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An experimental evaluation of substrate type and color selection by the endangered salamander *Ambystoma altamirani* (Caudata: Ambystomatidae)

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Abstract

An experimental evaluation of substrate type and color selection by the endangered salamander *Ambystoma altamirani* (Caudata: Ambystomatidae). The stream habitats of Mexican *Ambystoma* are being degraded by human activities, which can have detrimental effects on their populations. A better understanding of the microhabitat and habitat use of these amphibians is therefore important. We used preference experiments to examine the selection of substrate type (gravel, mud, sand, and stone) and color (dark brown, brown, light brown, and gray) by the endangered, endemic salamander *Ambystoma altamirani. Ambystoma altamirani* used mud substrates more than the other substrate types. Overall, *A. altamirani* showed no preference for substrate color; however, females used light brown substrates significantly more often and gray substrates less often than males and juveniles did. These experimental results are consistent with the observed substrate type use of *A. altamirani* in the field, but the lack of a substrate color preference differed from the observed preference for dark brown substrates in the field.

Keywords: Arroyo los Axolotes, Behavior, Mexico, Microhabitat, Mountain Stream Siredon.

Resumo

Uma avaliação experimental do tipo de substrato e da seleção de cores pela salamandra ameaçada de extinção *Ambystoma altamirani* (Caudata: Ambystomatidae). Os habitats de riacho das salamandras do gênero *Ambystoma* no México estão sendo degradados pelas atividades humanas, o que pode ter efeitos prejudiciais sobre suas populações. Assim, é importante compreender melhor o uso de habitas e micro-habitatas por parte desses anfíbios. Utilizamos experimentos de preferência para examinar a seleção do tipo (cascalho, lama, areia e pedra) e da cor (castanho-escuro, castanho, castanho-claro e cinzento) do substrato pela salamandra *A. altamirani*, uma espécie endêmica e ameaçada de extinção. *Ambystoma altamirani* utilizou mais os substratos de lama do que os outros

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tipos. De modo geral, *A. altamirani* não mostrou preferência pela cor do substrato; no entanto, as fêmeas usaram substratos castanhos claros com mais frequência e substratos cinzentos com menos frequência do que os machos e os juvenis. Esses resultados experimentais são consistentes com o uso do tipo de substrato observado em *A. altamirani* no campo, mas a ausência de preferência pela cor do substrato difere da preferência por substratos marrons escuros observada no campo.

Palavras-chave: Arroyo los Axolotes, Comportamento, México, Micro-habitat, Siredon-de-riachos-de-montanha.

Introduction

The quality of freshwater habitats is frequently negatively affected by humans (Carpenter *et al.* 2011, Oberdorff 2022). In particular, a substantial percentage of streams and rivers are degraded by human activities (Kaufmann *et al.* 2022b, McManamay *et al.* 2022). Indeed, higher human population densities are correlated with a loss of biodiversity in streams (Urban *et al.* 2006). Anthropogenic impacts on streams include sedimentation, changes in vegetation within or surrounding the stream, or changes in habitat complexity (Kaufmann *et al.* 2022a), water flow and hydrology (Poff *et al.* 2006, McManamay *et al.* 2022), and water quality (e.g., chemistry, temperature; Poole and Berman 2001, Ferreira *et al.* 2017, Vázquez *et al.* 2023).

Several species of salamanders use streams, either as places to breed or for their entire life cycle (Wells 2007). As such, the characteristics of stream habitats can be important in determining their distribution and abundance. Often, aspects of the stream substrate are important factors driving salamander abundance or distribution. In particular, substrate composition (e.g., gravel, sand, silt, mud, etc.) has been shown to determine the abundance or distribution of salamanders along streams (e.g., Bowles *et al.* 2006, Miller *et al.* 2007, Kroll *et al.* 2008, Rizzo *et al.* 2016). Because of the importance of substrate composition in determining the abundance and distribution of stream salamanders, the anthropogenic activities that influence the amount or composition of stream substrates can negatively affect populations of stream salamanders. In particular, increased sedimentation, often associated with alterations in water flow or changes in land use near streams, has been shown to negatively impact stream salamander populations, primarily by increasing sediment embeddedness (e.g., Lowe and Bolger 2002, Lowe *et al.* 2004, Moseley *et al.* 2008).

