Pleiotropic function of the *oca2* gene underlies the evolution of sleep loss and albinism in cavefish

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1   **Summary**

2   Adaptation to novel environments often involves the evolution of multiple morphological,

3   physiological and behavioral traits. One striking example of multi-trait evolution is the suite

4   of traits that has evolved repeatedly in cave animals, including regression of eyes, loss of

5   pigmentation, and enhancement of non-visual sensory systems [1,3]. The Mexican tetra,

6   *Astyanax mexicanus,* consists of fish that inhabit at least 30 caves in Northeast Mexico

7   and ancestral-like surface fish which inhabit the rivers of Mexico and Southern Texas [6].

8   Cave *A. mexicanus* are interfertile with surface fish and have evolved a number of traits

9   that are common to cave animals throughout the world, including albinism, eye loss, and

10   alterations to behavior [8–10]. To define relationships between different cave-evolved

11   traits, we phenotyped 208 surface-cave F2 hybrid fish for numerous morphological and

12   behavioral traits. We found significant differences in sleep between pigmented and albino

13   hybrid fish, raising the possibility that these traits share a genetic basis. In cavefish and

14   many other species, mutations in *oculocutaneous albinism 2* (*oca2*) cause albinism [11–

15   15]. Surface fish with CRISPR-induced mutations in *oca2* displayed both albinism and

16   reduced sleep. Further, this mutation in *oca2* fails to complement sleep loss when surface

17   fish harboring this engineered mutation are crossed to different, independently evolved

18   populations of albino cavefish with naturally occurring mutations in *oca2*, confirming that

19   *oca2* contributes to sleep loss. Finally, analysis of the *oca2* locus in wild caught cave and

20   surface fish suggests that *oca2* is under positive selection in at least three cave

21   populations. Taken together, these findings identify *oca2* as a novel regulator of sleep

22   and suggest that a pleiotropic function of *oca2* underlies the adaptive evolution of both of

23   albinism and sleep loss.

Results

24

25   Colonization of a novel environment often results in evolution of behavioral,

26   morphological, and physiological traits. Uncovering the relationships between these traits

27   is critical to understanding the mechanisms that drive adaptation. Multiple populations of

28   cave *A. mexicanus* have evolved a suite of traits that distinguish them from surface

29   dwelling counterparts. These include regression of eyes, albinism, elevated energy

30   stores, and sleep loss [4,16–19]. To investigate the relationship between evolved traits

31   in cave *A. mexicanus,* we generated hybrids from surface fish and cavefish from the

32   Pachón cave, which are highly troglomorphic. Surface-Pachón F2 hybrids possess

33   variable morphological phenotypes (Fig 1A). We quantified numerous traits associated

34   with cave evolution including eye size, albinism, adiposity, feeding and sleep [4,8,17–19].

35   At 20 dpf, surface fish, cavefish, and surface-cave F1 and F2 hybrids were assayed for

36   sleep over a 24hr period, followed by measurements of food consumption. Fish were then

37   stained with Nile Red, which labels adipocytes in larval fish [19,20] and imaged to score

38   pigmentation, adiposity, and eye diameter (Fig 1B). As individual fish were followed

39   throughout all phenotyping steps, this experimental design allows for determining the

40   relationship between traits in F2 hybrid fish.

41   Comparison of hybrids to pure cave and surface populations revealed variability

42   suggestive of both monogenic and polygenic inheritance of the various traits, largely in

43   agreement with previously published findings [4,21,22]. Sleep duration was significantly

44   reduced in Pachón cavefish compared to surface fish. F1 hybrids slept more than pure

45   Pachón cavefish and less than surface fish (Fig 1C, Fig S1A). F2 individuals were variable

46   in the amount they slept, with some F2 fish sleeping very little, similar to Pachón cavefish,

47   and other F2 fish sleeping at surface fish-like levels (Fig 1C). Additionally, sleep

48   architecture was different between the groups. Sleep loss in Pachón cavefish is due to

49   reductions in both the number and duration of sleep bouts relative to surface fish (Figure

50   S1B,C). The average duration of each sleep bout in F1 hybrid fish was similar to cavefish,

51   while bout length in F1 hybrids was intermediate between the surface fish and cavefish

52   (Figure S1B,C). The dominant cave phenotype for bout length, but not bout number,

53   raises the possibility that different components of sleep architecture are independently

54   regulated. Indeed, bout number and bout duration were weakly correlated in F2 fish,

55   (Spearman's rho = 0.36, p<0.0001), consistent with at least some different genetic factors

56   contributing to these different components of sleep architecture (Fig S1D).

57   One hypothesis for the evolution of sleep loss in cavefish is that altered foraging

58   and/or metabolic demands associated with living in a food-poor environment drive the

59   evolution of sleep loss [23]. In agreement with the previous findings that feeding does not

60   differ between surface fish and Pachón cavefish [18,24], we observed no significant

61   differences in total food consumption between surface fish, Pachón cavefish, F1 hybrids

62   or F2 hybrids (Fig 1D). These results suggest that in the Pachón population, sleep loss

63   is unlikely to be linked to changes in food consumption at the larval stage. The initial

64   production of adipose deposits is ontogenically regulated and occurs earlier in Pachón

65   cavefish relative to surface fish [19]. Within all four experimental groups, some fish had

66   developed adipose deposits by 22 dpf, while others had not (Fig 1E, Fig S1E,F). However,

67   a significantly larger percentage of cavefish had developed adipose deposits when

68   compared to surface fish at 22 dpf. An intermediate percentage of F1 and F2 hybrid fish

69   had developed adipose deposits at this stage relative to both cave and surface

70   populations (Fig 1E), suggesting that differences in adiposity have a genetic basis and

71   that regulation of adiposity shows partial or incomplete dominance.

