Metabolic shift toward ketosis in asocial cavefish increases social-like collective behavior.

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Abstract

Social affinity and collective behavior are nearly ubiquitous in the animal kingdom, but many lineages feature evolutionarily asocial species. These solitary species may have evolved to conserve energy in food-sparse environments. However, the mechanism by which metabolic changes regulate social affinity is not well investigated. In this study, we used the Mexican tetra (*Astyanax mexicanus*), which features riverine sighted surface (surface fish) and cave-dwelling populations (cavefish), to address the impact of metabolic shifts on asociality and other cave-associated behaviors in cavefish, including repetitive turning, sleeplessness, swimming longer distances, and enhanced foraging behavior. After 1 month of ketosis-inducing ketogenic diet feeding, asocial cavefish exhibited significantly higher social affinity, whereas social affinity regressed in cavefish fed the standard diet. The ketogenic diet also reduced repetitive turning and swimming in cavefish. No detectable behavioral shifts were found regarding sleeplessness and foraging behavior, suggesting that other evolved behaviors are not regulated by ketosis. We further examined the effects of the ketogenic diet via supplementation with extragenic ketone bodies, revealing that ketone bodies are pivotal molecules associated with social affinity. Our study indicated that fish that evolved to be asocial remain capable of exhibiting social affinity under ketosis.

Introduction

Wild animals experience frequent fasting because of daily, seasonal, and yearly changes in food availability. Physiologically, fasting can increase the secretion of appetite-related hormones (e.g., ghrelin, peptide Y, orexin) and induce a metabolic shift into nutritional ketosis (McCue, 2010). Concerning behavioral outputs, fasting also induces shifts including boldness in foraging involving risk-taking (Padilla et al., 2016) and a shift from avoiding to approaching prey (Filosa et al., 2016). Interestingly, fasting also induces non-foraging–related behaviors including aggression toward cohorts (Fokidis et al., 2013; Solianik et al., 2016) and engagement in social dominance (Nakajo et al., 2020). These non-foraging behaviors could be evoked by the metabolic changes that occur in a state of nutritional ketosis instead of the increased production of appetite-related hormones. However, it is not fully understood whether ketosis itself in the absence of hunger drives these non-foraging behaviors. Such knowledge will open a path to understanding the effects of different dietary intakes on changing environments, such as switching from ketosis-inducing very low-carbohydrate diets to glycolysis-inducing carbohydrate-rich diets or vice versa.

Recently, the ketosis-inducing ketogenic diet (KD), which contains a high amount of fat, sufficient protein, and very low amount of carbohydrates, gained popularity among humans because of its neuroprotective and anti-inflammatory effects without effects on appetite-related hormone levels (Deemer et al., 2020; Ludwig, 2020; Sumithran et al., 2013). The KD is an effective treatment for refractory seizures, and there is some evidence that it may be beneficial for other nervous system-based disorders such as Alzheimer's disease, Perkinson's disease, and autism (Lee et al., 2018; McDonald and Cervenka, 2018; Phillips et al., 2018; Ruskin and Masino, 2012). Because modern humans evolved to acquire resistance to starvation (Bellisari, 2008), our body physiology and behavioral tendencies possibly evolved to accommodate drastic metabolic changes. However, the major molecular mechanisms for these positive are largely unknown (Ludwig, 2020; Qin et al., 2021). We were therefore motivated to explore the effects of metabolic shifts, particularly from glycolysis to ketosis, on behavioral

75 outputs such as social affinity using a single species consisting of typical and starvation-resistant
76 populations.

77 A suitable model system for this purpose is the Mexican cavefish (*Astyanax mexicanus*). *A.*
78 *mexicanus* has emerged as a model system of diverse aspects of evolution and development, including
79 those with relevance to human medicine, e.g., cataract formation, diabetes, albinism-related syndrome,
80 and insomnia (Aspiras et al., 2015; Bilandžija et al., 2018, 2013; Duboué et al., 2012, 2011; Jaggard et
81 al., 2017; Keene et al., 2016; Ma et al., 2014; McGaugh et al., 2014; Riddle et al., 2018; Rohner et al.,
82 2013; Strickler et al., 2007). *A. mexicanus* consists of surface riverine epigean (surface fish) and cave-
83 dwelling hypogean (cavefish) populations. Cavefish diverged from their surface-dwelling relatives
84 20,000–200,000 years ago (Fumey et al., 2018; Herman et al., 2018), and they have subsequently
85 evolved many distinct morphological and behavioral phenotypes in the food-sparse cave environment,
86 including eye regression/loss, pigment reduction, increased mechanosensory lateral line activity,
87 adherence to vibration stimuli, sleeplessness, hyperactivity, repetitive circling, and reduced social
88 affinity (Iwashita and Yoshizawa, 2021; Keene et al., 2016; Yoshizawa, 2015; Yoshizawa et al., 2018).
89 Compared to cavefish, surface fish exhibit typical teleost phenotypes, including typical eyed and
90 pigmented morphologies, no strong adherence to vibration stimuli, nocturnal sleep patterns, and social
91 affinity. Many cavefish traits are believed to have evolved to adapt to food-sparse dark environments.
92 Indeed, wild cavefish were estimated to be exposed to approximately 6 months of food-sparse conditions
93 annually (Espinasa et al., 2021), and they are likely to have the ability to withstand starvation via
94 increased fat storage (Aspiras et al., 2015), slower weight loss during starvation (Huppop, 1986),
95 reduced energy-costing circadian activities, and lack of eyes (Moran et al., 2015, 2014).

