

**Convergence on reduced aggression through shared behavioral traits in multiple populations
of *Astyanax mexicanus***

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1 **Summary Statement**

2 Comparison of aggression between surface fish and cavefish demonstrates that multiple
3 complex behaviors compose aggression in surface fish and reveals heterogeneity in loss of
4 aggression in cave populations.

5 **Abstract**

6 Aggression is a complex behavior that is observed across the animal kingdom, and plays roles in
7 resource acquisition, defense, and reproductive success. While there are many individual
8 differences in propensity to be aggressive within and between populations, the mechanisms
9 underlying differences in aggression between individuals in natural populations are not well
10 understood. We addressed this using the Mexican tetra, *Astyanax mexicanus*, a powerful model
11 organism to understand behavioral evolution. *A. mexicanus* exists in two forms: a river-dwelling
12 surface form and multiple populations of a blind cave form. We characterized aggression in
13 surface fish and cavefish in a resident/intruder assay through quantifying multiple behaviors
14 occurring during social interactions. Surface fish, which are aggressive, display multiple social
15 behaviors in this context, which we characterized into two types of behaviors: aggression-
16 associated and escape-associated behaviors. The majority of these behaviors were reduced or
17 lost in Pachón cavefish. Further, both aggression-associated and escape-associated behaviors
18 were not dependent on the presence of light, and both surface fish and cavefish remained
19 aggressive or non-aggressive, respectively, when opposed to fish from a different population.
20 Additionally, we found that within populations, levels of stress response were not correlated with
21 aggression- or escape-associated behaviors. Finally, when we compared aggression- and escape-
22 associated behaviors across four cavefish populations, we found that both types of behaviors are
23 reduced in three cave populations, while still present in one. Together, these results reveal that
24 multiple cavefish populations have repeatedly evolved reduced aggression through shared
25 behavioral components, while other cavefish have retained aggression.

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30 Introduction

31 Aggression is broadly defined as hostile behavior that creates harm or damage from one
32 individual to another individual [1]–[3]. Motivation for aggressive behaviors can stem from
33 multiple factors, including resource acquisition, establishment of hierarchies, survival and
34 reproductive success [4]–[7]. Further, extreme levels of aggression can be detrimental for
35 survival, suggesting aggression might be under stabilizing selection in some species [8] and
36 highlighting the adaptive importance of regulating levels of aggression. Teleost fish are excellent
37 models for studying aggression, as multiple species of fish display aggressive behaviors, including
38 *Betta splendens* (Siamese fighting fish), multiple species of cichlids, sticklebacks, and the
39 zebrafish *Danio rerio* [9]–[13]. While significant work in fishes has focused on the neural
40 underpinnings of aggressive behaviors [14]–[16], the mechanisms contributing to evolution of
41 aggressive behaviors are less well understood.

42 The Mexican tetra, *Astyanax mexicanus*, is a powerful emerging model for investigating
43 the evolution of social behaviors [17]. *A. mexicanus* is a single species of fish consisting of river-
44 dwelling, eyed and pigmented surface fish and at least 30 populations of blind, cavefish exhibiting
45 reduced pigmentation or albinism [18], [19] Cavefish populations have evolved a number of
46 behavioral differences relative to surface fish, including reduced sleep and schooling [20]–[22]
47 increased vibration-attraction behavior (VAB) for prey detection [23], [24], and reduced
48 aggression [25]–[28], providing a basis to investigate the ecological and genetic factors that
49 underly the evolution of complex behavior. Cavefish and surface fish are interfertile, allowing for
50 assessment of the genetic basis of behavioral evolution in this species through crosses and
51 genetic mapping approaches [17]. Further, at least some cavefish populations have evolved
52 independently of each other, providing the opportunity to examine whether cave-associated
53 traits have evolved repeatedly [29], [30].

54 Teleost fish from other species exhibit a number of behaviors during aggressive
55 encounters, including biting, striking, circling, following, escaping, freezing and avoidance [13],
56 [27], [31]. While some of these behaviors have been reported in surface fish populations [32],
57 previous work in *A. mexicanus* quantified aggression as a single metric, the number of attacks
58 between pairs of fish [25], [26]. Thus, whether reduced aggression in cavefish is characterized by

59 reduction in all or a subset of the multiple social behaviors that are exhibited during aggressive
60 encounters in surface fish, and if these behaviors are under independent genetic control and thus
61 evolve independently, is currently unknown.

62 To characterize the reductions in aggression that have evolved in cavefish, we quantify
63 traits comprising aggressive-associated and escape-associated behaviors during social
64 encounters of *A. mexicanus* surface fish and cavefish. Specifically, we asked: (1) What are the
65 differences in the social behaviors that compose aggressive encounters in surface fish and
66 cavefish, and are these behaviors dependent on sex, social context, or immediate environment?
67 (2) Are the behaviors that occur during aggressive encounters repeatedly lost in multiple,
68 independently evolved cave populations? Our findings position *A. mexicanus* as a powerful model
69 for addressing how natural genetic variation contributes to a complex suite of aggressive
70 behaviors, and how aggressive behaviors evolve.

71

72 **Materials and methods**

73 *Fish Husbandry*

74 All animal husbandry was performed according to methods previously described [33], [34]. All
75 protocols were approved by the IACUC of Florida Atlantic University. Fish were raised at
76 $23 \pm 1^\circ\text{C}$. Adult *A. mexicanus* were housed in groups on a circulating filtration system in 18–37-
77 liter tanks on a 14:10 hour light cycle that was constant through the animal's lifetime. All fish
78 used in this study were bred and raised in the laboratory. There were no statistical differences
79 between surface fish from Río Choy and Texas lineages, and both populations were used in this
80 study. Cavefish originated from the Pachon, Molino, Tinaja or Los Sabinos caves. All fish were 6
81 months – 1-year adults, which ranged from 3 to 6 cm in length.

82

83 *Resident-Intruder assay*

84 All fish assayed for aggression were fed one hour before behavioral acclimation and assayed
85 between zeitgeber time (ZT) ZT0-ZT6 Aggressive behaviors were quantified using a resident-
86 intruder assay, which was previously shown to induce aggressive behavior in *A. mexicanus* and
87 other vertebrates [31]. Pairs of resident and intruder fish from the same home tank were

88 transferred to individual 2.5 L plastic tanks and acclimated for 18 hours in a dedicated behavioral
89 room in which the light: dark cycle was maintained. All pairs of fish were sex- and size-matched.
90 Following acclimation, the intruder fish was transferred to the tank of the resident fish and their
91 interaction was recorded for 1 hour using a Microsoft Studio Webcam (#Q2F-00013). All
92 recordings were performed from the front of the tank. For recordings in darkness, fish were
93 acclimated and assayed in the dark. Infrared (IR) lights (850 nM) and cameras that could detect
94 IR light were used during the resident-intruder assay. All resident-intruder recordings were
95 acquired at 15 frames per second using VirtualDub2 (Version 1.10.5), an open-source video-
96 capture and processing utility developed for Microsoft Windows
97 (<https://www.virtualdub.org/features.html>).

98

99 *Novel tank assay*

100 The novel tank assay, a well-established assay for assessing stress-like behaviors in fish, was
101 performed on a subset of fish that were subsequently assayed for aggression in light versus dark
102 conditions. All adult fish were of similar size (3-6cm). Stress assays were performed between
103 Zeitgeber (ZT)6-ZT7 (ZT0=start of the light phase) as previously described [35], [36] with minor
104 modifications. Groups of fish were transferred from their home tanks on the fish system into
105 tanks in a dedicated behavioral room and allowed them to acclimate to the room for at least 1
106 hr. Next, each fish was transferred to a 500mL plastic holding tank for 10-minute acclimation,
107 followed by gentle transfer into a 2.5 L tank containing 2 L of conditioned fish system water. Once
108 transferred, fish were filmed in the light for 10-minutes using a Microsoft Studio Webcam (#Q2F-
109 00013). All stress recordings were acquired at 30 frames per second using VirtualDub2. After
110 behavioral recording, the fish were housed individually in their respective tanks for acclimation
111 in the resident-intruder assay.

112

113 *Manual Behavior Annotations*

114 We annotated all staged-fights using the Behavioral Observation Research Interactive Software
115 (BORIS) event-logging program [37]. For all annotations, we used the following ethogram based
116 on previous behaviors observed in *A. mexicanus* and other fish species, and our own observations

117 [13], [38], [39] (Table 1). Some behaviors were scored as single events in time (point events =
118 biting, striking, circling) or continuous behavioral events (state events = following, escaping,
119 freezing, avoidance). Individual fish behavior was scored throughout the video to distinguish
120 between resident and intruder fish.

121

122 *Data Analysis*

123

124 *Manual Annotation in BORIS for Aggression*

125 All data was exported from BORIS as activity plots and time budgets for quantification as text files
126 (*.txt) [37]. Time budgets represented the number of events during which a given behavior
127 happened and the duration of such events, if applicable. For each time budget, the number of
128 times each behavior happened was recorded for all behaviors, while the total duration (in
129 seconds) was recorded only for the behaviors that had a time component (following, escaping,
130 freezing and avoidance).

131

132 *Automated Tracking for Stress*

133 The center position of each fish was tracked using automated tracking with Ethovision software, and x-y
134 displacement was calculated across all frames from the 10-minute recordings following previously
135 published protocols using Ethovision XT13 (version 13.0, Noldus, Inc., Leesburg, VA) [40], [41]. To quantify
136 bottom-dwelling for each fish, the arena was divided into three equal sections in Ethovision and the total
137 duration of time spent in the bottom third of the arena was calculated. Ethovision accurately tracked the
138 position of the fish using background subtraction.