72   Cave animals are characterized by loss of eyes and pigmentation [1]. Similar to

73   previously reported studies [25,26], we found that Pachón cavefish eyes were significantly

74   smaller than eyes in surface fish. Further, eyes in F1 hybrid fish were intermediate in size

75   and significantly different from both parental populations (Fig 1F). Eye size ranged in F2

76   hybrid fish from surface-like to cave-like (Fig 1F). Therefore, nearly all traits analyzed with

77   differences between surface fish and cavefish display intermediate phenotypes in F1

78   hybrids and variability in F2 fish consistent with a polygenic basis for evolved differences

79   between *A. mexicanus* cave and surface populations.

80   Finally, we analyzed albinism, the complete loss of melanin pigmentation. All

81   surface and F1 fish exhibited robust melanin pigmentation, while all cavefish were albino,

82   consistent with the recessive nature of this trait [11,17] (Fig 1A). In our F2 population, 61

83   of the 208 fish tested were albino (Fig 1A). To examine segregation of albinism in F2

84   individuals precisely, we also quantified the number of albino F2 fish from a single clutch

85   by collecting a random cohort of fish pre-pigmentation, and allowing these fish to develop

86   until 5 days post fertilization, when albinism can be easily scored by eye as complete

87   absence of melanin pigmentation. Of these fish, 66 out of 283 were albino (23.3%), which

88   was not significantly different from the expected ratios for a trait controlled by a single

89   gene (Chi square: p=0.69), consistent with the monogenic inheritance of albinism

90   previously reported [11,17].

91   Cave traits could have evolved independently from each other, or through a shared

92   genetic or functional basis. To determine if there is a relationship between cave-evolved

93   traits, we performed pairwise comparisons between the traits that were significantly

94   different between cavefish and surface fish: sleep, eye size, pigmentation and the

95   presence of adipose tissue, reasoning that traits that were co-inherited would be

96   significantly correlated with one another. We found three significant relationships from

97   this analysis. F2 fish with adipose deposits had significantly smaller eyes compared to

98   F2 fish without adipose (Cohen's d=0.31, Table 1). Further, albino F2 fish had significantly

99   smaller eyes than pigmented fish, raising the possibility that shared genetic architecture

100   regulates these morphological traits (Cohen's d=0.61, a moderate effect, Fig S2A, Table

101   1). Additionally, total sleep duration was significantly less in albino F2 fish compared to

102   pigmented F2 fish (Cohen's d=0.55, a moderate effect, Fig 2A,B). In albino F2 hybrid fish,

103   both bout duration and bout number were significantly reduced, raising the possibility of

104   shared genetic factors underlying the evolution of albinism and both sleep components

105   (Fig 2C,D). These results suggest that albinism is associated with multiple traits that are

106   present in cavefish populations. As little is known about the relationship between

107   morphological and behavioral evolution, we focused on the relationship between albinism

108   and sleep loss to determine if the co-heritability of these traits is due to closely linked loci

109   or pleiotropy.

110   In two independently evolved populations of cavefish, albinism is caused by

111   deletions in the coding sequence of the *oculocutaneous albinism 2* (*oca2*) gene [11,12].

112   The finding that albinism is associated with shortened sleep raises the possibility that

113   mutation of *oca2* also contributes to the evolution of sleep loss. To test this directly, we

114   quantified sleep in surface fish with a CRISPR/Cas9 engineered deletion in exon 21 of

115   the *oca2* gene (*oca2^{Δ2bp}*). A deletion of this entire exon is found in Molino cavefish [11],

116   suggesting the engineered mutation could phenocopy the naturally occurring mutation.

117   This engineered mutation, when homozygous, results in complete loss of melanin

118   pigmentation in surface fish, phenocopying albino cavefish (Fig 3A,B, [12]). Sleep was

119   significantly reduced during the day and night in *oca2$^{Δ2bp/Δ2bp}$* surface fish compared to

120   pigmented, wild-type *oca2$^{+/+}$* control siblings (Cohen's d=1.3, a large effect, Fig 3,A-D).

121   This reduction in sleep derives from reduced bout duration, which is significantly reduced

122   in *oca2$^{Δ2bp/Δ2bp}$* surface fish compared to wildtype *oca2$^{+/+}$* control siblings (Fig 3E).

123   Mutation of *oca2* did not result in a statistically significant change in bout number (Fig 3F).

124   Together, these findings demonstrate that mutations in the *oca2* gene can affect both

125   pigmentation and sleep, and raise the possibility that pleiotropy of the *oca2* gene

126   contributed to the evolution of both albinism and reduced sleep in cavefish.

127   Surface fish heterozygous at the *oca2* locus (*oca2$^{Δ2bp/+}$*) are pigmented and

128   presented an intermediate total sleep duration phenotype which did not differ significantly

129   from sleep in wild-type or *oca2* mutant siblings (Rerun with heterozygous individuals

130   included: Average total sleep duration for *oca2$^{+/+}$* = 8.42 hours (n=32), *oca2$^{Δ2bp/+}$* = 7.03

131   hours (n=47), *oca2$^{Δ2bp/Δ2bp}$* = 5.64 hours (n=53); One-way ANOVA: F= 6.89, p= 0.0014;

132   Tukey's posthoc test: *oca2$^{+/+}$* vs *oca2$^{Δ2bp/+}$* (p=0.1674), *oca2$^{+/Δ2bp}$* vs *oca2$^{Δ2bp/Δ2bp}$*

133   (p=0.1104), *oca2$^{Δ2bp/Δ2bp}$* vs *oca2$^{+/+}$* (p=0.0010)). To confirm that *oca2* regulates sleep in

134   cavefish, we investigated whether our engineered loss-of-function *oca2* alleles in surface

