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Acoustic signatures in cavefish populations inhabiting different caves

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22 Key words: acoustic signatures, blind cavefish, population accents

23

24 **Abstract** (150-200 words)

25

26 Complex patterns of acoustic communication exist throughout the animal kingdom, including
27 underwater. The river-dwelling and the Pachón cave-adapted morphs of the fish *Astyanax mexicanus*
28 share a repertoire of sounds, but their trigger, use and meaning has changed in cavefish after their
29 recent colonization of the subterranean environment. Here, we explored whether and how sounds
30 produced by blind cavefishes inhabiting different Mexican caves may have evolved, too. We
31 compared acoustic parameters of sounds produced by wild cavefish, recorded in their natural
32 settings, in six different caves located in three mountains ranges in North-East Mexico. Multi-
33 parametric analyses show that the six cavefish populations sampled present cave-specific acoustic
34 signatures, as well as possible individual signatures. The variations in acoustic parameters do not
35 seem related to fish phenotypes, phylogeography or ecological conditions. We propose that the
36 evolution of such acoustic signatures would be neutral and occur by drift, progressively leading to the
37 differentiation of local accents that may prevent interbreeding and thus contribute to speciation.

38 Introduction

39

40 Animal communication brings together all the information exchanges between individuals
41 of the same or different species. The transmitter produces a signal, which causes a change in
42 behavior or physiological state of the recipient. In the aquatic environment, where the speed of
43 sound propagation is approximately four times faster than in the air and travels long distances,
44 acoustic communication is widespread in mammalian and non-mammalian vertebrates such as
45 teleosts [1-3]. In fishes, acoustic signals play roles in feeding, reproduction, hierarchy, predator
46 detection, orientation and habitat selection.

47

48 Sonic animals have their own sound repertoires. The dolphin whistles, the whale clicks, or
49 the toadfish boat-whistles are emblematic for aquatic species. Then, among species repertoires,
50 individual signatures exist: Lusitanian toadfish males advertise their quality to females with boat-
51 whistle calling rate [4], and both male and female frogs produce individual signature calls,
52 suggesting that their acoustic communication may be more complex than expected [5]. Acoustic
53 signatures are also species-specific. Their evolution has a suggested role in the speciation process, as
54 proposed in cichlids [6, 7] or pipefishes [8]. In the later, differences in the structure of sound
55 producing apparatus including cranial bone morphology may explain the unique acoustic signatures
56 of the feeding clicks produced by closely related species. Also, within the piranha species *Serrasalmus*
57 *marginatus*, red- and yellow-eyed morphs produce sounds with different amplitude features [9]. A
58 mutation or different hormonal concentrations could explain both sound amplitude and eye color,
59 playing a role in animal communication. This is, to our knowledge, a rare case of within-species
60 acoustic signature in fish.

61

62 The fish *Astyanax mexicanus* (Mexican tetra) is also a sonic species [10]. Remarkably,
63 acoustic communication has evolved between *Astyanax* river-dwelling and blind cave-adapted
64 morphs, which have diverged about 20.000 years ago [11, 12]. Cavefish and river fish share a
65 repertoire of six sounds, but functionally the trigger, the use, and the meaning of sounds has
66 changed between cavefish from the Pachón cave and river fish [10]. Thus, acoustic communication
67 has evolved after colonization of the subterranean habitat, opening avenues for the exploration of
68 the evolution of acoustic signatures within the species.

