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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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 ± 1°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–OOO13). 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 that

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 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 *Phrynus*

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.

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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).
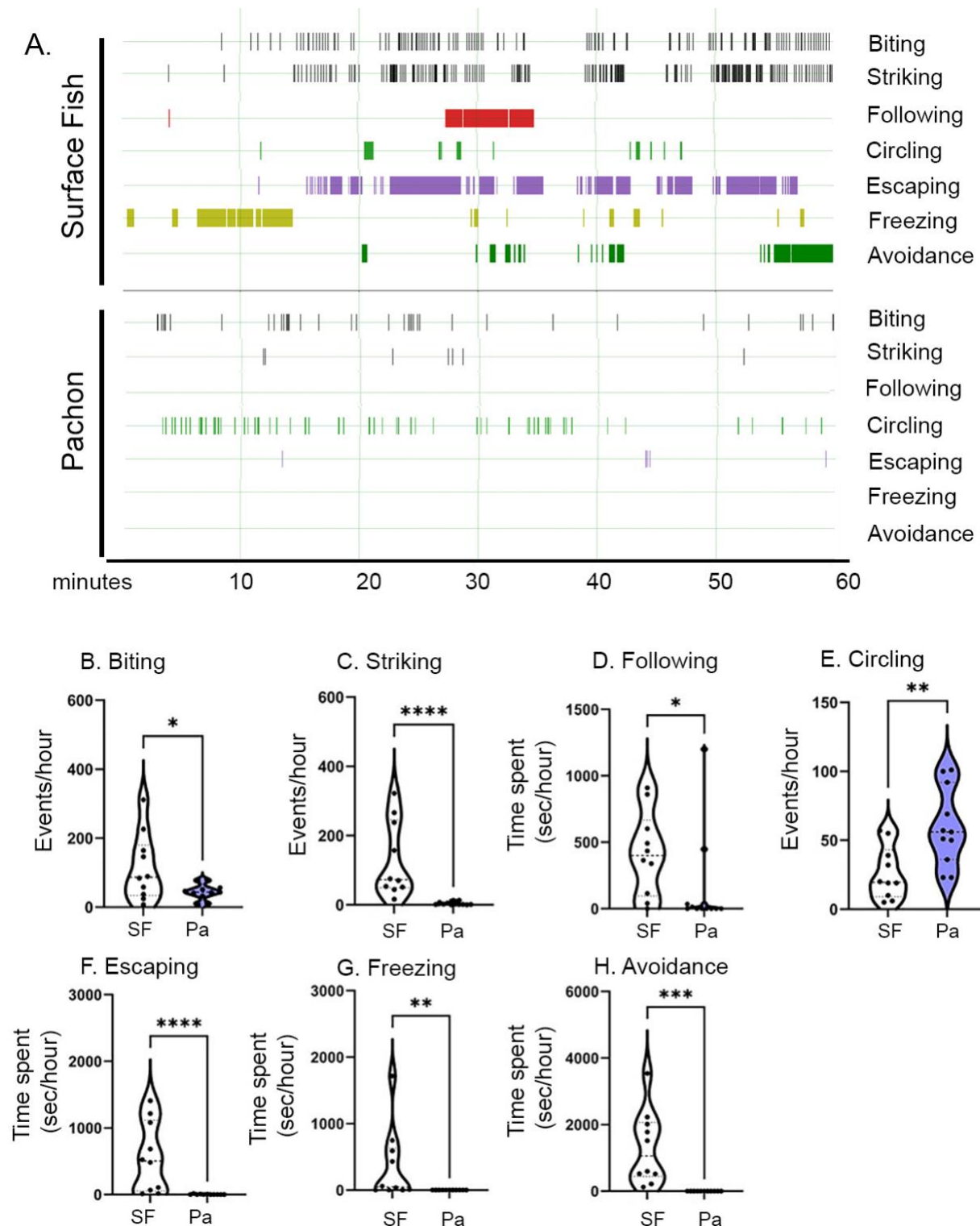
Figure 1. Quantification of social behaviors in the resident/intruder assay for surface fish and Pachón cavefish. (A) Representative ethograms for pairs of surface fish (top) and Pachón cavefish (bottom) during the resident/intruder 1-hour assay. Seven behaviors were annotated: biting, striking, following, 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).