135   fish complemented naturally occurring deletions in *oca2* in albino cavefish. We crossed

136   surface fish heterozygous at the *oca2* locus (*oca2$^{Δ2bp/+}$*) to Pachón or Molino cavefish,

137 both of which harbor natural coding mutations in *oca2* [11]. The presence of the

138 engineered *oca2^{Δ2bp}* allele resulted in albino offspring in surface-Pachón and surface-

139 Molino crosses (genotype *oca2$^{Δ2bp/ΔPa}$* and *oca2$^{Δ2bp/ΔMo}$*, respectively), whereas offspring

140 that inherited the wild-type allele from the surface parent (genotype *oca2$^{+/ΔPa}$* and

141 *oca2$^{+/ΔMo}$*) were pigmented (Fig 4A,B,D,E), confirming this mutation is sufficient to induce

142 albinism in multiple cave populations and consistent with previous studies [12]. Further,

143 albino *oca2$^{Δ2bp/ΔPa}$* and *oca2$^{Δ2bp/ΔMo}$* hybrid fish slept significantly less than their wildtype

144 *oca2$^{+/ΔPa}$* and *oca2$^{+/ΔMo}$* hybrid pigmented siblings (Fig 4C,F). These findings demonstrate

145 that the engineered *oca2* mutant allele fails to complement the sleep phenotype in

146 Pachón and Molino cavefish, supporting the notion that loss of *oca2* contributes to sleep

147 loss in multiple independently evolved cavefish populations. Bout duration was reduced

148 in surface-Pachón *oca2$^{Δ2bp/ΔPA}$* hybrid fish compared to *oca2$^{+/ΔPA}$* siblings (Fig S3A). Both

149 bout duration and bout number in surface-Molino *oca2$^{Δ2bp/ΔMo}$* hybrid fish were lower

150 relative to siblings that inherited one wild-type surface allele, but they did not reach

151 significance (Fig S3A,B,D,E). Finally, we crossed *oca2$^{Δ2bp/+}$* surface fish to Tinaja

152 cavefish, which are not albino, and do not harbor known loss of function mutations in

153 *oca2,* but which also present a reduced sleep phenotype [27]. We found no visible

154 difference in melanin pigmentation between *oca2$^{Δ2bp/Ti}$* and *oca2$^{+/Ti}$* hybrid fish, confirming

155 a lack of effect of mutations in *oca2* on the presence of melanin pigmentation in this

156 population (Fig 4G,H). Further, sleep in the *oca2$^{Δ2bp/Ti}$* hybrid fish was not significantly

157 different from sleep in the *oca2$^{+/Ti}$* siblings, suggesting *oca2* mutations do not play a role

158 in sleep loss in this population (Fig 4I, Fig S3C,F).

159   A central question in evolutionary biology is the extent to which selection or drift

160   has driven trait evolution. Our findings that a single gene contributes to both albinism

161   and sleep loss in multiple populations raises the possibility that selection for one or both

162   of these traits leads to evolution of both traits in cave populations harboring *oca2*

163   mutations. To test directly if *oca2* is under selection in cavefish, we conducted genome

164   scans for selection using hapFLK (v1.4), software that allows for detection of genomic

165   signatures of selection based on population genotyping [7]. The hapFLK statistic

166   provides a powerful approach to detect regions of the genome under selection by

167   testing for differentiation among populations in haplotype cluster frequencies exceeding

168   what is expected by neutral evolution. We analyzed whole genome resequencing data

169   from individuals of multiple populations of cave and surface fish collected previously [5].

170   In addition to cavefish populations from Molino (n=9), Pachón (n=10) and Tinaja (n=8),

171   we analyzed surface *A. mexicanus* populations from two different localities: Rascón

172   (n=7) and the Río Choy (n=9), the surface population used for behavioral data collection

173   in this study. We also included a single individual from *Astyanax aeneus,* a closely

174   related species*,* to serve as an outgroup. We observed statistically significant hapFLK

175   values (1% FDR cutoff, p-values < 4.11e-05) within the *oca2* region, consistent with

176   positive selection at this locus (Fig S4A). To identify the populations that experienced

177   positive selection, branch lengths were re-estimated by building a local population tree

178   for *oca2* using Reynolds distances based on haplotype frequencies. We observed

179   significant p-values in branches corresponding to both surface and all three cave

180   populations, Pachón, Molino, and Tinaja, (Fig S4B, Table S1), indicative of selection.

181   This analysis reveals that population-specific *oca2* alleles are under positive selection

182   across *A. mexicanus* populations. Therefore, these findings support the notion that

183   selection for loss of *oca2* is a critical contributor to the evolution of sleep loss and

184   albinism in cave habitats.

185

186   **Discussion**

187   Understanding the relationships between evolved traits is a central goal of

188   evolutionary biology. The interfertility of independently evolved *A. mexicanus*

189   populations provides a system to investigate the genetic and functional relationships

190   between traits. Previous studies of morphological trait evolution in *A. mexicanus* found

191   that quantitative trait loci (QTL) for many seemingly unrelated morphological traits

192   cluster in the genome more than expected by chance [22,28]. Further, evolved

193   differences in sensory systems are thought to be critical drivers of the behavioral

194   changes observed in cavefish, and in some cases, QTL for sensory traits and behaviors

195   overlap [29,30]. However, none of these studies have addressed which genes and

196   genetic lesions underlie these relationships. Thus, it is still unclear if these observed

197   relationships are due to linkage, pleiotropy, or both. The finding that a single gene,

198   *oca2*, underlies albinism and contributes to the reduction in sleep in two independently

199   evolved cave populations of *A. mexicanus,* definitively demonstrates a pleiotropic role of

200   a naturally occurring genetic variant in a morphological and a behavioral trait. To our

201   knowledge, these findings represent the first association between monoallelic albinism

202   and the evolution of an ecologically-relevant behavior in any cave animal.

