12:12 hour light-dark cycle and six in constant darkness (for details see [13]). Two weeks after hatching, the percentages of surviving fish and of those with spinal deformities were quantified.

**(b) *Spinal deformities in* P. mexicana**

We surveyed our records of *P. mexicana* collected from surface or cave populations between 2005 and 2014 [14-16] (*n*=1084), or raised in a common-garden study in 2009/2010 (*n*=140), which included raising half the individuals in perpetual darkness and the other half under a 12:12 hr light:dark cycle [9,10]. Of these, *n*=819 originated from surface streams or were reared under a light:dark regime, while *n*=405 originated from cave environments or were reared in permanent darkness.

**(c) *Statistical analyses***

All analyses were conducted in IBM SPSS Statistics Version 21. We first analysed mortality in our *A. citrinellus* experiment by means of a Generalized Linear Mixed Model (GLMM) with a binominal error distribution and a logit-link function, and included ‘light regime’ (normal light:dark cycle vs. permanent darkness) and ‘replicate-nested-within-light-regime’ [henceforth: replicate(treatment)] as factors.

To analyse the occurrence of spinal deformities (binary data: 1 = scoliosis; 0 = normal spinal curvature; Fig. 1) between fish exposed to different light regimes, we applied two species-specific GLMMs with a binominal error distribution and a logit-link function, and included ‘light regime’ as a fixed factor. The *P. mexicana*-based model further included ‘ecotype’ (cave vs. surface) as another factor.

**3. Results**

**(a) *Mortality and spinal deformities in* Amphilophus citrinellus**

When analysing mortality of *A. citrinellus* (44% of *n*=120), neither treatment (Wald *χ*2=2.69, *df*=1, *p*=0.10) nor replicate(treatment) had a significant influence (Wald *χ*2=11.97, *df*=10, *p*=0.29). Visual inspection of the data (Fig. 1a) suggests slightly higher mortality in the light-treatment.

When analysing spinal deformation among surviving individuals (*n*=65), none of the fish raised in the light-treatment exhibited any spinal deformities (*n*=28) while all-but-one individuals raised in permanent darkness did (i.e.,36 out of 37; Figs. 1b, 2a, c). Almost complete separation of the data precluded our planned analysis. An alternative Chi-square goodness-of-fit test revealed a significant effect of darkness on the occurrence of spinal deformities (*χ*2=14.22, *df*=1, *p*<0.001).

**(b) *Spinal deformities in* Poecilia mexicana**

In total, we found 11 cases of spinal deformities in our survey of *P. mexicana* (*n*=1224; Table 1; Fig. 2b, d). Light regime had a statistically significant effect on the occurrence of scoliosis (Wald *χ*2=5.48, *df*=1, *p*=0.019), while ecotype did not (Wald *χ*2=0.05, *df*=1, *p*=0.82). The light-effect was not surprising given that almost all cases of scoliosis were found either in wild-caught cave fish (*n*=6) or in fish raised in experimental permanent darkness (*n*=4), the only exception being a single cave fish that was raised under light:dark conditions (Table 1).

**4. Discussion**

We uncovered a high incidence of spinal deformities in the cichlid *A. citrinellus* raised in permanent darkness, while none of the fish raised under normal light conditions developed similar deformities. Congruently, no cave cichlid is known despite their widespread and frequent occurrence in the Americas [1]. Mortality rates in our *A. citrinellus* common-garden experiment were relatively high throughout, corroborating previous reports [17], but did not differ significantly between treatments. An extensive survey of our data on cave- and surface-forms of *P. mexicana*—another widespread Central American freshwater fish—also revealed an association between darkness and spinal deformities, albeit at a much lower overall rate of occurrence. This aligns with the fact that *P. mexicana* evolved two cave forms [12]. Our results support the notion that exposure to light at least during critical stages of teleost spinal system development might be more important than previously assumed [18]. Previous studies in fish demonstrated that spinal deformities have a strong negative impact on locomotor abilities (e.g., [19]), likely resulting in strongly reduced fitness under natural conditions.