The degradation of stream habitats due to human activities—such as deforestation, livestock, and agriculture—is detrimental to populations of Mexican *Ambystoma* Tschudi, 1838 due to changes in water chemistry and water flow, the introduction of nonnative species, and increased substrate embeddedness (e.g., Estrella Zamora *et al.* 2018, Guerrero de la Paz 2020, Piñon-Flores *et al.* 2021). Many populations and species of Mexican *Ambystoma* are threatened (Heredia-Bobadilla and Sunny 2021). It is therefore important to better understand their microhabitat and habitat use in the streams and lakes they inhabit.

In field studies of the substrate use of Mexican *Ambystoma,* several species have been found to frequently use mud substrates (*A. rivulare*: Bille 2009, Lemos-Espinal *et al.* 2015; *A. leorae:* Lemos-Espinal *et al.* 2017). Other species of Mexican *Ambystoma* use multiple substrate types, including rock, sand, and mud substrates (*A. leorae:* Sunny *et al.* 2014, Monroy-Vilchis *et al.* 2015), but others do not (*A. rosaceum* Taylor, 1941: Anderson 1961).

Ambystoma altamirani Dugès, 1895 is an endangered, endemic salamander found in streams in the mountains near Mexico City (Lemos-Espinal *et al.* 1999, Woolrich-Piña *et al.*

2017). *Ambystoma altamirani* lives most, if not all, of its life in streams or immediately adjacent to streams (< 5 m; Lemos-Espinal *et al.* 1999). The dorsal color of *A. altamirani* is usually olive-green with black or yellow markings or solid black (Villarreal Hernández *et al.* 2020b). In-stream characteristics appear to be more important than terrestrial characteristics (e.g., presence of livestock, distance to forest) in determining the abundance of *A. altamirani* along a stream (Gómez-Franco *et al.* 2022). *Ambystoma altamirani* in the Arroyo Los Axolotes selected sites with mud and avoided sites with gravel or bedrock (Lemos-Espinal *et al.* 2016, Villarreal Hernández *et al.* 2020a). In nature, *A. altamirani* was found on dark brown substrates more than brown, light brown, or gray substrates (Villarreal Hernández *et al.* 2020a; see also Lemos-Espinal *et al.* 2016), and this selection did not differ with the color of the salamander (Villarreal Hernández *et al.* 2020b). Despite these field studies, there have been no experimental investigations of substrate selection in *A. altamirani.* Such experiments would help inform our understanding of the basis for the field observations (i.e., Is it a true preference or simply a reflection of availability?), which can inform our knowledge of what aspects of a stream, especially its substrates, are needed for the persistence of these endangered salamanders.

Here, we report on the results of experiments designed to determine the preferences of *A. altamirani* for substrate type and color. We also determined whether these preferences differed among adult males, adult females, and juveniles.

Materials and Methods

We collected 135 individuals of *A. altamirani* from multiple sites along a 1 km section of the Arroyo del Axolotes, Isidro Fabela municipality, Mexico, using a dipnet and transported them to a nearby facility for the preference experiments. We collected salamanders and conducted experiments in December 2021 (*N* = 2 salamanders), February 2022 (*N* = 4), March 2022 (*N* = 13), April 2022 (*N* = 32), May 2022 (*N* = 19), June 2022 (*N* = 35), July 2022 (*N* = 8), August 2022 (*N* = 10), September 2022 (*N* = 5), October 2022 (*N* = 4), and November 2022 (*N* = 3). We categorized individuals as adult males, adult females, or juveniles. We used the presence of a bulge on the tail near the cloaca to identify adult males, whereas adult females lack this bulge (Brandon and Altig 1973). We classified a salamander as a juvenile if it possessed gills (see Villarreal Hernández *et al.* 2020a).

We conducted two preference experiments: one for substrate type and one for substrate color (see Mushinsky 1976, Rittenhouse *et al.* 2004, Martin *et al.* 2012, and Sánchez-Sánchez *et al.* 2023 for similar experiments). The experiments were performed one to four hours after capture. We kept the salamanders in plastic containers between capture and experimentation. For each experiment, we established multiple test arenas using 36 L aquaria (40 × 30 × 30 cm) with each aquarium divided into sections as described below. At the start of each trial, we placed a single *A. altamirani* in the center of an aquarium and allowed it to acclimate for 5 min prior to data collection. During the acclimation period, the salamanders were allowed to move about the aquarium. After the acclimation period, we recorded the substrate type or color on which the salamander was located every minute for 15 minutes. Each salamander was tested only once in each experiment.