203   The protein encoded by *oca2* functions at the first step of the melanin synthesis

204   pathway in *A. mexicanus*, the conversion of L-tyrosine to L-DOPA [12,31]. Further,

205   mutations in *oca2* have been linked to catecholamines. Morpholino knockdown of *oca2*

206   in larval surface fish increases levels of dopamine, and albino cavefish have evolved

207   higher levels of the catecholamines dopamine and norepinephrine [32–34]. This raises

208   the possibility that OCA2 could modulate behaviors through regulating catecholamine

209   levels [32,34]. Catecholamines regulate a number of behaviors, including sleep, feeding

210   and social behaviors [35–37]. Further, many of these behaviors have evolved in

211   cavefish [4,18,30,38] and reductions in schooling behavior, anesthesia resistance, and

212   loss of sleep have all been linked to catecholamines in *A. mexicanus* through

213   pharmacological analyses [30,34,39]. Thus, our observations that *oca2* contributes to

214   sleep loss in cavefish could be due to effects of *oca2* on catecholamine levels. Further

215   analyses to determine if loss of function mutations in *oca2* are sufficient to alter

216   catecholamines are needed to examine this relationship.

217   Multiple adaptive roles for pigmentation have been shown across a variety

218   species, including protection against ultraviolet radiation, parasitism, and

219   thermoregulation [40]. In line with this, we found that *oca2* shows signatures of positive

220   selection in both populations of surface fish. Further, all three cave populations

221   examined here also show signatures of positive selection at the *oca2* locus. That a

222   signature of selection is present in all populations regardless of whether they are albino

223   or not suggests two non-mutually exclusive possibilities: First, that pigmentation is

224   under selection across populations and is an adaptive trait in surface fish, offering

225   protection against harmful UV radiation or thermal regulation. Second, other traits

226   controlled by *oca2* are under selection across populations, supporting the hypothesis

227   that *oca2* is pleiotropic.

228   While adaptive roles for pigmentation are well documented in surface

229   populations, the evolutionary drivers of reductions of pigmentation in subterranean

230   habitats are poorly understood. In cave populations, reduction or loss of melanin

231   pigmentation in *A. mexicanus* cave populations has occurred through at least three

232   mechanisms: reduction in the number of melanin producing melanophores, reduction in

233   amount of melanin pigment produced, and the complete loss of melanin pigmentation,

234   albinism, caused by mutations in *oca2* [11,16,17,41]. For decades, it has been argued

235   that loss of pigmentation in cave animals is due to reduced selective pressure to

236   maintain pigmentation within the dark cave environment [16,21]. Further, previous

237   studies on the effects of QTL for melanophore number suggest that reductions of

238   pigmentation alone may not be under direct selection [21]. Here, we provide genomic

239   evidence that *oca2* is under selection in cave populations of *A. mexicanus*. Further, we

240   demonstrate that loss of function alleles of *oca2* reduce sleep. Together, these results

241   support the hypothesis that albinism has evolved through selection, not drift, in cave

242   populations, and raise the possibility that selection has acted on another trait, distinct

243   from albinism, sleep.

244   In conclusion, we report three complementary findings that suggest *oca2*

245   contributes to sleep loss in cavefish. First, in F2 hybrid fish, sleep is lower in albino fish

246   compared to pigmented fish from the same brood. Second, CRISPR-mediated mutation

247   of *oca2* in surface fish reduces sleep. And finally, these CRISPR-mediated mutations

248   fail to complement the sleep phenotypes of Pachón and Molino cavefish that harbor

249   endogenous *oca2* mutations. Finally, we find that *oca2* is under selection in two albino

250   cavefish populations, Pachón and Molino, as well as the hypopigmented Tinaja

251   population. These findings raise the possibility that selection drove the evolution of

252   albinism and sleep loss observed in multiple independently evolved cavefish

253   populations.

254

255   **Materials and Methods**

256   **Husbandry**

257   Animal husbandry and breeding were carried out as described previously [42]. All

258   procedures were done in accordance with the IACUC committee at Florida Atlantic

259   University. Adult fish were housed in a fish facility with an Aquaneering flow-through

260   system maintained at 23°C with a 14:10 hr light: dark cycle. Fish were bred by feeding

261   frozen blood worms 2-3 times for the duration of breeding, and heating the water to 26-

262   28°C to induce spawning. Larvae were raised in groups of 50 in stand-alone tanks, and

263   fed brine shrimp once daily until 20 days post fertilization (dpf) for all assays described

264   here.

265

266   ***oca2* mutant fish**

267   The $oca2^{\Delta 2bp}$ allele was previously isolated following CRISPR/Cas9 induced

268   mutagenesis at the *oca2* locus [12]. Founder fish from this cross were from a surface

269   fish population originated in Texas. These fish were outcrossed for 1-2 generations to

270   surface fish from Mexico (Río Choy). For the experiments described here, we crossed

271   heterozygous (*$oca2^{\Delta 2bp/+}$*) F2 or F3 to one another, or crossed $oca2^{\Delta 2bp/+}$ F2 or F3 to

272   cavefish. All crosses were performed using pairs of fish, with each assay being

273   performed on fish from multiple crosses.