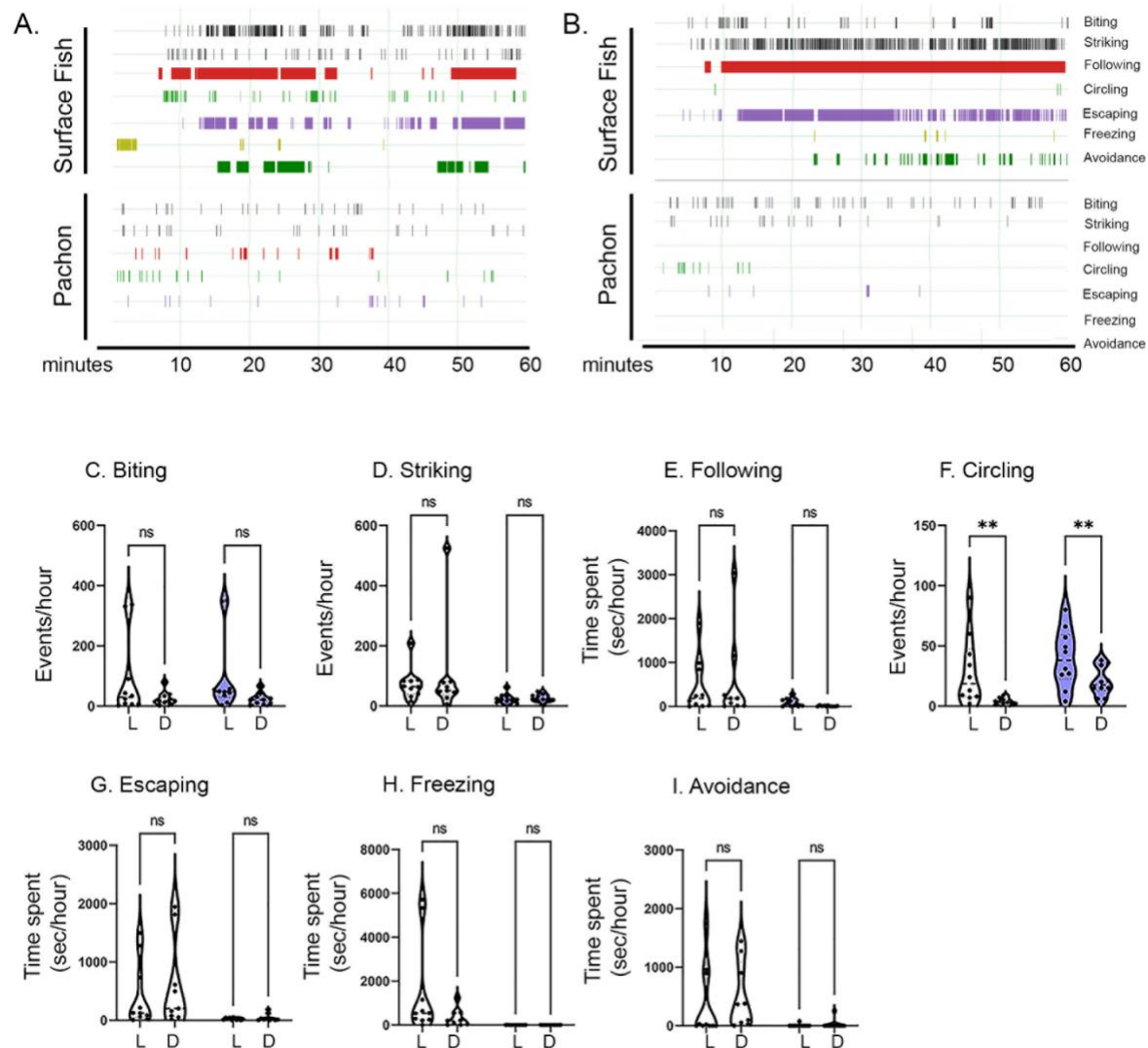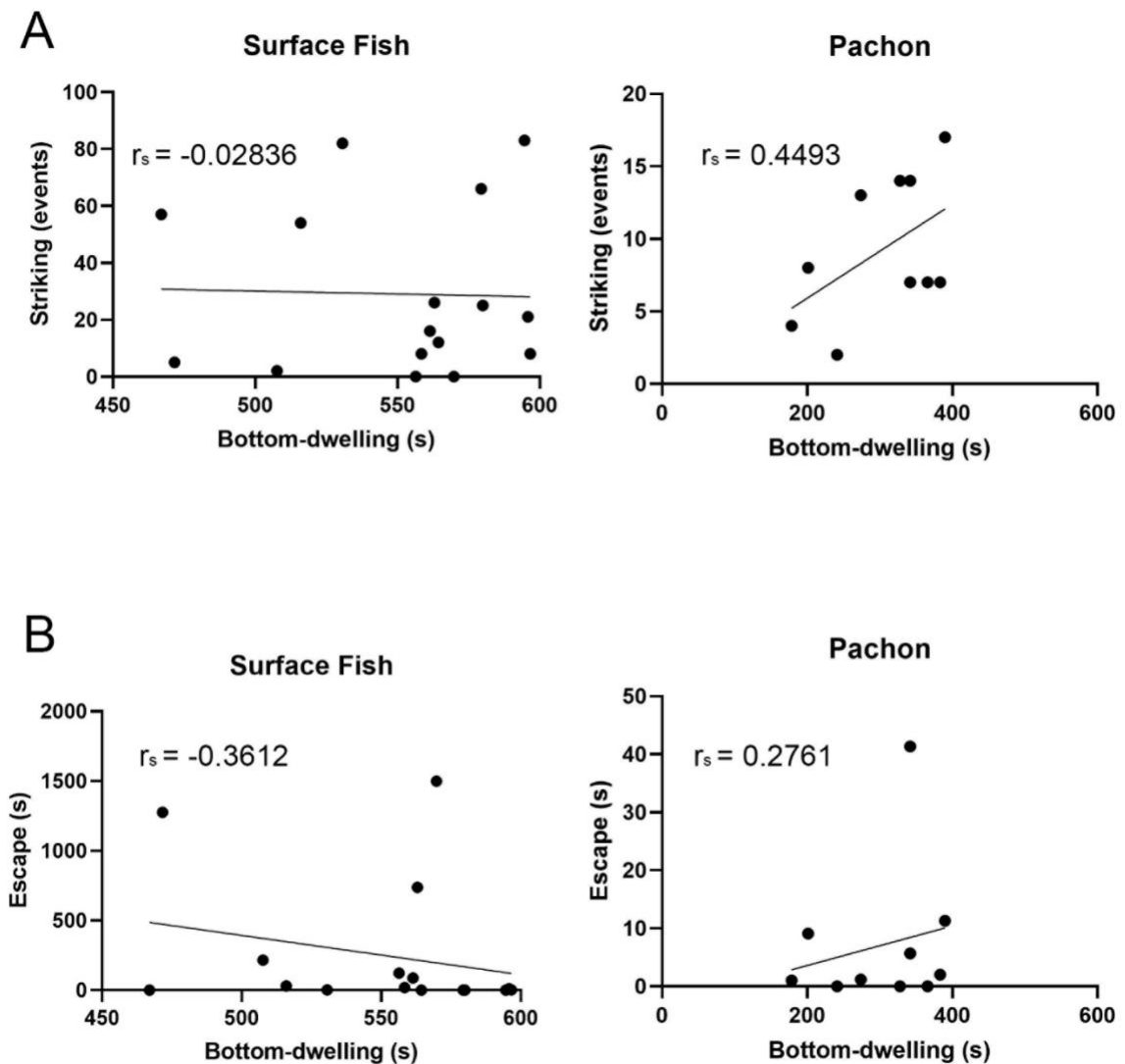
Figure 2. Social Behaviors in a Resident/Intruder Assay Under Light/Dark Conditions. (A-B) Representative merged resident/intruder activity plots for surface fish (top) and Pachón cavefish (bottom) in the light (A) or dark (B) during resident/intruder interactions. (C-I) Quantifications of behaviors 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).