69

70 Results

71

72 In North-Eastern Mexico, there are more than 30 caves where cavefish populations leave [13,
73 14] and show signs of ongoing genetic differentiation [15-17]. We performed acoustic recordings in
74 March 2016 and 2017 in six different caves, in natural settings. We chose the Molino, Pachón, Los
75 Sabinos, Tinaja, Chica, and Subterráneo caves because they are distributed throughout the 3
76 geographically distinct mountains ranges where *A. mexicanus* cavefish populations leave (**Fig. 1A**).
77 Because of the topography and technical constraints encountered in each cave, the recording
78 conditions, the number of fish recorded and the length of audio bands varied between caves [Molino
79 (03/2017): 4 fish in openwork crate, 1h30; Pachón (03/2016): 10 fish in net, 11h; Los Sabinos
80 (03/2017): 10 fish in net, 9h; Tinaja (03/2016), 25 fish in natural pool, 1h30; Chica (03/2017), 20 fish
81 in natural pond, 11h; Subterráneo (03/2016): 12 fish in net, 10 hours; **Fig. 1B** and see Methods]. In
82 agreement with known genetic or ecological conditions previously reported across caves e.g.[18], the
83 phenotypes (size, level of troglomorphy) of the recorded fish were variable. For example, the fish
84 recorded in Pachón cave were 2.7 to 6.7 cm in length (including one showing juvenile traits) and
85 were fully troglomorphic, while the fish recorded in Subterráneo cave were 4.5 to 8 cm in length and
86 among them two had tiny eyes (**Fig. 1C**).

87

88 A first, global analysis of audio bands (total 44h of recordings) showed that Clicks and Serial
89 Clicks were the most represented sounds produced by cavefish in their natural environment, while
90 the other sounds of the repertoire (Clocs, Serial Clocs, Sharp Clicks and Rumbings [10]) were rarer.
91 We therefore focused on these two sounds, which are those showing the largest frequency
92 bandwidth (500-10,000Hz) (**Fig. 1D,E**). We extracted and selected Clicks and Serial Clicks (n=50-100
93 each) for each cavefish population and compared their acoustic parameters (**Supplemental data,**
94 **Table 1**).

95

96 Concerning single Clicks, we found that the sound duration, the dominant frequency, the
97 signal to noise ratio/RMS power all varied significantly among the six caves (**Fig. 2A**). Clicks were
98 longer in Tinaja (and Chica), more high-pitched in Molino (and Chica), and deeper and more powerful
99 in Subterráneo. The signal to noise ratio was high in Chica and low in Tinaja. Of note, the duration
100 and SNR variances were highest in Chica, suggesting less homogeneity in the sound production,
101 possibly related to the hybrid genetic background of the fish in this cave. For Serial Clicks, we
102 focused on the parameters related to their multi-pulse nature and the sound envelope. Pulse
103 duration, number and rate, as well as interpulse and total sound duration were also variable among

104 populations (**Fig. 2B**). Consistent with the single Click data, the pulse duration in Serial Clicks were
105 longer in Tinaja. In Pachón, the pulse rate was impressively high, about ten times higher on average
106 than in the other caves, accompanied by a number of pulses that was also twice higher, together
107 with a shorter interpulse duration and a lesser total duration of the sound. Other significant features
108 were high pulse numbers and long total sound durations in Subterráneo, and long interpulses in
109 Molino. Together, these data show that sounds produced differ among caves, but with no apparent
110 correlation or order relative to fish phenotypes (e.g., small Pachón fish can produce high pulse rates)
111 or cave phylogeography (e.g., Pachón and Subterráneo fish share a high number of pulses in their
112 Serial Clicks).

113

114 We next performed principal component analyses (PCA) to evaluate the possibility of a cave
115 acoustic signature (**Fig. 2CD**). For both sounds, Clicks and Serial Clicks, confidences ellipses around
116 centroids on the PCA showed little overlap (except Chica) and were mostly well separated.
117 Moreover, pDFA (permutated Discriminant Function Analysis) generated confusion matrixes with
118 good scores of correctly reclassified sounds, strongly suggesting that the sounds produced in each
119 cave carry a specific acoustic signature (**Fig. 2CD**, insets; $p=0.001$). Molino (80% reclassification),
120 Pachón (46%) and Tinaja (91%) Clicks were particularly distinctive, and Serial Clicks from all caves
121 except Chica showed significant reclassification scores (**Fig. 2CD**). Again, using a hierarchical
122 clustering tree onto geographical coordinates of caves in the PCA (**Fig. 2E**, Single Clicks), we found
123 some grouping of caves that does not fit with phylogeography. The apparent closeness of
124 Subterráneo (Micos group) and Sabinos (El Abra group), or Molino (Guatemala group) and Chica (El
125 Abra) rather suggests independent evolution of sound production in these different caves. In sum,
126 our data provide strong evidence for the evolution of an “accent” in the different cavefish
127 populations.