274

275   Genotyping was performed as described previously [12,43,44]. DNA was isolated from

276   whole larval fish or from adult fin clips by incubating larvae or fin clips in 50 mM NaOH

277   for 30 minutes at 95°C. Following addition of 1/10th volume Tris-HCl pH 8.0, PCRs were

278   run using forward primers specific to the alleles: 5'-CTGGTCATGTGGGTCTCAGC- 3'

279   was used for the wild type surface *oca2* allele and 5'-TCTGGTCATGTGGGTCTCATT-3'

280   was used for the 2 base pair mutant allele. The same reverse primer, 5'-

281   TGTCAAGATATGTGATCTTTGGAAA-3' was used for both reactions.

282

283   **Hybrid fish**

284   To obtain F2 hybrid fish, a single surface fish female from a Mexican population was

285   crossed with a single Pachón cavefish male to obtain cave-surface F1 hybrids. A single

286   pair of F1 hybrid fish was crossed to produce all of the F2 hybrids described here. Wild-

287   type surface and cave larvae were produced by group crosses of surface fish and

288   Pachón cavefish. F1 fish for behavioral assays were progeny of a single pairwise cross

289   between a female Río Choy surface fish and a male Pachón cavefish.

290

291   **Phenotyping**

292   Sleep experiments were carried out as described previously [27,45]. Briefly, 20 dpf

293   larvae were acclimated in 24-well plates for ~15 hours prior to recording. Following

294   feeding with artemia for 10 minutes, fish were recorded for 24 hours (14L:10D) starting

295   at ZT0, lit from the bottom with LED white and IR lights. Videos were recorded at 15

296   frames per second using the video capturing software, VirtualDub (Version 1.10.4).

297   Videos were then subsequently tracked using Ethovision XT 13.0 (Noldus, IT) software.

298   Sleep behavior parameters were defined from raw data using a custom-written Perl

299   script and MS Excel macros. Sleep is defined by periods of inactivity during which

300   individuals experience an increased arousal threshold [2]. Periods of inactivity that were

301   of 60 seconds or greater were defined as sleep, as this period of immobility is

302   associated with an increase in arousal threshold in *A. mexicanus* [4]. Total sleep

303   duration, number of sleep bouts, and sleep bout length were quantified for each fish.

304

305   At 21 dpf, following the 24-hour sleep recording, fish were analyzed for feeding

306   behavior. They were transferred to a 24-well plate pre-filled with approximately 70

307   *Artemia* naupili and were well and recorded for two hours. Total feeding was quantified

308   by counting artemia before and after the feeding using Fiji [46] and subtracting to get

309   the total number of artemia consumed.

310

311   Following feeding, fish were fasted for 20 hours (to limit autofluorescence), and at 22

312   dpf, fish were stained with Nile red (Sigma Aldrich 19123). The stock solution was

313   prepared by dissolving Nile Red in acetone at a concentration of 1.25 mg/mL and stored

314   in the dark at -20°C. Prior to staining the stock solution was diluted with fish water to a

315   final working concentration of 1/1000. Fish were stained in a 24-well plate with 1mL of

316   the working solution in each well, and placed in a 28°C incubator for 30 minutes, as

317   previously described [19]. Following staining, fish were euthanized in 100 ug/mL MS-

318   222 and imaged on a Nikon SMZ25 stereoscope using an GFP filter. Fish were scored

319   for presence or absence of Nile Red staining.

320

321   Measurements of standard length and eye diameter were taken from these images,

322   using Fiji [46]. Eye diameter was measured from anterior edge of the eye to the

323   posterior edge. Standard length was measured from snout to caudal peduncle. Eye

324   diameter throughout the paper was corrected for standard length by dividing eye

325   diameter by standard length.

326

327   Color images of individuals of a subset of representative larval fish taken at 21 dpf using

328   a Canon Rebel t6i camera on a clear background, illuminated from above and below.

329

330   **Statistical analysis for trait comparisons**

331   All statistical analyses were performed using Graphpad Prism 8.4.3. All data was tested

332   for normality using Shapiro-Wilk tests. Data which did not pass the normality test were

333   analyzed using the nonparametric Kruskal-Wallis test. Where statistical significance was

334   indicated, post-hoc comparisons were completed using Dunn's multiple comparisons

335   test. Normally distributed data with multiple groups were analyzed using an ANOVA,

336   with post-hoc comparisons completed using a Tukey post-hoc test. For continuous traits

337   which did not pass the normality test, relationships between these continuous traits

338   were analyzed using the Spearman's rank correlations. Relationships between binary

339   and continuous traits were analyzed for normality, and then analyzed using a t-test if

340   they passed the normality test, or a Mann-Whitney test if they did not pass the normality

341   test. For adipose proportion, data error bars were calculated using z*-value of 1.96 and

342   denote the margin of error of the sample proportion and data was analyzed using

343 Fisher's Exact Test, and a post-hoc of pairwise Fisher's Exact Test was performed

344 where significance was indicated. Effect size tests were also performed on F2 and *oca2*

345 comparisons, an odds ratio effect size test was performed for adipose proportion data,

346 all other effect size tests for comparisons were Cohen's D-test.

347

348 **Test for selection**

349 To test for signatures of selection on the *oca2* gene we used hapFLK (v1.4) [7] on *A.*

350 *mexicanus* whole genome resequencing data from two surface populations, Rascón

351 (n=7) and Río Choy (n=9), and three cave populations, Tinaja (n=8), Molino (n=9), and

352 Pachón (n=10). We also included a single *Astyanax aeneus* surface fish as an

353 outgroup. Details on sequencing and genotyping can be found in Herman et al. (2018)

354 [5]. Briefly, samples were sequenced as 100 bp paired end reads on an Illumina

355 HiSeq2000 at The University of Minnesota Genomics Center. Raw sequencing data for

356 these samples was downloaded from NCBI (available under SRA Accession Numbers

357 SRP046999, SRR4044501, and SRR4044502). Three samples (Rascon_6, Tinaja_6,

358 and Tinaja_E) that were included in [5] were excluded from this analysis due to putative

359 recent hybrid ancestry.