To test for substrate type preferences, we established four equal sections on the bottom of the aquaria with mud, sand, gravel, and rock using material collected from the stream (Figure 1). The mud substrate was dark brown in color, the sand was brown, the gravel was grey, the rock was light brown or light grey. We used small rectangular plastic trays to keep the substrate types separate. Since color and substrate type were confounded in the substrate type experiment, we conducted a second experiment to specifically study the effect of substrate color. For the substrate color experiment, we divided the aquarium floor into four equal sections with colored paper under the clear bottom of the tank. We chose colors to approximate the four most common substrate colors in the Arroyo Los Axolotes using the COMEX color palette (brown color family): dark brown, brown, light brown, and grey (Villarreal Hernández *et al.* 2020a,b). We replaced the water in the aquaria between trials and the orientations of the experimental aquaria were arbitrarily established prior to each set of experiments (i.e., each monthly trial).

We used two-way repeated measures ANOVAs to analyze substrate type and color preferences, with sex/stage (male, female, juvenile) and season (wet, dry) as the independent variables and substrate type or color as the repeated measures. We analyzed the number of observations of each individual found in a substrate or color. We used Wilcoxon signed-rank tests to compare means for significant terms.

Results

The results of the two-way repeated measures ANOVA for the substrate type and for the substrate color experiments are given in Table 1. The only significant effect was that mud was used more than the other substrate types (Figure 2A). There was a significant interaction between color used and the sex/stage of the salamander, with females using light brown substrate more and gray substrates less than males and juveniles (Figure 2B). No other terms were significant.

Discussion

Ambystoma altamirani individuals used mud substrates more often than stone, gravel, or sand substrates. This pattern did not differ among males, females, and juveniles or between the wet and dry seasons. Our experimental results are generally consistent with field studies on substrate use by *A. altamirani* which found that

Sand Gravel Mud Rock

Figure 1. A photograph showing the different substrate types used in the experiment.

Table 1. Results of a two-way repeated measures analysis of variance on substrate type/color preference of *Ambystoma altamirani*, with sex/stage (male, female, juvenile) and season (wet, dry) as independent variables and substrate type/color as the repeated measure.

	Substrate type			**Substrate color**		
	dfs	*F*	*p*	dfs	*F*	*p*
Sex/stage	2, 129	1.86	0.16	2, 129	0.87	0.42
Season	1, 129	2.83	0.10	1, 129	1.00	0.32
Sex/stage * season	2, 129	0.85	0.43	2, 129	0.28	0.76
Substrate type	3, 387	3.35	0.019	3, 387	0.80	0.49
Substrate type * sex/stage	6, 387	0.90	0.49	6, 387	2.52	0.022
Substrate type * season	3, 387	0.85	0.47	3, 387	2.18	0.10
Substrate type * sex/stage * season	6, 387	1.08	0.38	6, 387	1.19	0.26

sections of streams with mud were used more often than sections of streams with other substrates were (Lemos-Espinal *et al.* 2016, Villarreal Hernández *et al.* 2020a). Mud is also a commonly used substrate for other Mexican *Ambystoma,* including *A. rivulare* (Bille 2009, Lemos-Espinal *et al.* 2015) and *A. leorae* (Sunny *et al.* 2014, Lemos-Espinal *et al.* 2017). All of these results suggest that mud substrates have some quality that is attractive or beneficial to these salamanders. For example, mud substrates may provide salamanders with refugia from predators or other disturbances by allowing them to bury themselves (see Bille 2009, Lemos-Espinal *et al.* 2015).