128

129 Finally, in our Molino dataset where only 4 fish were recorded, we noticed, using
130 unsupervised hierarchical clustering on single Clicks, the presence of 4 different groups of sounds
131 (**Fig. 2F**). Likewise, in Pachón (10 fish in net) and Subterráneo (12 fish in net) the same method
132 yielded 8 groups and 9 groups of sounds, respectively (not shown). These results strongly suggest
133 that besides their cave-specific acoustic signature, cavefish may also exhibit individual signatures.

134

135 Discussion

136

137 The existence of acoustic signatures in recently evolved populations of cavefish was
138 unexpected. Our findings provide an outstanding model to study the proximal and distal mechanisms
139 for the evolution of acoustic communication in a species on its way to diversification and speciation
140 [19] – even though *Astyanax* morphs still belong to the same species and show little genetic
141 differentiation. The most likely origins for observed differences between caves include plasticity in
142 response to specific local biotic and abiotic ecological conditions [13] see also [20], or the
143 independent and subtle morphological evolution of facial and jaw bones in relation with the loss of
144 eyes in cavefishes [21]. The latter would imply that the developmental evolution of the cavefish head
145 not only affects the visual, olfactory and mechano-sensory but also the acoustic facet of their
146 communication modalities, in a pleiotropic manner. We propose that the evolution of such acoustic
147 signatures would be neutral and occur by drift, progressively leading to the differentiation of local
148 accents that may ultimately prevent interbreeding and contribute to speciation.

149

150 In the subterranean environment too, the soft chirps of naked mole-rats encode individual
151 identity as well as colony identity and they are culturally transmitted as colony vocal dialects,
152 carrying information about group membership [22]. Although cavefish are supposed to be asocial,
153 they are capable of social-like interactions in familiar environments [23]. The individual and cave-
154 specific acoustic signatures, or accent, we have discovered may well participate in such sociality
155 when thriving in their natural caves.

156

157

158 **Materials and Methods**

159

160 **Fish samples**

161 Field recordings were obtained during three field expeditions in the states of San Luis Potosi and
162 Tamaulipas, Mexico, in March 2016 and March 2017, under the auspices of the field permit 02438/16
163 delivered by the Mexican Secretaria de Medio Ambiente y Recursos Naturales. We recorded from 6
164 caves hosting *Astyanax mexicanus* troglomorphic cavefish populations (map on Fig.1A).

165

166 **Sound recordings and analyses**

167 For natural field recordings, cavefish were directly recorded in their hosting pools in 6 different
168 natural caves, in the dark. This was done either from 10-12 fish maintained inside a large net (approx.

169 1m³ free water volume, Fig.1B) installed in their natural pool, or from freely swimming fish in the
170 case of small natural pools (Tinaja and Chica caves). Hydrophones (Aquarian audio H2a XLR,
171 Anacortes, WA, USA) were connected to portative pre-amplifiers (ART Dual Pre USB, NY 14305, USA)
172 and recorders (Zoom H4n, NY 11788, USA) with SD cards, and recording parameters were adjusted
173 with direct audio listening depending on environmental acoustic characteristics of each cave. They
174 were left on sites for overnight recordings, except in the Molino cave: there, the entrance being a 70
175 meters vertical pit, the cave could not be visited on two consecutive days and the recording was
176 limited to 1h30, during the afternoon. The four fish taped were nevertheless productive, as 50 clicks
177 and 50 serial clicks could be extracted from this short period for analysis. Natural soundscape in
178 Tinaja (heavy and continuous dripping limestone ceiling) prevented to exploit overnight recordings
179 and made us seek for a quiet pond with specimens on the second day, leading to shorter recording as
180 well (1h30). We therefore analyzed ≤100 sounds from the audio bands from other caves, to
181 equilibrate the comparisons and statistical analyses with the more limited Molino and Tinaja dataset.
182 Of note, for each cave the number of sounds analyzed (50) corresponded to minimum ten times the
183 number of variables studies, i.e., 5 variables for the serial clicks.