360

361 The haplotype-based hapFLK statistic is an extension of the SNP-based FLK statistic

362 [7]. hapFLK provides a powerful approach to detect regions of the genome under

363 selection by using a model that incorporates linkage disequilibrium to test for

364 differentiation in haplotype clusters among populations. Unlike F_{ST}, the hapFLK statistic

365 accounts for hierarchical population structure and for the effects of recombination [7].

366   We first ran hapFLK in SNP mode to calculate FLK tests and to generate a kinship

367   matrix based on the entire genome. The whole genome kinship matrix was then used to

368   calculate haplotype-based hapFLK statistics on a 5 Mb region of chromosome 13 (35

369   Mb - 40 Mb) containing *oca2* (37,904,635-38,048,888 bp). For the haplotype-based

370   tests, we used a K of 10 based on cross validation with fastPHASE (v1.4.8) and ran 20

371   expectation maximization iterations. hapFLK P-values were calculated using a chi-

372   square distribution with the script scaling_chi2_hapflk.py. We used the R package

373   qvalue to set a q-value cutoff of 0.01 and apply for a 1% false discovery rate (FDR)

374   correction based on p-values present within the 35 Mb - 40 Mb regions of chromosome

375   13. To identify population-specific signatures of selection at *oca2*, we built local

376   population trees using Reynolds distances based on haplotype frequencies with the

377   script local_reynolds.py. We visualized changes in haplotype clusters across *oca2* in

378   each population using the script hapflk-clusterplot.R. P-values were computed with the

379   script local_trees.R by comparing the Reynolds distances among populations for the

380   local tree compared to those from the whole genome tree. R and python scripts used in

381   this analysis were downloaded from the hapFLK developers' website https://forge-

382   dga.jouy.inra.fr/projects/hapflk/documents.

383

Acknowledgements

390 **Figures**
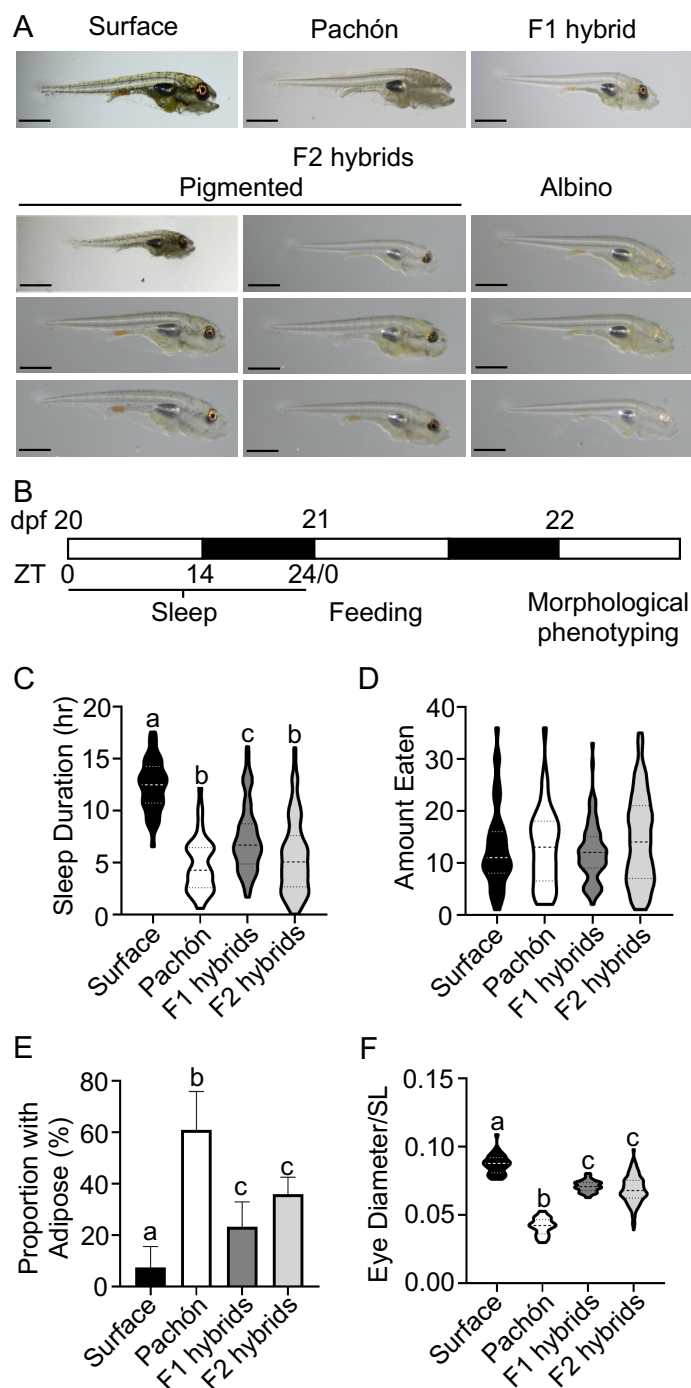
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392 Figure 1 - Genetic analysis of multiple traits evolved in cave *A. mexicanus*. (A) Images
393 of 22dpf fish (scale = 1mm). (B) Timeline for phenotypic analysis. Surface fish (n=40),
394 Pachón cavefish (n=41), surface-Pachón F1 hybrids (n=73) and surface-Pachon F2
395 hybrid fish (n=208) were phenotyped for (C) Total sleep duration (Kruskal-Wallis:
396 H_2=97.37, p <0.0001; Dunn's multiple comparison: Pa vs. F1: z=3.55, p= 0.002, F1 vs.