139

140 Quantifications of all behaviors can be found in the supplementary materials.

141

142 *Statistical Analysis*

143 We imported all data extracted from BORIS to GraphPad Prism 9. All data was tested for
144 normality using Shapiro-Wilk test and parametric (t-tests for 2 group comparisons and One-Way-
145 ANOVA for multiple group comparisons of a single variable) or non-parametric (Mann-Whitney
146 for 2 group comparisons and Kruskal Wallis for multiple group comparisons) tests were used

147 when appropriate. When analyzing more than one variable, such as the case when comparing
148 the variation between light and dark conditions in surface fish versus cavefish populations, we
149 used 2-Way-ANOVAs or Kruskal Wallis. Data was considered statistically significant if $p < 0.05$ (*),
150 $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

151 We used the Spearman's rank-order correlation test to measure the association between all
152 aggressive behaviors annotated with stress, and we calculated the rho (r_s) for each correlation.

153

154 Outputs from statistical tests can be found in the supplementary materials.

155

156 **Results**

157

158 *Aggression-associated Behaviors Are Observed in Surface Fish and Are Reduced in Pachón* 159 *Cavefish*

160 To characterize the behavioral repertoire that composes aggressive interactions in *A.*
161 *mexicanus*, we performed a resident/intruder assay in surface fish ($n = 10$ pairs) and Pachón
162 cavefish ($n = 11$) and annotated multiple behaviors displayed during aggressive interactions (Fig
163 1). Surface fish are highly aggressive, and pairs of fish exhibit a number of aggressive interactions
164 throughout the course of the behavioral trial [25], [31], [42]. Surface fish display a number of
165 behaviors observed in other fish species during aggressive interactions, including biting, striking,
166 circling, and following (Fig 1A, B). While behaviors like fin fanning have been observed in other
167 fish species, like in the Siamese fighting fish [43], we did not observe instances of these behaviors
168 in *A. mexicanus* surface fish. In addition to these aggressive behaviors, fish exhibited a number
169 of behaviors typically associated with subordinate/defeated status [13], [33], [44], including
170 escaping, freezing and avoidance (Fig 1A, B). We quantified these behaviors in pairs surface fish
171 and Pachón cavefish assayed in the resident/intruder assay. While Pachón cavefish exhibit some
172 behaviors associated with both aggression and escape, most of these were significantly reduced
173 in Pachón cavefish compared to surface fish. Pachón cavefish perform fewer aggression-
174 associated behaviors compared to surface fish, including biting ($p < 0.05$), striking ($p < 0.0001$), and
175 following ($p < 0.05$) (Fig 1B-D). While surface fish also exhibit escape-associated behaviors, many

176 of these were either reduced or absent in Pachón cavefish, including escaping ($p < 0.0001$),
177 freezing ($p < 0.001$) and avoidance ($p = 0.01$) (Fig 1F-H). Interestingly, both surface fish and Pachón
178 cavefish exhibited circling behavior, and Pachón cavefish performed significantly more circling
179 than surface fish ($p < 0.01$) (Fig 1E), suggesting circling could be an aggression-associated behavior
180 conserved in Pachón cavefish, or a social behavior serving another purpose in one or both
181 populations of *A. mexicanus*. To assess whether sex played a role in the number of aggression-
182 or escape associated behaviors observed in this assay, we performed a 2 way-ANOVA and found
183 no significant effect of sex on aggression- or escape-associated behaviors, and no significant
184 interaction between sex and population for any behavior, except for avoidance, where surface
185 fish males were performing more avoidance than females (Fig S1, Supplementary Data sheets
186 1&3). Together, this suggests that reduced aggression in Pachón cavefish is characterized by
187 reductions in a number of aggression-associated behaviors observed in surface fish.

188

189 Next, we asked whether aggression-associated behaviors were similar in quantity in both
190 fish in each assay, or whether there was an asymmetry in how fish behaved, with one fish
191 consistently acting as the aggressor and the other fish consistently escaping. As we tracked
192 individual fish during our behavioral annotations, we examined whether there were quantitative
193 differences in behaviors associated with resident/intruder status. We found no significant effects
194 of resident/intruder status on any aggression- or escape-associated behaviors, or statistically
195 significant interactions between resident/intruder status and population (Fig S2, Supplementary
196 Data sheets 1&3). Next, we assessed whether within these assays, we could quantify behavioral
197 symmetry between both contenders in each fight, regardless of resident/intruder status. To do
198 so, we designated the fish in each pair that exhibited more strikes that aggressor, and the other
199 fish the non-aggressor. When we compared aggression-associated and escape-associated
200 behaviors for the aggressor versus non-aggressor in surface fish, we found there is a significant
201 asymmetry in most aggression- and escape-associated behaviors in surface fish, with the
202 aggressor performing significantly more biting, striking and following than the non-aggressor,
203 and the non-aggressor performing significantly more escaping and avoidance than the aggressor
204 (Fig S3). This pattern was not present in Pachón cavefish, consistent with the reduced aggression

205 observed in fish from this population (Fig. S3). Together, these data suggest that, within pairs of
206 surface fish, one fish is quantitatively more aggressive, and that this asymmetry is not observed
207 in cavefish which have evolved reduced aggression.

208

209 *Aggressive Behaviors in Surface Fish and Pachón Cavefish Remain Constant under Light or Dark* 210 *Conditions*

211 Some social behaviors in surface fish, including schooling and shoaling, are reduced or absent
212 when visual cues are not available [21]. To determine if this is the case for the aggression- and
213 escape-associated behaviors quantified here, we performed resident/intruder assays under both
214 light and dark conditions. Surface fish and Pachón cavefish exhibited similar behavior under light
215 and dark conditions for the majority of the behaviors quantified (Fig 2). However, both surface
216 fish and Pachón cavefish performed less circling in the dark relative to in the light (SF = 27.51 vs.
217 5.432, $p < 0.01$, Pa = 41.19 vs. 19.11, $p < 0.01$), suggesting that there is an effect of light dependency
218 for at least one of these social behaviors (Fig 2F). Taken together, our data suggests that cavefish
219 did not lose aggression simply due to the loss of the ability to receive visual cues to induce this
220 behavior.

221

222 *Surface fish Demonstrate Inter-population Aggression Towards Pachón Cavefish*

223 It is possible that cavefish do not exhibit aggressive or escape-associated behaviors when they
224 are interacting with other cavefish, but that these behaviors are inducible in the presence of
225 another individual that exhibits them. To examine this possibility, we quantified behavior
226 between inter-population pairs of fish in the resident/intruder assay under two conditions: (1)
227 Surface fish-resident vs. Pachón-intruder ($n = 8$ pairs/each), and (2) Pachón-resident vs. Surface
228 fish-intruder ($n = 8$ pairs/each). Surface fish exhibited aggression-associated behaviors when
229 paired with a Pachón cavefish opponent (Fig. 3A-B), suggesting aggression is not associated with
230 the identity the contender. These interactions induced one escape-associated behavior in
231 Pachón cavefish, escaping (Fig. 3F). When surface fish were residents, they performed more
232 striking and following than Pachón cavefish intruders, but this difference did not reach statistical
233 significance (Fig. 3D, E). When Pachón cavefish were the residents, by contrast, most of the

234 behavioral differences between resident and intruder observed were significant, with surface fish
235 biting ($p < 0.01$, Fig.3C), striking ($p < 0.01$, Fig.3D) and following ($p < 0.001$, Fig.3E) more than
236 Pachón cavefish residents, and escaping less ($p < 0.01$, Fig.3F). Taken together, this suggests that
237 surface fish remain aggressive when opposed to cavefish, becoming even more aggressive when
238 introduced as the intruders. Although Pachón cavefish do not become aggressive when opposed
239 to a surface fish opponent, their interaction with surface fish induced escape-like responses,
240 reminiscent of the profile of less-aggressive fish during surface fish contests (Fig S3).

241

242 *Stress is Unrelated to Aggressive Displays in Surface Fish*

243 Previous work suggested stress is an influencing factor on the onset of aggression [45]–[47]. To
244 test this, we subjected surface fish and Pachón cavefish to an assay that has been used to quantify
245 stress-related behaviors in multiple fish species [48]–[51], the novel tank assay [35], [52], prior
246 to the resident/intruder assay acclimation for the comparisons of aggression in the light and the
247 dark (Fig 2). As a proxy for stress, we measured the amount of time spent bottom-dwelling upon
248 introduction to a novel environment, which was previously reported as a behavior exhibited
249 when fish are stressed [53]. As reported previously, surface fish spend significantly more time at
250 the bottom of the tank relative to cavefish (Fig S4). These observations confirmed previous
251 findings that suggest surface fish are inherently more prone to stress than cavefish [41]. To
252 examine whether some individuals within each of these populations exhibited more aggression-
253 associated behaviors because they were more stressed, we compared the amount of time spent
254 bottom-dwelling in the novel tank assay with the number of the aggression- or escape-associated
255 behaviors we observed in fish in the light. We found no significant correlations between bottom
256 dwelling and of the aggression- or escape-associated behaviors in either surface fish or in cavefish
257 (Fig.4 and Fig.S5). Taken together, aggression appears to be unrelated to the stress profile within
258 parental populations of fish, which suggests that differences in stress between cavefish and
259 surface fish do not drive the differences in aggression observed.