184 Sounds were extracted by ear from audio bands recordings. Each audio track was carefully
185 scrutinized manually from sonograms magnified at a 3-4 seconds temporal window and further at a
186 0.2 and 1 second bins (Fig.1D) allowing a visual control of each extracted sound motif while listening
187 from T0 to end, including tracks of 11h. Clicks and serial clicks were not extracted in an exhaustive
188 but in a systematical manner: only sounds previously described in the acoustic range of single click or
189 serial click produced by fish in the lab or in the wild were considered, and replicates of each
190 identified sound motif were randomly extracted. This allowed to increase the stringency and to
191 capture the diversity of clicks and serial clicks for a comprehensive view of each cave soundscape.

192 Sounds were digitized at 44.1 kHz (16-bit resolution) and analysed using fast Fourier transform (FFT)
193 with Avisoft SAS Lab Pro 5.2.07 software (Avisoft bioacoustics, Glienicke, Germany) [24]. The acoustic
194 structure of single clicks was analyzed using a set of:

195 -1 temporal parameter, the duration, measured from the envelop of the oscillogram,

196 -2 parameters measured from the oscillogram: RMS amplitude, and signal to noise ratio (SNR; [RMS
197 amplitude of the signal-RMS amplitude of noise]/RMS amplitude of noise),

198 -5 spectral parameters obtained from power spectra (FFT, window type: Hann, window size: 512;
199 time overlap: 90%) within a 0-22.5 kHz bandwidth. Spectral parameters were: 1) peak frequency of
200 the frequency spectrum (dominant frequency), 2) first quartile of energy (Q25), i.e. the frequency

201 value corresponding to 25% of the total energy spectrum, 3) second quartile of energy (Q50), 4) third
202 quartile of energy (Q75), and 5) interquartile, i.e. difference between Q75 and Q25.

203 Serial clicks were examined using 5 fine temporal parameters including sound duration, pulse
204 number, mean inter-pulse duration, mean pulse duration, and pulse rate (= sound duration/pulse
205 number) using personal routines developed with R package Seewave [25].

206 Pulses were considered “single” if they were of short duration (<20msec) and separated by >1sec
207 interval from the next pulse (threshold defined from the histogram of the inter-pulse durations).
208 After calculating the correlation coefficients between the variables, we excluded highly correlated
209 variables ($r > 0.65$ or < -0.65), retaining 3 uncorrelated variables for single clicks (duration, SNR,
210 dominant frequency) and 5 uncorrelated variables for serial clicks. A principal component analysis
211 (PCA; R package FactoMineR) was performed using the 3 variables retained for single clicks and the 5
212 variables retained for serial clicks, allowing to draw the centroids of the 6 caves, surrounded by their
213 95% confidence circles (Fig.2CD). A permutated discriminant function analysis (pDFA; R routine from
214 Bertucci et al. 2010 [24]) performed on the principal components axis of the previous PCA provided a
215 classification procedure that assigned each sound to its appropriate cave (correct assignment) or to
216 one of the others (incorrect assignment) (Fig. 2CD, insets).

217 Acoustic distances between caves plotted on a plan according to their actual GPS coordinates were
218 calculated using an agglomerative hierarchical clustering on principle components (Euclidean metric,
219 Ward method), for Single Clicks (Fig. 2E).

220 An unsupervised hierarchical clustering method was used on the Single Click Molino dataset and
221 displayed on the ACP plan to estimate a potential individual acoustic signature in this cave (Fig.2F).

222

223 **Statistics**

224 Normality was assessed with a Kolmogorov-Smirnov test. Kruskal-Wallis tests followed with Dunn’s
225 *post hoc* were performed on non-normally distributed data sets. In figures, box plots show the
226 distribution, median and extreme values (top and bottom whiskers) of samples. Statsoft Statistica 6,
227 GraphPad Prism 9 and R 3.1.3 [26] were used for statistical analyses and graphical representations.