96 Concerning social-like behavior, cavefish exhibit no detectable schooling behavior (Kowalko et
97 al., 2013; Patch et al., 2020; Pierre et al., 2020) or hierarchal dominance (Elipot et al., 2013). By
98 contrast, surface fish school/shoal with cohorts and model fish (Kowalko et al., 2013) exhibit group
99 hierarchical dominance (Elipot et al., 2013). Because social behaviors in many fish (*e.g.*, zebrafish) are

100 promoted by visual stimuli, blind cavefish might not express social-like activities because of the absence
101 of visual acuity. However, a recent detailed study illustrated that surface fish exhibit a high level of
102 social-like nearby interactions (one-by-one affinity) in the dark, and these of which were promoted by
103 mechanosensory lateral line inputs (Iwashita and Yoshizawa, 2021). Interestingly, blind cavefish
104 displayed much lower levels, albeit significant, of nearby interactions than surface fish (Iwashita and
105 Yoshizawa, 2021). Further, cavefish exhibited plasticity in the level of nearby interactions in which they
106 increased plasticity in a familiar environment in comparison with an unfamiliar environment (Iwashita
107 and Yoshizawa, 2021), which is similar to the findings in patients with autism (Helt et al., 2020; Runco
108 et al., 1986).

109 Thus far, similarities between cavefish and patients with autism have been investigated in terms
110 of gene regulation- and innate behavior-profiles. First, the cavefish gene expression profile is closer to
111 that of patients with autism than to that of other known model systems (cavefish and surface fish
112 transcriptomes exhibited the same directional gene expression changes observed in the brains of patients
113 with autism [>58.5% of 3152 cavefish orthologs]). Conversely, other proxy systems (BTBR mice
114 [classic autism model] and *shank3* knockout mice) exhibit much less overlap (<11%) (Lee et al., 2019;
115 Provenzano et al., 2016; Yoshizawa et al., 2018). Second, cavefish's evolved behaviors—asociality,
116 repetitive behavior, sleeplessness, higher swimming activity, adherence to a particular vibration
117 stimulus, and higher anxiety-related plasma cortisol levels—are similar to those in patients with autism
118 (Yoshizawa et al., 2018). Third, cavefish and human ancestors are starvation-resistant, and they could
119 share some metabolic pathways (Aspiras et al., 2015; Bellisari, 2008; Huppop, 1986; Riddle et al.,
120 2018). These similarities and a fact that KD feeding increases sociality in patients with autism
121 (Evangeliou et al., 2003; Lee et al., 2018; Li et al., 2021; Napoli et al., 2014) prompted us to study the
122 effects of ketosis on social affinity in asocial cavefish.

123 In this study, we assessed the effects of the KD on an evolutionarily asocial cave population of *A.*
124 *mexicanus*. The time-course experiment revealed that 1 month of KD feeding promoted and sustained

125 the juvenile level of nearby interactions, whereas control diet (CD)-fed cavefish exhibited diminished
126 nearby interactions. KD feeding also reduced repetitive turning and swimming activity, which are the
127 hallmarks of the autism-like condition. However, the effects of the KD were limited. For example,
128 sleeplessness and high adherence to a particular vibrating stimulus were not detectably changed under
129 the 1-month KD treatment. To reveal the molecular basis of the effects of the KD, we provided
130 supplementation with a major ketone body, beta-hydroxybutyrate (BHB). This experiment indicated that
131 BHB supplementation promoted nearby interactions and reduced repetitive turning, covering the major
132 effect of the KD.
133 Overall, ketosis appears to be capable of significantly shifting the asociality of evolved cavefish
134 toward the surface fish phenotype, providing new insights into the contribution of the diet to the
135 evolution of behavior.
136

137 **Results**

138 From our observation in their wild habitat (the Mexican cave Pachón, Supplemental Movie 1),
139 cavefish swam slower and remained near each other more frequently than the lab population. Because
140 the cave environment has a limited diet compared to that of the surface, we predicted that cavefish
141 experience frequent ketosis induced by fasting.