260

261 *Reduced Aggression is observed in cavefish from independently evolved cave populations*

262 Populations of organisms that evolve under similar conditions often repeatedly evolve the same
263 traits. *A. mexicanus* cavefish provide a powerful opportunity for studying repeated evolution, as
264 multiple cavefish populations exist that have independently evolved a number of traits [17], [30],
265 [54]. After assessing the repertoire of aggressive-like and escape-like behaviors which were
266 present in surface fish and absent in Pachón cavefish, we asked if other cavefish populations have
267 evolved reductions in aggression through modulation of the same aggression-associated
268 behaviors. We quantified aggression in fish from four cavefish populations: Pachón (n = 5 pairs),
269 Tinaja (n = 9 pairs) and Los Sabinos (n = 7 pairs) cavefish from the Sierra del Abra, and Molino
270 cavefish (n = 12 pairs) from the Sierra de Guatemala. We found that Tinaja and Los Sabinos
271 cavefish exhibited reduced or no instances of most aggression-associated and escape-associated
272 behaviors, similar to the patterns found in Pachón cavefish (Fig.5). However, Molino cavefish
273 exhibited a different set of behaviors compared to fish from these three cavefish populations.
274 Specifically, Molino cavefish displayed more striking and more escaping than Pachón cavefish (Fig
275 5). Further, the increase in circling behavior we observed in Pachón cavefish relative to surface
276 fish (Fig 1E), was not present in other cavefish populations (Fig 5A, E). These results suggest that
277 reduced aggression has evolved in multiple, although not all, cavefish populations through
278 reductions in multiple aggression-associated behaviors, and that some cave environments might
279 favor the conservation of aggression-associated and escape-associated behaviors.

280

281 Discussion

282 *Astyanax mexicanus* offers an opportunity to interrogate how complex behaviors, like
283 aggression, evolve in closely related populations of fish. We took advantage of this and tested
284 four blind cave populations (Pachón, Tinaja, Los Sabinos and Molino), which have evolved many
285 traits independently, as well as sighted surface fish to probe for differences in aggression.

286 External factors from the environment can play a role in levels of aggression exhibited by
287 individuals [55], [56]. Here, we examined whether morphological adaptations in cavefish,
288 specifically loss of eyes and vision [26], were contributing to these differences in behavior. In
289 other fish species, like the Coho Salmon, aggression is reduced in the dark, while in juvenile
290 Atlantic Salmons serial reductions in light intensity decreased aggression [57], [58]. In *A.*

291 *mexicanus*, there is some degree of controversy regarding aggression in the dark, as some studies
292 report reduced aggression in surface fish in the dark [27], whereas others found that vision was
293 dispensable for aggression in sighted surface fish [25], [26], and that surface fish raised following
294 a lensectomy early in development are highly aggressive [26]. Our findings were in line with this
295 latter work and further expanded this to demonstrate that multiple aggression-associated
296 behaviors are observed under dark conditions. These differences in findings may be due to
297 differences in the type of assay conditions [25], as well as the behaviors scored, and underscore
298 the importance of quantifying a robust number of aggression-associated behaviors in this
299 species.

300 Circling behavior has been associated with aggression in zebrafish [13], [59], sound-
301 producing piranhas [60] and in *A. mexicanus* [31]. Our results were intriguing in the sense that
302 Pachón cavefish perform fewer of all aggression- and escape-associated behaviors, except for
303 circling. When compared with other cave populations, including Molino cavefish which exhibit
304 aggression-associated behaviors, we found that increased circling is unique to Pachón cavefish.
305 This behavior may not necessarily be aggression-associated, but instead serve a different
306 purpose. For example, previous reports have found that Pachón cavefish perform stereotypic
307 repetitive circling and that this behavior decreases under conditions that increase social
308 interactions [61]. Thus, the circling behavior observed here in Pachón cavefish may not be used
309 for social purposes. Alternatively, circling could be a social behavior in these fishes that is not
310 associated with aggression.

311 Another question was if the presence of a fish from a different population triggered or
312 suppressed aggression- and escape-associated behaviors. We tested this by adapting the
313 resident/intruder assay for inter-population fights of surface fish and Pachón cavefish. These
314 experiments led to two main findings: (1) surface fish were more aggressive than Pachón cavefish
315 in intra-population assays, and (2) these interactions induced escape-associated behaviors in
316 Pachón cavefish. While surface fish were overall more aggressive than Pachón cavefish
317 whether they were residents or intruders, these differences between surface fish and cavefish
318 were larger when surface fish were intruders in the assay. This might be due to the existence of
319 territoriality in surface fish, which has been proposed before [25], [27], [31], [62], and could result

320 from surface fish intruders seeking to establish a territory. This finding was in contrast to our
321 resident/intruder analysis of intra-population fights (surface versus surface, cave versus cave),
322 where the fish's designated role was not associated with whether the fish was ultimately the
323 more aggressive or less aggressive fish in the assay. Hence, resident/intruder status does matter
324 in this species, but this status appears to matter more when one fish is opposed to a fish from a
325 different population. Our second finding here suggests while Pachón cavefish do not perform
326 aggression- and escape-associated behaviors when interacting with other cavefish, they retain
327 strategies to escape from an aggressive fish. Thus, aggression-associated behaviors may have
328 been lost in Pachón cavefish while their escape-associated responses are still inducible. Evidence
329 of cannibalism has been reported for *A. mexicanus* cavefish from the Micos cave [63]; thus,
330 escape-like behaviors may be critical for survival in at least some cave environments.

331 Previous work introduced the notion that aggression and stress might be co-dependent
332 behaviors in some fish species, or that at the very least, one of these behaviors could modulate
333 the other [47], [64]. For example, in zebrafish, unpredictable chronic stress (UCS), as well as
334 increases in stress-associated cortisol levels, increased aggression in male fish [64]. In *A.*
335 *mexicanus*, stress-associated behaviors are reduced in the multiple populations of cavefish,
336 including Pachón, Tinaja and Molino, relative to surface fish [36]. Further, intra-population
337 differences in stress-levels, defined here as behavioral response to stress, were not correlated
338 with levels of aggression- or escape-like behaviors in either cavefish or in surface fish, which
339 suggests that, within *A. mexicanus* populations, individual differences in stress do not predict
340 levels of aggression. Whether evolved differences in response to a stressful environment
341 between populations is related to the evolution of reduced aggression in cavefish of this species
342 remains to be determined.

343 Loss of aggression is observed in other animals that have evolved to live in cave
344 environments, including other cavefish [65] and other cave species, like the whip spider *Phrynos*
345 *longipes* [66]. To determine if and how aggression-associated behaviors have evolved across
346 closely related cave populations, we examined whether repeated loss of aggression-associated
347 behaviors has evolved in multiple cave populations of *A. mexicanus*. Studies in *A. mexicanus* using
348 microsatellite and mitochondrial DNA suggest that cave populations are derived from at least

349 two colonization events [67]–[70]. Surface fish of the “old stock” inhabited the Sierra de El Abra
350 region and gave rise to the “old stock” of cavefish, including Pachón, Los Sabinos, Tinaja and
351 others [71]. A different wave of surface fish gave rise to the present surface fish in the region and
352 the “new stock” of cavefish, including Molino and Escondido [71]. Genetic studies suggest that
353 many traits have evolved repeatedly in these different cave populations, whether they derive
354 from these different colonization events, or even between cave populations from the El Abra
355 caves. These traits including genetically encoded morphological traits such as the size of the eye
356 primordia [72], [73], and behavioral traits, including foraging behaviors [74].

357 Recent work suggested Molino cavefish were not aggressive, and behaved similarly to
358 Pachón cavefish, differing only in their patterns of attacks [25]. We observed that Molino fish
359 show increases in at least one aggression-associated behavior relative to Pachón cavefish, which
360 is in line with a previous study that found that Molino cavefish are aggressive [26]. This result
361 could mean the ecological environment of the Molino cave favors the conservation of aggression-
362 and escape-associated behaviors. Ultimately, these findings pose several new questions: (1) is
363 “cavefish aggression” unique to Molino, or have other *A. mexicanus* cavefish conserved these
364 behaviors? (2) Are these conserved aggressive behaviors specific to the cavefish derived from
365 this colonization, or are other, currently untested cavefish populations from the Sierra de El Abra
366 aggressive? (3) Do the same genes underlie reduced aggression in the Pachón, Tinaja and Los
367 Sabinos populations? Sampling fish from more caves will provide answers to some of these
368 questions. Ultimately, identifying and functionally interrogating the genes that are contributing
369 to the loss of aggression in *A. mexicanus* will provide additional insight into the genetic factors
370 contributing to natural variation in aggression in this species. Methods such as QTL analysis and
371 functional interrogation of candidate genes using CRISPR-Cas9 that are available in this species
372 could be used in the future to answer these questions [75]–[78]. Thus, this work provides a
373 platform for investigating the extent to which heredity and/or environmental pressures inform
374 the evolution of aggression across closely related populations in a same species.