228

229 **A Source data file** (Supplemental data Table 1) containing all the raw data presented and analyzed in
230 this paper is available as Supplemental Information.

231

232

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235

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240 Garcia for obtaining shared fieldwork permits.

241

242 **Competing Interests**

243

244 The authors declare no competing interests.

245

246 **References**

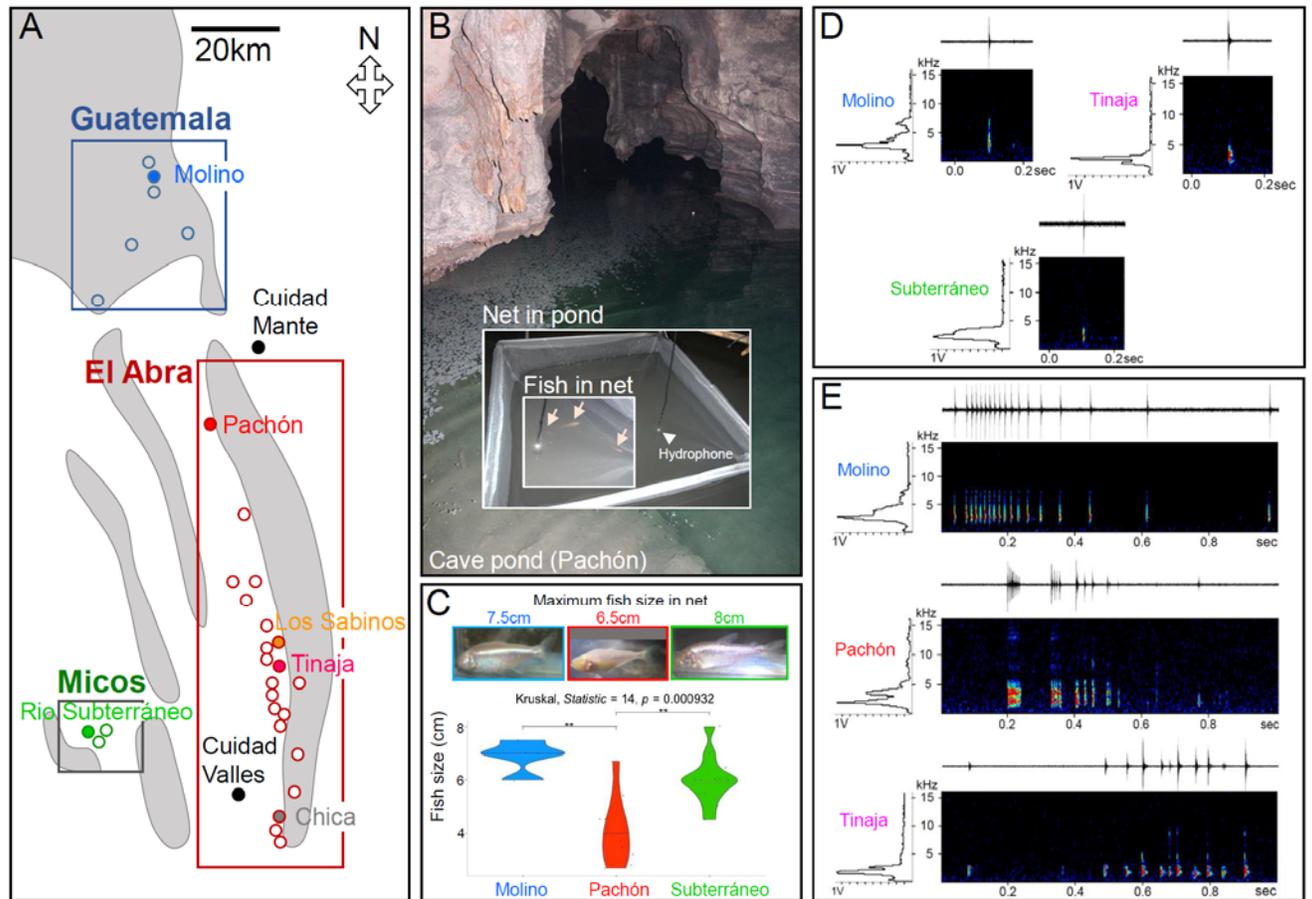
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309 **Legends and Figures**

310



311

312

313 **Figure 1:** Sampling sounds in *A. mexicanus* caves.