In our experiments, *A. altamirani* did not show any overall preference for substrate color. However, females differed from males and juveniles in their use of substrate colors, with females using light brown substrates more and gray substrates less than males and juveniles. It is unclear why such a difference might exist. Previous field studies have shown that *A. altamirani* uses darker substrates, such as dark brown or black, more than lighter substrates, such as grey (Lemos-Espinal *et al.* 2016, Villarreal Hernández *et al.* 2020a,b). These previous field studies also did not distinguish between males and females (Lemos-Espinal *et al.* 2016, Villarreal Hernández *et al.* 2020a,b). It is possible that the differences in the color of substrates used between our experimental study and the previous field results may reflect differences in salamander color or salamander perception of risk, or perhaps reflect differences in the sex or stage of the individuals observed in the field.

In other *Ambystoma,* there can be a relationship between individual color and substrate color, where individuals physiologically adjust their color to match the substrate color (Garcia and Sih 2003) or choose substrates to match their color (Garcia *et al.* 2003). In addition, the substrate color selected can depend on the presence of predator cues (Garcia and Sih 2003). There is also some evidence that substrate color choice can be conditioned by early experience (Garcia and Sih 2003). However, in at least one field study, *A. altamirani* showed no evidence of substrate color matching (Villarreal Hernández *et al.* 2020b). Future experiments that examine substrate color choice by *A. altamirani* in the presence of predator cues or after conditioning on particular substrate colors might help elucidate the factors driving substrate color selection in this endangered salamander. In addition, manipulating the color of the mud while retaining other characteristics could also

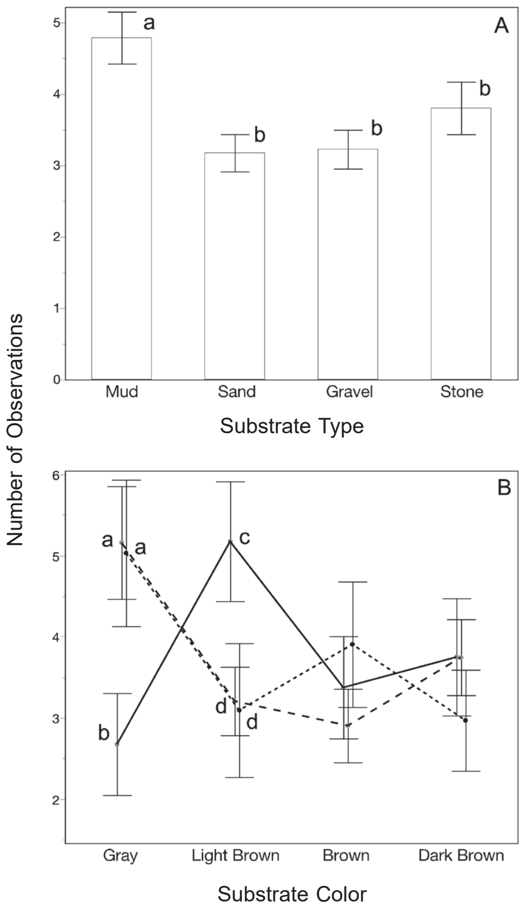

Figure 2. The mean number of times a individual *Ambystoma altamirani* used (**A**) a particular substrate type and (**B**) male (dotted line), female (solid line), and juvenile (dashed line) *A. altamirani* used a particular substrate color in the dry and wet seasons. Means sharing a letter are not significantly different (Wilcoxon signed rank test, $p > 0.05$). For (B) post-hoc tests were applied to each substrate color separately.

elucidate whether *A. altamirani* prefers the color or the mud.

Overall, our experiment results and similar results from field observations suggest that mud substrate is an important characteristic of streams used by the endangered salamander, *A. altamirani* (Lemos-Espinal *et al.* 2016, Villarreal Hernández *et al.* 2020). Taken together, these results indicate that preventing the loss of mud substrates is important when establishing conservation or management plans for these streams. In particular, this means maintaining stream flows that prevent the scouring of mud substrates, replacing mud substrates with other types of sediments (e.g., sand or other sediments with different particle sizes), or channelizing streams. Similar future experiments should focus on other aspects of the stream environment that have been shown to be related to the distribution and abundance of *A. altamirani,* such as dissolved oxygen, water velocity, water depth, refuse and garbage, and water temperature (Lemos-Espinal *et al.* 2016, Villanueva Camacho *et al.* 2020, Gómez Franco *et al.* 2022). In addition, experiments that use individuals from more than one stream would allow for greater generalization of the results since our study only included individuals of *A. altamirani* from a single study stream.

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