References

- [1] J. Archer, "The nature of human aggression," *Int. J. Law Psychiatry*, vol. 32, no. 4, pp. 202–208, 2009, doi: 10.1016/j.ijlp.2009.04.001.
- [2] J. Van Honk, E. Harmon-Jones, B. E. Morgan, and D. J. L. G. Schutter, "Socially explosive minds: The triple imbalance hypothesis of reactive aggression," *J. Pers.*, vol. 78, no. 1, pp. 67–94, 2010, doi: 10.1111/j.1467-6494.2009.00609.x.
- [3] R. M. M. de Almeida, J. C. C. Cabral, and R. Narvaes, "Behavioural, hormonal and neurobiological mechanisms of aggressive behaviour in human and nonhuman primates," *Physiol. Behav.*, vol. 143, pp. 121–135, 2015, doi: 10.1016/j.physbeh.2015.02.053.
- [4] A. Takahashi and K. A. Miczek, "Neurogenetics of Aggressive Behavior – Studies in Rodents," *Curr. Top. Behav. Neurosci.*, vol. 17, p. 3, 2014, doi: 10.1007/7854_2013_263.
- [5] E. Bath, E. R. Biscocho, A. Easton-Calabria, and S. Wigby, "Temporal and genetic variation in female aggression after mating," *PLoS One*, vol. 15, no. 4, Apr. 2020, doi: 10.1371/JOURNAL.PONE.0229633.
- [6] K. E. Holekamp, L. Smale, and M. Szykman, "Rank and reproduction in the female spotted hyaena," *J. Reprod. Fertil.*, vol. 108, no. 2, pp. 229–237, 1996, doi: 10.1530/JRF.0.1080229.
- [7] P. Stockley and J. Bro-Jørgensen, "Female competition and its evolutionary consequences in mammals," *Biol. Rev.*, vol. 86, no. 2, pp. 341–366, May 2011, doi: 10.1111/J.1469-185X.2010.00149.X.
- [8] R. R. H. Anholt and T. F. C. MacKay, "Genetics of aggression," *Annu. Rev. Genet.*, vol. 46, no. August, pp. 145–164, 2012, doi: 10.1146/annurev-genet-110711-155514.
- [9] P. H. Klopfer, "Aggression and avoidance by *Betta splendens* toward natural and artificial stimuli," 1975.
- [10] V. Nijman and B. A. Heuts, "Aggression and dominance in cichlids in resident-intruder tests: the role of environmental enrichment," *Neotrop. Ichthyol.*, vol. 9, no. 3, pp. 543–545, Sep. 2011, doi: 10.1590/S1679-62252011005000031.
- [11] T. C. M. Bakker, "Aggressiveness in Sticklebacks (*Gasterosteus Aculeatus* L.): a Behaviour-Genetic Study," *Behaviour*, vol. 98, no. 1–4, pp. 1–144, Jan. 1986, doi: 10.1163/156853986X00937.
- [12] L. Yong, B. Lee, and J. S. Mckinnon, "Variation in female aggression in 2 three-spined stickleback populations with female throat and spine coloration," *Curr. Zool.*, vol. 64, no. 3, pp. 345–350, Jun. 2018, doi: 10.1093/CZ/ZOY020.
- [13] K. N. Zabegalov *et al.*, "Understanding zebrafish aggressive behavior," *Behav. Processes*, vol. 158, no. April 2018, pp. 200–210, 2019, doi: 10.1016/j.beproc.2018.11.010.
- [14] M. F. Scaia, I. Akinrinade, G. Petri, and R. F. Oliveira, "Sex Differences in Aggression Are Paralleled by Differential Activation of the Brain Social Decision-Making Network in Zebrafish," *Front. Behav. Neurosci.*, vol. 16, p. 5, Feb. 2022, doi: 10.3389/FNBEH.2022.784835/BIBTEX.
- [15] J. M. Butler, S. M. Whitlow, D. A. Roberts, and K. P. Maruska, "Neural and behavioural correlates of repeated social defeat," *Sci. Reports 2018 81*, vol. 8, no. 1, pp. 1–13, May 2018, doi: 10.1038/s41598-018-25160-x.

- [16] A. L. Filby, G. C. Paull, T. F. A. Hickmore, and C. R. Tyler, "Unravelling the neurophysiological basis of aggression in a fish model," *BMC Genomics*, vol. 11, no. 1, p. 498, Sep. 2010, doi: 10.1186/1471-2164-11-498.
- [17] J. Kowalko, "Utilizing the blind cavefish *Astyanax mexicanus* to understand the genetic basis of behavioral evolution," *J. Exp. Biol.*, vol. 223, Feb. 2020, doi: 10.1242/JEB.208835.
- [18] W. R. Jeffery, "Astyanax surface and cave fish morphs," *Evodevo*, vol. 11, no. 1, Jul. 2020, doi: 10.1186/S13227-020-00159-6.
- [19] "Mexican eyeless Characin fishes, genus *Astyanax*: environment, distribution, and evolution." <https://digital.lib.usf.edu//SFS0052637/00001/pdf> (accessed Aug. 19, 2021).
- [20] A. K. R. B. ER Duboué, "Evolutionary convergence on sleep loss in cavefish populations," *Curr Biol*, vol. 21, no. 8, pp. 671–676, Apr. 2011, doi: 10.1016/j.cub.2011.03.020.
- [21] J. E. Kowalko *et al.*, "Loss of Schooling Behavior in Cavefish through Sight-Dependent and Sight-Independent Mechanisms," *Curr. Biol.*, vol. 23, no. 19, pp. 1874–1883, Oct. 2013, doi: 10.1016/J.CUB.2013.07.056.
- [22] A. Patch *et al.*, "Kinematic analysis of social interactions deconstructs the evolved loss of schooling behavior in cavefish," *PLoS One*, vol. 17, no. 4, p. e0265894, Apr. 2022, doi: 10.1371/JOURNAL.PONE.0265894.
- [23] J. PARZEFALL, "Field observations in epigeal and cave populations of the mexican characid *Astyanax mexicanus* (Pisces, Characidae)," *Mémoires de biospéologie*, vol. 10, pp. 171–176, 1983.
- [24] M. Yoshizawa, Š. Gorčiki, D. Soares, and W. R. Jeffery, "Evolution of a behavioral shift mediated by superficial neuromasts helps cavefish find food in darkness," *Curr. Biol.*, vol. 20, no. 18, p. 1631, 2010, doi: 10.1016/J.CUB.2010.07.017.
- [25] Y. Elipot, H. Hinaux, J. Callebert, and S. Rétaux, "Evolutionary shift from fighting to foraging in blind cavefish through changes in the serotonin network," *Curr. Biol.*, vol. 23, no. 1, pp. 1–10, 2013, doi: 10.1016/j.cub.2012.10.044.
- [26] L. Espinasa, Y. Yamamoto, and W. R. Jeffery, "Non-optical releasers for aggressive behavior in blind and blinded *Astyanax* (Teleostei, Characidae)," *Behav. Processes*, vol. 70, no. 2, pp. 144–148, Sep. 2005, doi: 10.1016/J.BEPROC.2005.06.003.
- [27] B. H. D. A. and P. J., "Aggressive behaviour of an epigeal population of *Astyanax mexicanus* (Characidae, Pisces) and some observations of three subterranean populations," *Behav. Processes*, vol. 11, no. 3, pp. 225–235, 1985, doi: 10.1016/0376-6357(85)90017-8.
- [28] J. Parzefall and C. Hausberg, "Ontogeny of the aggressive behaviour in epigeal and hypogean populations of *Astyanax fasciatus* (Characidae, Teleostei) and their hybrids," *undefined*, 2001.
- [29] G. JB, "The complex origin of *Astyanax* cavefish," *BMC Evol. Biol.*, vol. 12, no. 1, 2012, doi: 10.1186/1471-2148-12-105.
- [30] A. Herman *et al.*, "The role of gene flow in rapid and repeated evolution of cave related traits in Mexican tetra, *Astyanax mexicanus*," *Mol. Ecol.*, vol. 27, no. 22, p. 4397, Nov. 2018, doi: 10.1111/MEC.14877.
- [31] H. Hinaux, S. Rétaux, and Y. Elipot, "Social Behavior and Aggressiveness in *Astyanax*," *Biol. Evol.*

- Mex. Cavefish*, no. November, pp. 335–359, 2016, doi: 10.1016/B978-0-12-802148-4.00017-7.
- [32] “Ontogeny of the aggressive behaviour in epigeal and hypogeal populations of *Astyanax fasciatus* (Characidae, Teleostei) and their hybrids | Semantic Scholar.” <https://www.semanticscholar.org/paper/Ontogeny-of-the-aggressive-behaviour-in-epigeal-and-Parzefall-Hausberg/fe56ed44e0ba778d5e3256fb74389d2a1748ec73> (accessed Mar. 18, 2022).
- [33] B. A. Stahl, J. B. Jaggard, J. S. R. Chin, J. E. Kowalko, A. C. Keene, and E. R. Duboué, “Manipulation of Gene Function in Mexican Cavefish,” *JoVE (Journal Vis. Exp.)*, vol. 2019, no. 146, p. e59093, Apr. 2019, doi: 10.3791/59093.
- [34] R. Borowsky, “Handling *Astyanax mexicanus* Eggs and Fry,” *Cold Spring Harb. Protoc.*, vol. 2008, no. 11, p. pdb.prot5093, Nov. 2008, doi: 10.1101/PDB.PROT5093.
- [35] J. Cachat *et al.*, “Measuring behavioral and endocrine responses to novelty stress in adult zebrafish,” *Nat. Protoc.* 2010 511, vol. 5, no. 11, pp. 1786–1799, Oct. 2010, doi: 10.1038/nprot.2010.140.
- [36] J. S. R. Chin *et al.*, “Convergence on reduced stress behavior in the Mexican blind cavefish,” *Dev. Biol.*, vol. 441, no. 2, pp. 319–327, Sep. 2018, doi: 10.1016/J.YDBIO.2018.05.009.
- [37] O. Friard and M. Gamba, “BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations,” *Methods Ecol. Evol.*, vol. 7, no. 11, pp. 1325–1330, 2016, doi: 10.1111/2041-210X.12584.
- [38] G. P. Way, N. Ruhl, J. L. Sneker, A. L. Kiesel, and S. P. McRobert, “A comparison of methodologies to test aggression in zebrafish,” *Zebrafish*, vol. 12, no. 2, pp. 144–151, 2015, doi: 10.1089/zeb.2014.1025.
- [39] H. Wilkens, “Evolution and Genetics of Epigeal and Cave *Astyanax fasciatus* (Characidae, Pisces),” *Evol. Biol.*, pp. 271–367, 1988, doi: 10.1007/978-1-4613-1043-3_8.
- [40] N. LP, S. AJ, and T. RA, “EthoVision: a versatile video tracking system for automation of behavioral experiments,” *Behav. Res. Methods. Instrum. Comput.*, vol. 33, no. 3, pp. 398–414, 2001, doi: 10.3758/BF03195394.
- [41] J. S. R. Chin *et al.*, “Convergence on reduced stress behavior in the Mexican blind cavefish,” *Dev. Biol.*, vol. 441, no. 2, p. 319, Sep. 2018, doi: 10.1016/J.YDBIO.2018.05.009.
- [42] L. TG, N. B, H. C, and P. J, “Evolution of the optical releasers for aggressive behavior in cave-dwelling *Astyanax fasciatus* (Teleostei, Characidae),” *Behav. Processes*, vol. 34, no. 2, pp. 161–167, 1995, doi: 10.1016/0376-6357(94)00063-M.
- [43] D. Romano, G. Benelli, E. Donati, D. Remorini, A. Canale, and C. Stefanini, “Multiple cues produced by a robotic fish modulate aggressive behaviour in Siamese fighting fishes,” *Sci. Rep.*, vol. 7, no. 1, Dec. 2017, doi: 10.1038/S41598-017-04840-0.
- [44] R. F. Oliveira, J. M. Simes, M. C. Teles, C. R. Oliveira, J. D. Becker, and J. S. Lopes, “Assessment of fight outcome is needed to activate socially driven transcriptional changes in the zebrafish brain,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 113, no. 5, pp. E654–E661, 2016, doi: 10.1073/pnas.1514292113.