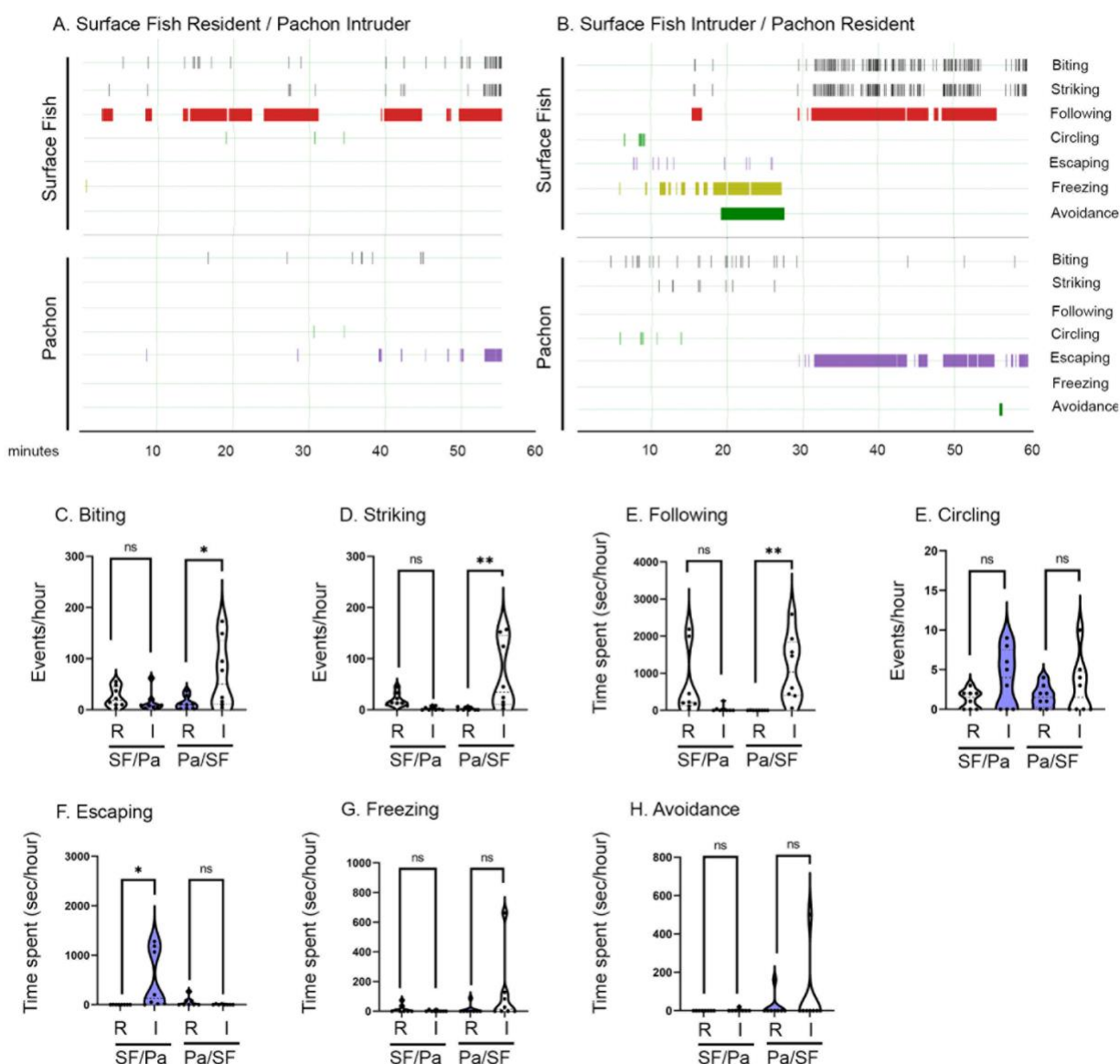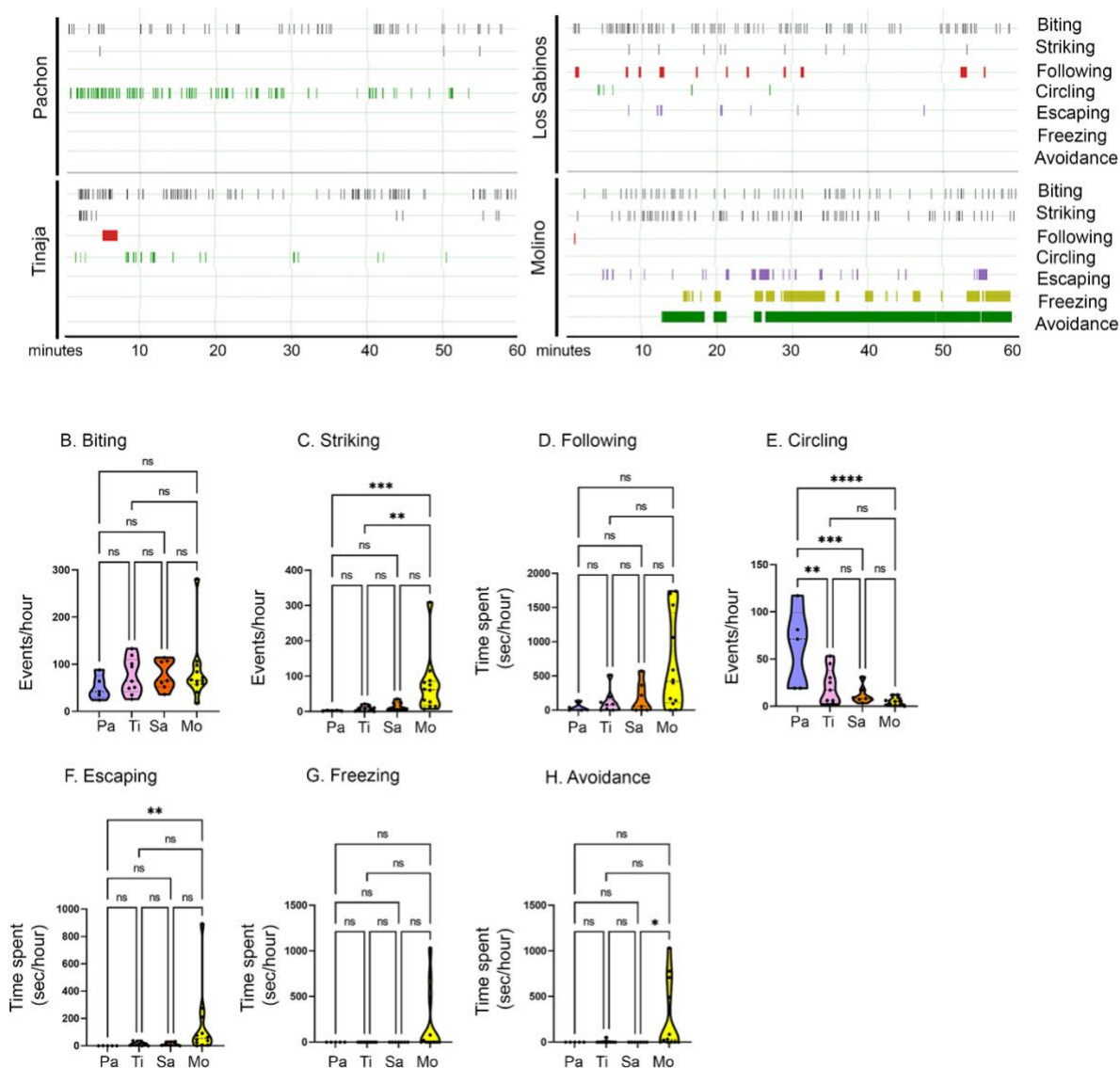
Figure 3. Resident/Intruder dynamics in surface fish versus Pachón cavefish fights. (A-B) Resident/intruder activity plots for surface fish-residents with Pachón-intruders (A) and Pachón-residents with surface fish-intruders (B) during staged fights. (C-I) Quantifications of behaviors annotated during 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).