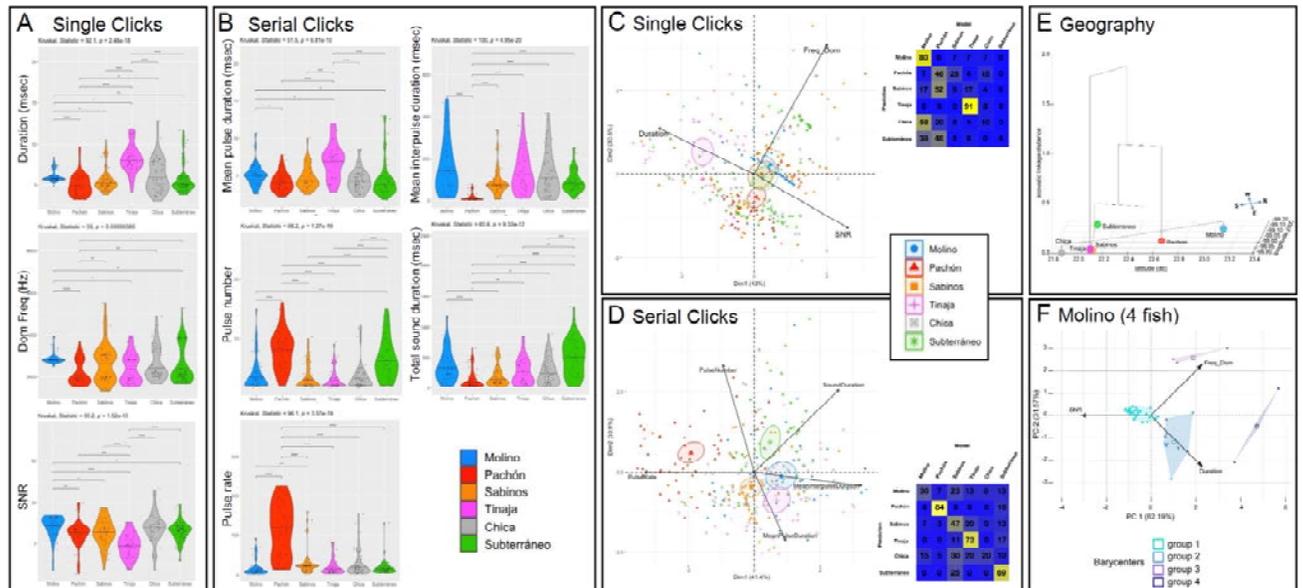
314 **A,** Map showing geographical localization of the 6 caves (color-coded) in 3 mountain ranges
315 (rectangles) where acoustic production was recorded.

316 **B,** Recording setup in natural settings.

317 **C,** Comparison of the sizes of the fish recorded in different caves, with photograph of the largest
318 individual present in the recording net.

319 **D, E,** Examples of sonograms of Clicks (D) and Serial Clicks (E) recorded in the wild, showing
320 substantial level of variations across caves.

321



322

323

324 **Figure 2:** Comparing acoustic parameters of Single Clicks and Serial Clicks in *A. mexicanus* caves.

325 **A-B,** Univariate analysis of acoustic parameters in different caves (color-coded).

326 **C-D,** Multivariate analysis of acoustic parameters using PCA, followed by pDFA (insets).

327 **E,** Hierarchical clustering of acoustic parameters of Single Clicks (height axis) projected onto a map of
 328 geographical coordinates of caves (NSWE in x and y-axes).

329 **F,** Unsupervised clustering on PCA analysis for Single Clicks in the Molino cave (4 fish, 4 groups).

330