- [45] “Gender differences in aggression and cortisol levels in zebrafish subjected to unpredictable chronic stress | Elsevier Enhanced Reader.” <https://reader.elsevier.com/reader/sd/pii/S0031938416305698?token=2B61B6A94BE2A000CE8C6EA87519ECE281FE789C54A150852D171C2A70109CC6C883C231B06C945DC8AA49B1F9567B1D&originRegion=us-east-1&originCreation=20210819161036> (accessed Aug. 19, 2021).
- [46] C. H. Summers and S. Winberg, “Interactions between the neural regulation of stress and aggression,” *J. Exp. Biol.*, vol. 209, no. 23, pp. 4581–4589, Dec. 2006, doi: 10.1242/JEB.02565.
- [47] E. RL *et al.*, “Social interactions tune aggression and stress responsiveness in a territorial cichlid fish (*Archocentrus nigrofasciatus*),” *Physiol. Behav.*, vol. 88, no. 4–5, pp. 353–363, Jul. 2006, doi: 10.1016/J.PHYSBEH.2006.04.002.
- [48] G. Audira, B. P. Sampurna, S. Juniardi, S. T. Liang, Y. H. Lai, and C. Der Hsiao, “A Versatile Setup for Measuring Multiple Behavior Endpoints in Zebrafish,” *Invent. 2018, Vol. 3, Page 75*, vol. 3, no. 4, p. 75, Nov. 2018, doi: 10.3390/INVENTIONS3040075.
- [49] “Measures of Anxiety in Zebrafish (*Danio rerio*): Dissociation of Black/White Preference and Novel Tank Test.” <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3355173/> (accessed Mar. 18, 2022).
- [50] R. E. Blaser and D. B. Rosemberg, “Measures of Anxiety in Zebrafish (*Danio rerio*): Dissociation of Black/White Preference and Novel Tank Test,” *PLoS One*, vol. 7, no. 5, p. 36931, May 2012, doi: 10.1371/JOURNAL.PONE.0036931.
- [51] G. Audira *et al.*, “Interspecies Behavioral Variability of Medaka Fish Assessed by Comparative Phenomics,” *Int. J. Mol. Sci.*, vol. 22, no. 11, Jun. 2021, doi: 10.3390/IJMS22115686.
- [52] E. D. Levin, Z. Bencan, and D. T. Cerutti, “Anxiolytic effects of nicotine in zebrafish,” *Physiol. Behav.*, vol. 90, no. 1, pp. 54–58, Jan. 2007, doi: 10.1016/J.PHYSBEH.2006.08.026.
- [53] “Novel Tank Diving Assay: A Method to Study Innate Stress Responses in Zebrafish | Protocol.” <https://www.jove.com/v/20178/novel-tank-diving-assay-method-to-study-innate-stress-responses> (accessed Oct. 04, 2021).
- [54] M. Bradic, H. Teotónio, and R. L. Borowsky, “The population genomics of repeated evolution in the blind cavefish *astyanax mexicanus*,” *Mol. Biol. Evol.*, vol. 30, no. 11, pp. 2383–2400, Nov. 2013, doi: 10.1093/MOLBEV/MST136.
- [55] E. Iwata, K. Masamoto, H. Kuga, and M. Ogino, “Timing of isolation from an enriched environment determines the level of aggressive behavior and sexual maturity in Siamese fighting fish (*Betta splendens*),” *BMC Zool.*, vol. 6, no. 1, pp. 1–11, Dec. 2021, doi: 10.1186/S40850-021-00081-X/TABLES/3.
- [56] M. L. Brandão, G. Colognesi, M. C. Bolognesi, R. S. Costa-Ferreira, T. B. Carvalho, and E. Gonçalves-de-Freitas, “Water temperature affects aggressive interactions in a Neotropical cichlid fish,” *Neotrop. Ichthyol.*, vol. 16, no. 1, 2018, doi: 10.1590/1982-0224-20170081.
- [57] L. P. Gaffney, B. Franks, D. M. Weary, and M. A. G. von Keyserlingk, “Coho Salmon (*Oncorhynchus kisutch*) Prefer and Are Less Aggressive in Darker Environments,” *PLoS One*, vol. 11, no. 3, p. e0151325, Mar. 2016, doi: 10.1371/JOURNAL.PONE.0151325.
- [58] S. K. Valdimarsson and N. B. Metcalfe, “Is the level of aggression and dispersion in territorial fish

- dependent on light intensity?," *Anim. Behav.*, vol. 61, no. 6, pp. 1143–1149, Jun. 2001, doi: 10.1006/ANBE.2001.1710.
- [59] M. C. Teles and R. F. Oliveira, "Chapter 20 Quantifying Aggressive Behavior in Zebrafish," vol. 1451, pp. 293–305, 2016, doi: 10.1007/978-1-4939-3771-4.
- [60] S. Millot, P. Vandewalle, and E. Parmentier, "Sound production in red-bellied piranhas (*Pygocentrus nattereri*, Kner): an acoustical, behavioural and morphofunctional study," *J. Exp. Biol.*, vol. 214, no. 21, pp. 3613–3618, Nov. 2011, doi: 10.1242/JEB.061218.
- [61] M. Iwashita and M. Yoshizawa, "Social-like responses are inducible in asocial Mexican cavefish despite the exhibition of strong repetitive behavior," *Elife*, vol. 10, Sep. 2021, doi: 10.7554/ELIFE.72463.
- [62] J. Torres-Paz, C. Hyacinthe, C. Pierre, and S. Rétaux, "Towards an integrated approach to understand Mexican cavefish evolution," *Biol. Lett.*, vol. 14, no. 8, 2018, doi: 10.1098/RSBL.2018.0101.
- [63] L. Espinasa, N. Bonaroti, J. Wong, K. Pottin, E. Queinnec, and S. Rétaux, "Contrasting feeding habits of post-larval and adult *Astyanax* cavefish," *Subterr. Biol.*, vol. 21, no. 1, pp. 1–17, 2017, doi: 10.3897/SUBTBIOL.21.11046.
- [64] R. CL *et al.*, "Gender differences in aggression and cortisol levels in zebrafish subjected to unpredictable chronic stress," *Physiol. Behav.*, vol. 171, pp. 50–54, Mar. 2017, doi: 10.1016/J.PHYSBEH.2016.12.032.
- [65] M. L. Niemiller and D. Soares, "Cave environments," *Extrem. Fishes Ecol. Evol. Physiol. Teleosts Extrem. Environ.*, pp. 161–191, Jan. 2015, doi: 10.1007/978-3-319-13362-1_8.
- [66] K. J. Chapin, "Cave-epigeal behavioral variation of the whip spider *Phrynos longipes* (Arachnida: Amblypygi) evidenced by activity, vigilance, and aggression," <https://doi.org/10.1636/J15-04>, vol. 43, no. 2, pp. 214–219, Aug. 2015, doi: 10.1636/J15-04.
- [67] U. Strecker, V. H. Faúndez, and H. Wilkens, "Phylogeography of surface and cave *Astyanax* (Teleostei) from Central and North America based on cytochrome b sequence data," *Mol. Phylogenet. Evol.*, vol. 33, no. 2, pp. 469–481, Nov. 2004, doi: 10.1016/J.YMPEV.2004.07.001.
- [68] S. U. B. L. and W. H., "Genetic divergence between cave and surface populations of *Astyanax* in Mexico (Characidae, Teleostei)," *Mol. Ecol.*, vol. 12, no. 3, pp. 699–710, Mar. 2003, doi: 10.1046/J.1365-294X.2003.01753.X.
- [69] D. TE, M. DP, and J. WR, "Evidence for multiple genetic forms with similar eyeless phenotypes in the blind cavefish, *Astyanax mexicanus*," *Mol. Biol. Evol.*, vol. 19, no. 4, pp. 446–455, 2002, doi: 10.1093/OXFORDJOURNALS.MOLBEV.A004100.
- [70] C. P. Ornelas-García, O. Domínguez-Domínguez, and I. Doadrio, "Evolutionary history of the fish genus *Astyanax* Baird & Girard (1854) (Actinopterygii, Characidae) in Mesoamerica reveals multiple morphological homoplasies," *BMC Evol. Biol.*, vol. 8, no. 1, p. 340, 2008, doi: 10.1186/1471-2148-8-340.
- [71] B. M, B. P, G. L. FJ, E.-B. S, and B. RL, "Gene flow and population structure in the Mexican blind cavefish complex (*Astyanax mexicanus*)," *BMC Evol. Biol.*, vol. 12, no. 1, 2012, doi: 10.1186/1471-2148-12-9.