397   F2: z=3.95, p=0.0016). All other: p<0.0001) (D) Total brine consumed (Kruskal-Wallis:
398   H_2=4.36, p=0.2256 (E) Proportion of individuals with adipose. Error bars calculated
399   using z*-value of 1.96 and denote the margin of error of the sample proportion. Fishers
400   Exact tests: SF v Pa p<0.0001, SF v F1 p=0.0408, SF v F2 p=0.0002, Pa v F1
401   p=0.0001, Pa v F2 p=0.0048, F1 v F2 p=0.0590). (F) Eye diameter, corrected for
402   standard length (ANOVA: F=197.4, p<0.0001; Tukey's: F1 vs. F2 q=5.76, p=0.1970. all
403   others p<0.0001). Statistical differences are indicated by different letters in (C)-(F).
404
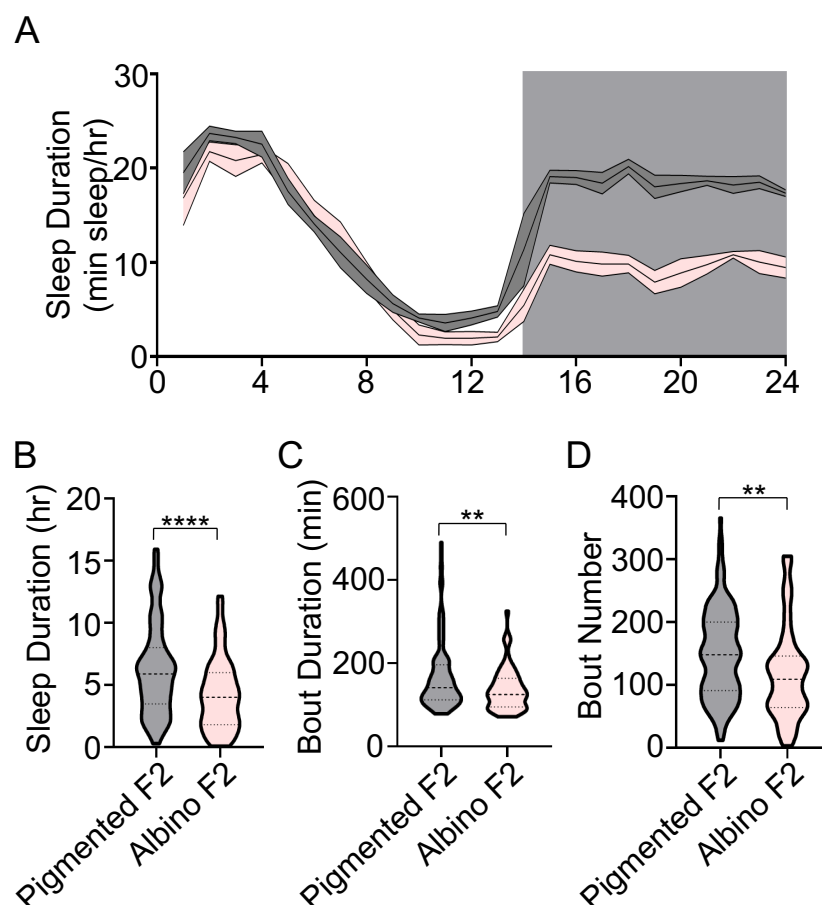
Figure 2 - Relationship between sleep and pigmentation in cave-surface hybrid fish. (A) 24-hour sleep profile of F2 hybrid crosses depicting the amount slept at 1 hour intervals over a 24-hour time frame. The gray area of each graph represents laboratory night, when lights were off. The gray line represents pigmented F2s while the pink line represents albino F2s (B) Total sleep duration in albino compared to pigmented F2 hybrids (Mann-Whitney, U=3379, p<0.0001). (C) Bout duration of albino compared to pigmented F2 hybrids (t-test, t=2.67, p=0.0079). (D) Bout number in albino versus pigmented F2 hybrids (t-test, t=3.24, p=0.0043). For (A)-(D), pigmented F2 hybrids (n = 147), and albino F2 hybrids (n = 61). Graph (A) represents the average and standard deviation. Graphs (B)-(D) represent the median ± the quartile
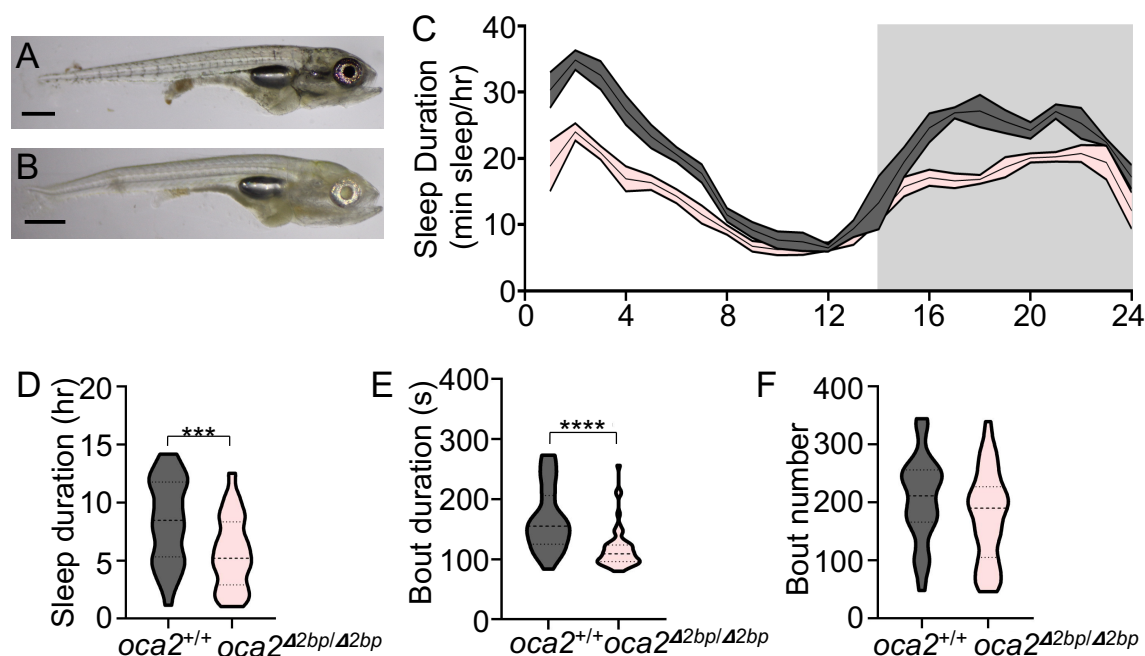
Figure 3 - *oca2* mutant surface fish sleep less. (A) Pigmented wild-type and sibling (B) albino *oca2$^{Δ2bp/Δ2bp}$* 22 dpf surface fish. (C) 24-hour sleep profile of oca2$^{+/+}$ and *oca2$^{Δ2bp/Δ2bp}$* surface fish siblings depicting the amount slept at 10-minute intervals over a 24-hour time frame. (D) Total sleep duration in *oca2$^{Δ2bp/Δ2bp}$* compared to wild-type oca2$^{+/+}$ siblings (t-test, t=3.71, p=0.0004). (E) Bout duration in *oca2$^{Δ2bp/Δ2bp}$* compared to wild-type oca2$^{+/+}$ siblings (Mann-Whitney, U=309, p<0.0001). (F) Bout number in *oca2$^{Δ2bp/Δ2bp}$* compared to wild-type oca2$^{+/+}$ siblings (Mann-Whitney, U=662, p=0.092). For (C)-(F), pigmented wild-type (n = 32), and albino *oca2$^{Δ2bp/Δ2bp}$* (n = 53).
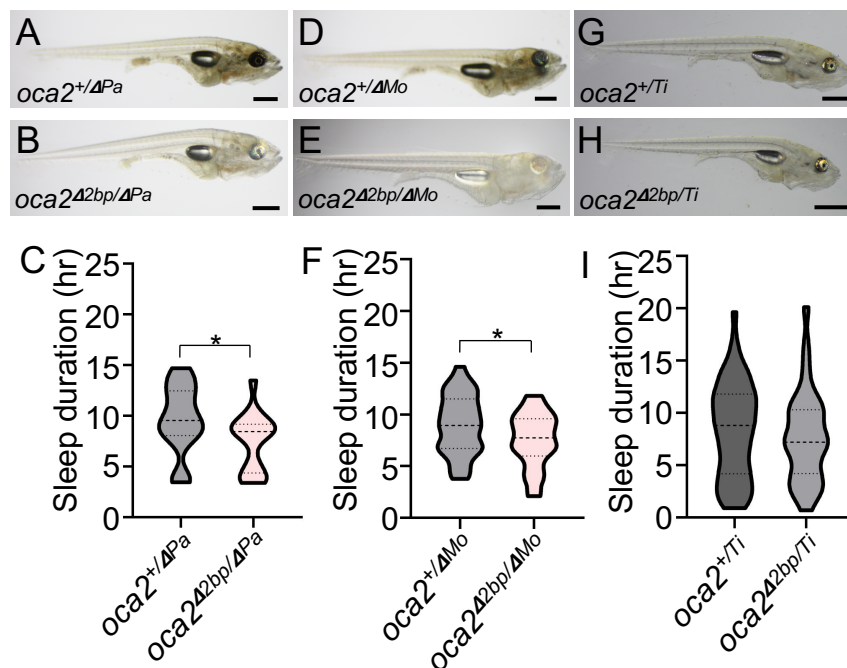
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Figure 4 - Lack of complementation in cave-surface F1 hybrid fish with two mutant *oca2* alleles. Melanin pigmentation (A, B, D, E, G, H) and sleep duration (C, F, I) were assessed in Pachon-surface F1 hybrid (A, B, C), Molino-surface F1 hybrid (D,E,F) and Tinaja-surface F1 hybrid fish (G,H,I). Images of 22 dpf (A) *oca2$^{+/\Delta PA}$* (B) *oca2$^{\Delta 2bp/\Delta PA}$* (D) *oca2$^{+/\Delta Mo}$* (E) *oca2$^{\Delta 2bp/\Delta Mo}$* (G) *oca2$^{+/Ti}$* (H) *oca2$^{\Delta 2bp/Ti}$* F1 hybrid fish.