As of yet, the exact mechanisms underlying our results remain elusive. Low levels (i.e., 1-2%) of spinal deformities are common in teleost fish even under seemingly benign conditions [20], but previous studies reported an association of increased rates of spinal deformities with, e.g., nutrient deficiencies or heavy metal contamination [21]. Nutrient deficiencies might also play a role in cave-dwelling *P. mexicana* (compare [22]), but all common garden-reared fish received the same diet, rendering this explanation unlikely. We propose that the complete absence of light in both, our experimental treatment and in natural cave populations causes a disruption of melatonin secretion. As a component of the circadian clock, melatonin is known to be dependent on regular light stimuli [18,23]. Melatonin is involved in the regulation of growth and development, and minimal light thresholds are needed for proper larval development in most fishes [18]. Indeed, dark-raised *A. citrinellus* have a disrupted thyroid metabolism [13], a downstream target of melatonin [18]. Not surprisingly, surgical removal of the pineal gland has been reported to cause spinal deformities in vertebrates [23]. Scoliosis in vertebrates has also been associated with distorted vitamin D3 levels [24], which could be caused by lack of light [25], although photosynthesis of vitamin D3 might be insignificant in fish [26].

We have previously reported that in *P. mexicana*, juvenile development in the absence of light leads to an increased incidence of stress-related columnaris disease, failure to reproduce and mortality [9,10]. All effects were stronger in surface-dwelling than in cave fish. Given that we are not aware of similar negative impacts from other, phylogenetically older cave fish, our data on spinal deformities suggests that cave-adapted *P. mexicana*, as phylogenetically young cave fish, have not yet evolved full independence of light (supported by the non-significant ‘ecotype’-effect in our analysis).

Altogether then, our present study provides further evidence for the strong negative selection imposed by permanent darkness on fish adapted to surface habitats, and helps explain why taxa whose surface-dwelling ancestors are adapted to bright-light environments are usually underrepresented among obligate cave fauna [27]. Clearly, successful colonization of a subterranean habitat is a relatively rare event, and as our data from *P. mexicana* suggest, the negative impacts of the transition into permanent darkness can still be measured in species that have successfully undergone the transition towards cave-dwelling, i.e., that show some degree of local adaptation to their cave environment. Future studies will need to elaborate on why *P. mexicana*—on a species level—shows comparatively stronger independence of light than *A. citrinellus*, and ask whether this independence is even stronger in fish families that have more cave representatives or inhabit aphotic environments, e.g., in the deep sea.

Ethics. The experiments comply with the current laws on animal experimentation of the United States of America (AUS-IACUC approved protocol: R06-026) and of Baden-Württemberg, Germany (approved protocol: 35-9185.81/G-16/07).

Data accessibility. The datasets supporting this article have been uploaded as part of the supplementary material.

Authors’ contributions. All authors collected data; J.T.-D. and R.R. conceived the idea for the analysis; R.R. analysed data; all authors wrote the manuscript, gave final approval for publication, and agree to be held accountable for the content herein.

Competing interests. The authors declare no competing interests.

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Table 1.Occurrence of spinal deformities (scoliosis) based on a survey of *n*=1224 *P. mexicana* that were dissected for life-history studies between 2005-2014; CdA = Cueva del Azufre, CLA = Cueva Luna Azufre, AB = Arroyo Bonita, RA = Río Amatán.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Year | ID | Population | Sex | Treatment |
| 1. wild-caught |  |  |  |  |
| 2007 | MT07-032, #8 | CdA X | female | — |
|  | MT07-033, #8 | CLA | female | — |
| 2010 | 08/01/10, #10 | CdA V | female | — |
|  | 10/01/10, #9 | CdA V | female | — |
| 2014 | 14PSVIII, #15 | CdA VIII | male | — |
|  | 14PSII, #13 | CdA II | female | — |
| 1. common-garden |  |  |  |  |
| 2009 | #f48 | CdA V | female | darkness |
|  | #d9 | AB | female | darkness |
|  | #m48 | RA | male | darkness |
|  | #m60 | CdA V | male | light:dark cycle |
|  | #m27 | CdA V | male | darkness |

Figure 1. Proportion (mean ± s.d.) of (a) mortality and (b) spinal deformities of *A. citrinellus* reared under natural light (white bars) and in permanently dark (black bars) conditions.



Figure 2. (a) Normally-developing *A.**citrinellus* embryo reared in light compared to (c) an embryo with spinal deformities (indicated by arrows) reared in darkness. Scoliosis in (b) a wild-caught *P. mexicana* from CdA V (ID 10/01/10, #9), and (d) a common-garden reared *P. mexicana* from CdA V (ID #f48) reared in darkness (see Table 1 for IDs).