- [72] W. R. Jeffery, "Regressive Evolution in *Astyanax* Cavefish," *Annu. Rev. Genet.*, vol. 43, p. 25, Dec. 2009, doi: 10.1146/ANNUREV-GENET-102108-134216.
- [73] H. Wilkens, "Genes, modules and the evolution of cave fish," *Hered. 2010 1055*, vol. 105, no. 5, pp. 413–422, Jan. 2010, doi: 10.1038/hdy.2009.184.
- [74] J. E. Kowalko *et al.*, "Convergence in feeding posture occurs through different genetic loci in independently evolved cave populations of *Astyanax mexicanus*," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 110, no. 42, pp. 16933–16938, Oct. 2013, doi: 10.1073/PNAS.1317192110/-/DCSUPPLEMENTAL.
- [75] S. E. McGaugh *et al.*, "The cavefish genome reveals candidate genes for eye loss," *Nat. Commun. 2014 51*, vol. 5, no. 1, pp. 1–10, Oct. 2014, doi: 10.1038/ncomms6307.
- [76] K. E. O'Quin, M. Yoshizawa, P. Doshi, and W. R. Jeffery, "Quantitative genetic analysis of retinal degeneration in the blind cavefish *Astyanax mexicanus*," *PLoS One*, vol. 8, no. 2, Feb. 2013, doi: 10.1371/JOURNAL.PONE.0057281.
- [77] M. R. Riddle, A. Aspiras, F. Damen, S. McGaugh, J. A. Tabin, and C. J. Tabin, "Genetic mapping of metabolic traits in the blind Mexican cavefish reveals sex-dependent quantitative trait loci associated with cave adaptation," *BMC Ecol. Evol.*, vol. 21, no. 1, pp. 1–22, Dec. 2021, doi: 10.1186/S12862-021-01823-8/TABLES/8.
- [78] H. Klaassen, Y. Wang, K. Adamski, N. Rohner, and J. E. Kowalko, "CRISPR mutagenesis confirms the role of *oca2* in melanin pigmentation in *Astyanax mexicanus*," *Dev. Biol.*, vol. 441, no. 2, pp. 313–318, Sep. 2018, doi: 10.1016/J.YDBIO.2018.03.014.

Table 1. Definitions for all aggression- and escape-associated behaviors scored in the resident/intruder assay.

Behavior	Description
Biting	Focal fish physically makes contact with another fish with its mouth while performing an opening and closing motion with its mouth.
Circling	Both fish engage in a circular motion, typically with one head facing the tail of the other fish and vice versa.
Following	Focal fish follows the trajectory of another.
Escaping	Focal fish accelerates away from the other fish.
Freezing	Focal fish stops moving for greater than 5 seconds in any position within the tank.
Avoidance	Focal fish localizes in a corner of the tank for greater than 5 seconds.
Striking	Focal fish accelerates towards another fish ending in contact (but not necessarily biting).

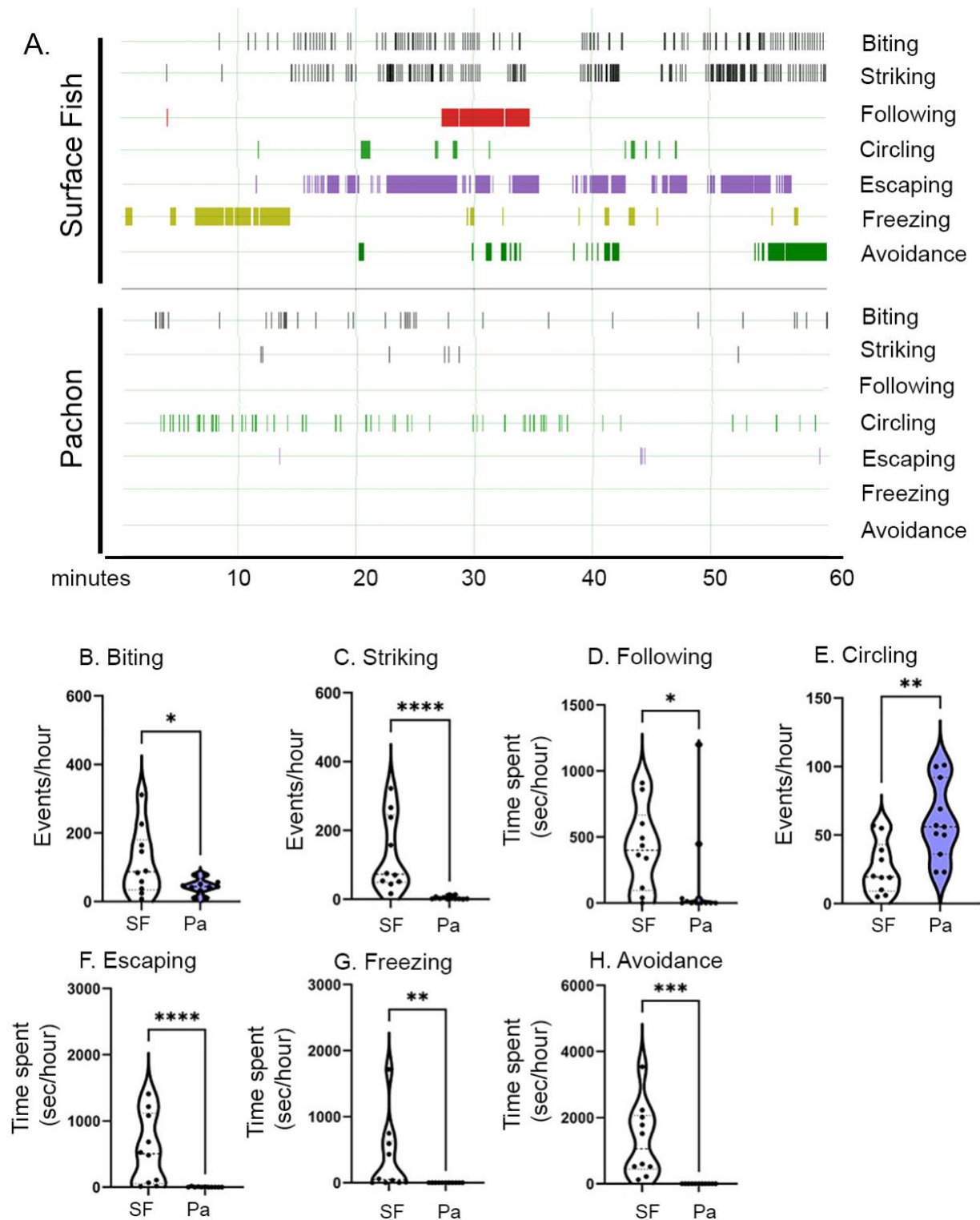


Figure 1. Quantification of social behaviors in the resident/intruder assay for surface fish and Pachón cavefish. (A) Representative ethograms for pairs of surface fish (top) and Pachón cavefish (bottom) during the resident/intruder 1-hour assay. Seven behaviors were annotated: biting, striking, following, circling,

escaping, freezing, and avoidance (Table 1) over the 60 min assay period. Behaviors were quantified for each fish, but were pooled for both fish in each resident/intruder assay here (surface: n=10, Pachón: n=11). (B-H) Quantifications of behaviors annotated during the resident/intruder assay. All behaviors were scored for both individuals in the tank, and each data point represents either the number of behavioral events (biting (B), striking (C), circling (E)) or the time spent in a behavioral state (following (D), escaping (F), freezing (G), avoidance (H)) for one trial. Unpaired t-tests were calculated for biting ($p < 0.05$), circling ($p < 0.01$) and freezing ($p < 0.001$). Mann-Whitney statistical tests were performed for striking ($p < 0.0001$), following ($p < 0.05$), escaping ($p < 0.0001$) and avoidance ($p < 0.01$). Significance: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) , $p < 0.0001$ (****), not significant (ns).