Total sleep duration in (C) *oca2$^{+/\Delta PA}$* (n=21) compared to *oca2$^{\Delta 2bp/\Delta PA}$* (n=17) siblings (t-test, t=2.210, p=0.033), (F) *oca2$^{+/\Delta Mo}$* (n=38) compared to *oca2$^{\Delta 2bp/\Delta Mo}$* (n=32) siblings (t-test, t=2.231, p=0.029) and (I) *oca2$^{+/Ti}$* (n=56) compared to *oca2$^{\Delta 2bp/Ti}$* (n=33) siblings (t-test, t=0.7128, p=0.478).

435

436 **Tables**

437

438 Table 1 – Pairwise comparisons of phenotypes in F2 individuals

F2 Comparisons	Test	p-value	Test Statistic
Adipose Proportion in Albino vs. Pigmented	Fisher's exact test	0.0805	
Total sleep in Albino vs. Pigmented	Mann-Whitney test	<0.0001	u=3379
Eye Diameter in Albino vs. Pigmented		0.0001	u=6211
Total sleep vs. Eye Diameter	Spearman-rank correlation	0.2628	r=0.078
Total sleep vs. Adipose Proportion		0.4460	r=0.05312
Eye Diameter in Adipose +/- fish	T-test	0.0333	t=2.143

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