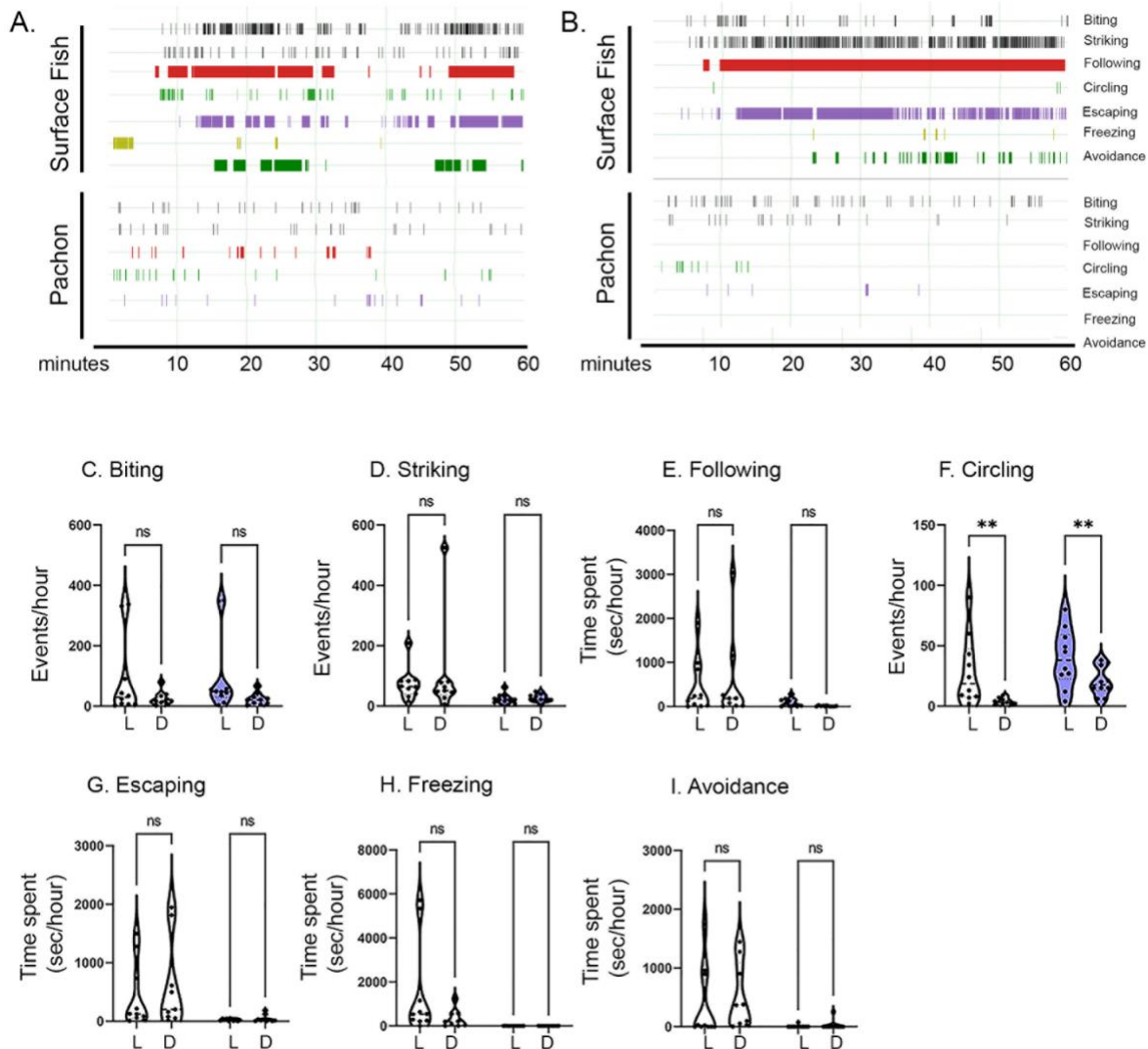


Figure 2. Social Behaviors in a Resident/Intruder Assay Under Light/Dark Conditions. (A-B) Representative merged resident/intruder activity plots for surface fish (top) and Pachón cavefish (bottom) in the light (A) or dark (B) during resident/intruder interactions. (C-I) Quantifications of behaviors annotated during each assay with light (L) versus dark (D) intra-population comparisons. 2-Way ANOVAs were performed for all behaviors in the light (surface fish, $n = 10$; Pachón cavefish, $n = 10$) and dark (surface fish, $n = 9$; Pachón cavefish, $n = 10$), followed by Tukey's multiple comparison's test for within surface or cave populations for light vs dark comparisons for biting ($p=0.1169$), striking ($p=0.9446$), circling ($p=0.0063$), following ($p=0.9999$), escaping ($p=0.9020$), freezing ($p=0.4333$), and avoidance ($p=0.9337$). Significance is reported only for comparisons within populations between light and dark: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****), not significant (ns).

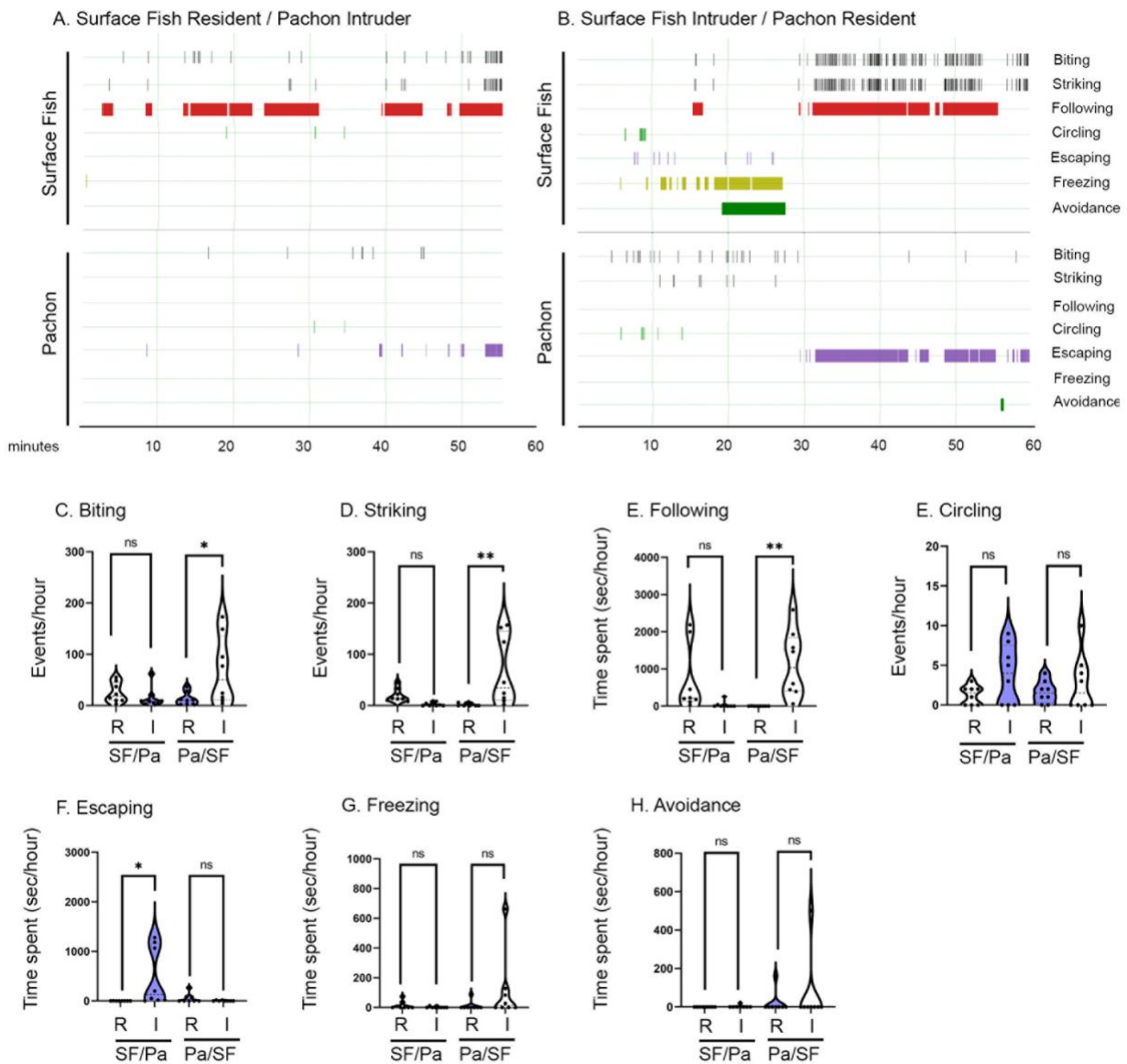


Figure 3. Resident/Intruder dynamics in surface fish versus Pachón cavefish fights. (A-B) Resident/intruder activity plots for surface fish-residents with Pachón-intruders (A) and Pachón-residents with surface fish-intruders (B) during staged fights. (C-I) Quantifications of behaviors annotated during staged fights with resident (R) and intruder (I) intra-population comparisons. 2-Way ANOVAs were performed for all behaviors, followed by Tukey's multiple comparison's test for resident versus intruder comparisons: When surface fish were residents: biting ($p=0.9513$), striking ($p=0.7403$), circling ($p=0.9935$), following ($p=0.1689$), escaping ($p=0.9865$), freezing ($p>0.9999$) and avoidance ($p=0.9712$). When Pachón cavefish were residents: biting ($p=0.0293$), striking ($p=0.0060$), circling ($p=0.8589$), following ($p=0.0086$), escaping ($p=0.0245$), freezing ($p=0.2423$) and avoidance ($p=0.5680$). Significance: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****), not significant (ns).

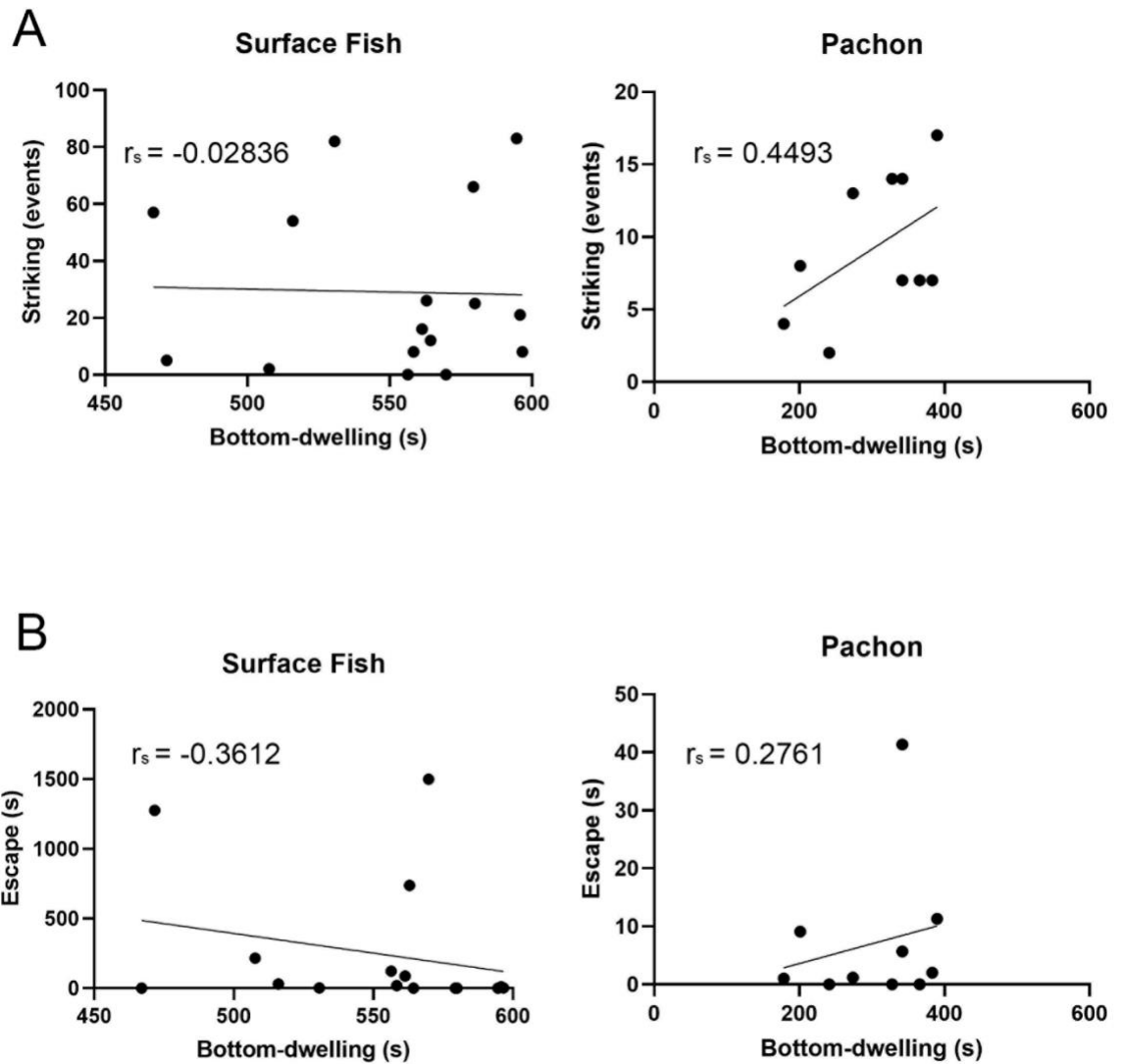


Figure 4. Correlation Between two social behaviors during a resident-intruder assay and bottom-dwelling. Correlations between number of strikes and time escaping during the resident/intruder assays and time spent in the bottom third of the tank in the novel tank assay were performed using Spearman's rank correlation test for striking (A, surface, $p = 0.9170$, Pachón, $p = 0.1941$) and escaping (B, surface, $p = 0.1694$, Pachón, $p = 0.4416$).

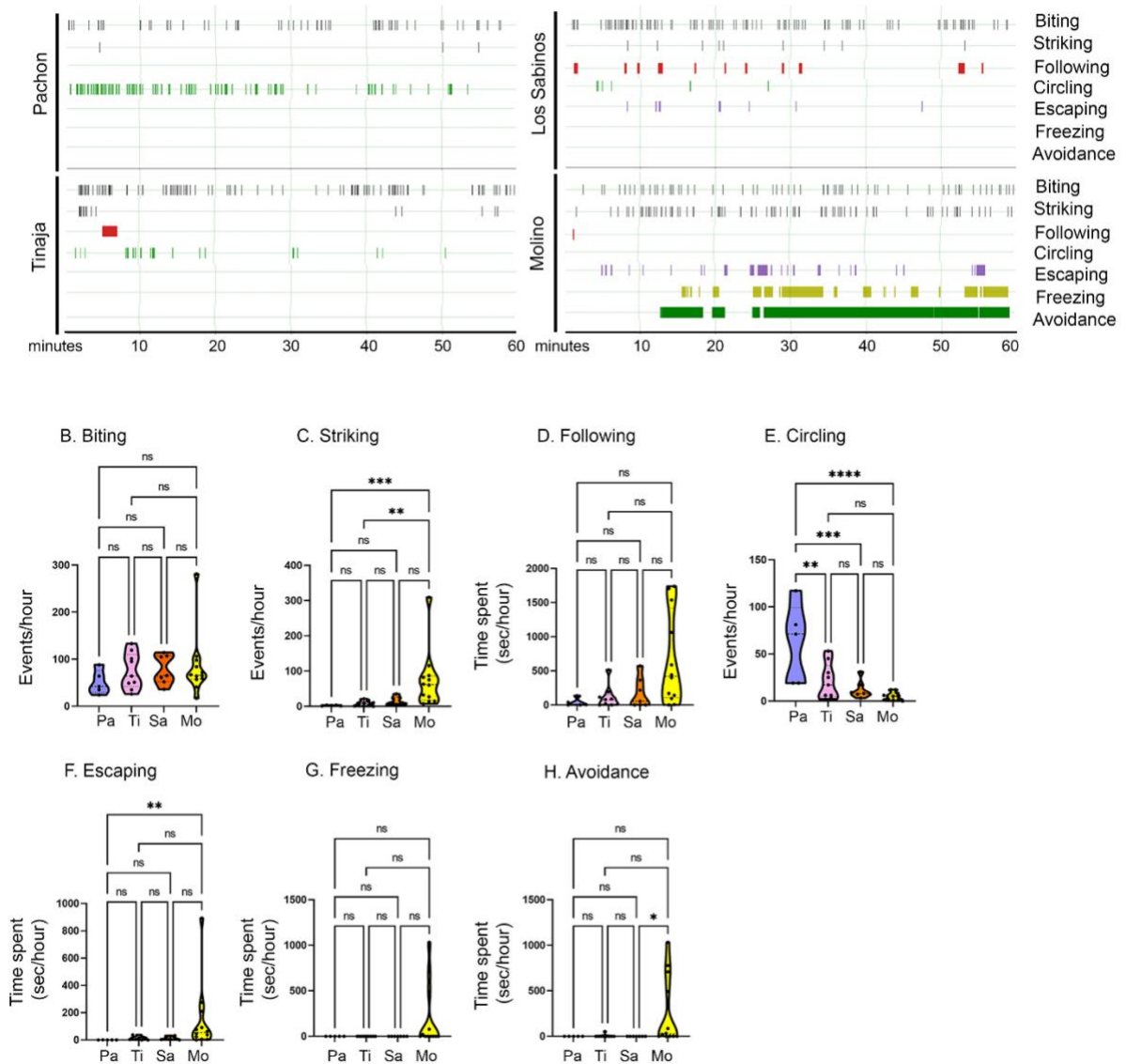


Figure 5. Social behaviors in a resident/intruder assay across multiple cave populations. (A) Representative resident/intruder activity plots for Pachón (top left), Tinaja (bottom left), Los Sabinos (top right) and Molino (bottom right) cavefish during the resident/intruder assay. The number of total behaviors for both the resident and the intruder were combined. All fish were sex and size matched, and sex was not used as a variable given the lack of effect of sex on seven behaviors in Pachón cavefish (Fig S1). (B-H) Quantifications of behaviors annotated during fights with comparisons across populations (Pachón = Pa, Tinaja = Ti, Los Sabinos = Sa, and Molino = Mo). One-way ANOVA followed by Tukey's multiple comparisons test was performed for circling (Pachón-Molino, $p < 0.0001$, Pachón-Tinaja, $p < 0.05$, Pachón-Los Sabinos, $p < 0.001$, Molino-Tinaja, $p = 0.2887$, Molino-Los Sabinos, $p = 0.885$, Tinaja-Los Sabinos, $p = 0.7954$). Kruskal-Wallis with Dunn's multiple comparisons test were performed for biting (Pachón-

Molino, $p=0.9213$, Pachón-Tinaja, $p>0.9999$, Pachón-Los Sabinos, $p=0.7564$, Molino-Tinaja, $p>0.9999$, Molino-Los Sabinos, $p>0.9999$, Tinaja-Los Sabinos, $p>0.9999$), striking (Pachón-Molino, $p<0.001$, Pachón-Tinaja, $p>0.9999$, Pachón-Los Sabinos, $p=0.2443$, Molino-Tinaja, $p=0.0057$, Molino-Los Sabinos, $p=0.3528$, Tinaja-Los Sabinos, $p>0.9999$), escaping (Pachón-Molino, $p<0.01$, Pachón-Tinaja, $p=0.409$, Pachón-Los Sabinos, $p=0.4466$, Molino-Tinaja, $p=0.206$, Molino-Los Sabinos, $p=0.341$, Tinaja-Los Sabinos, $p>0.9999$), following (Pachón-Molino, $p=0.0585$, Pachón-Tinaja, $p>0.9999$, Pachón-Los Sabinos, $p>0.9999$, Molino-Tinaja, $p=0.2307$, Molino-Los Sabinos, $p=0.3641$, Tinaja-Los Sabinos, $p>0.9999$), freezing (Pachón-Molino, $p=0.1938$, Pachón-Tinaja, $p>0.9999$, Pachón-Los Sabinos, $p>0.9999$, Molino-Tinaja, $p=0.0586$, Molino-Los Sabinos, $p=0.0995$, Tinaja-Los Sabinos, $p>0.9999$), and avoidance (Pachón-Molino, $p=0.0706$, Pachón-Tinaja, $p>0.9999$, Pachón-Los Sabinos, $p>0.9999$, Molino-Tinaja, $p=0.0702$, Molino-Los Sabinos, $p=0.0289$, Tinaja-Los Sabinos, $p>0.9999$). Significance: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****), not significant (ns).