142 To avoid appetite-related behavior, we developed a ketogenic diet (KD) based on a human milk
143 formula (KetoCal3:1® with Zeigler zebrafish standard irradiated diet at a 5:1 weight ratio [nutritionally
144 complete, ketogenic medical food; Nutricia North America, Inc. Gaithersburg, MD, USA]; Table 1;
145 Materials and Methods). To test whether our KD could induce a shift in the balance of ketone body and
146 glucose levels, we measured blood ketone and glucose levels after chronic dietary treatment. Three-
147 month-old fish (juvenile–young adult stage) were used in this study because many adult-type behaviors
148 of cavefish emerge in this stage, including higher adherence to a vibration stimulus (vibration attraction
149 behavior [VAB]) (Yoshizawa et al., 2010), less social affinity, and longer swimming distances compared
150 to the findings in surface fish. After 5 weeks of KD feeding, both ketone and glucose concentrations
151 were decreased compared to the findings in CD-fed fish (KetoCal3:1 and Zeigler zebrafish diet at a 1:5
152 weight ratio; Table 1; Fig. 1A–C). For both diets, surface fish exhibited a significantly higher ketone
153 body level than cavefish (Fig. 1B), whereas cavefish exhibited a higher glucose level than surface fish
154 (Fig. 1C). The glucose ketone index (GKI = $\frac{glucose\ (mM)}{ketone\ (mM)}$), a medical index proposed for brain cancer
155 diagnosis (Meidenbauer et al., 2015), was lower in surface fish than in cavefish, and the value was
156 reduced under KD feeding in both surface fish and cavefish compared to that in their CD-fed
157 counterparts (Fig. 1D). This result indicated that KD feeding more strongly reduced glucose levels than
158 ketone body levels, resulting in a lower GKI in KD-fed fish than in CD-fed fish (Fig. 1D) and
159 suggesting that KD feeding could shift the metabolic state from glycolysis toward ketosis.
160

161 Table 1. Nutrient composition of each diet used in the study

%	Brine shrimp	Zeigler zebrafish standard diet	KetoCal3:1	Control diet (CD)	Ketogenic diet (KD)
Protein	58.4	54.0	15.3	47.6	21.8
Lipid/fat	14.7	14.4	67.7	23.3	58.8
Carbohydrate	5.2	11.6	7.2	10.9	7.9
Ash	7.2	15.5	NA	NA	NA
Calories (kcal/g)	5.9	3.9	7.0	4.4 (20% w/v in agar)	6.5 (20% w/v in agar)

162 (Paffenhöfer, 1967; Panda, 2016)

163

164 Regarding this dietary treatment, we first examined its ontogenic (developmental) effects on
165 collective social-like behavior (Iwashita and Yoshizawa, 2021). Many adult behaviors emerge in the
166 transition from juvenile to young adult (adolescent) in 3–4-month-old *A. mexicanus* fish, including
167 foraging behavior, VAB (Yoshizawa, 2015; Yoshizawa et al., 2010), adult-type regulation of sleep
168 (independent from catecholamine) (Duboué et al., 2012; Jaggard et al., 2020, 2017; Yoshizawa et al.,
169 2015), and the collective behavior in young adults (under higher Reynold's number; Iwashita and
170 Yoshizawa, 2021). We therefore investigated the shift of collective behavior in 3–4-month-old fish using
171 criteria based on the vicinity of two fish (≤5 cm) and duration of nearby interactions (≥4 s) during
172 tracking in a four-fish group (Iwashita and Yoshizawa, 2021) (Fig. 2B). At 3 months old, ('Pre-
173 treatment' in Fig. 2), surface fish exhibited social-like nearby interactions for 17.0 ± 4.4 s (Fig. 2C) and
174 3.1 ± 0.4 bout number of nearby interactions (Fig. 2D) during the 5-min assay. Conversely, cavefish
175 exhibited an approximately 50% shorter interaction duration (8.3 ± 1.5 s; Fig. 2C) and a smaller bout
176 number of interactions (1.8 ± 0.3; Fig. 2D). To track the effect of the KD treatment, fish were fed the

177 KD for 5 weeks, followed by CD feeding during weeks 6–9 to assess the persistence of the effects of the
178 KD (Fig. 2A, C, and D).

179 The nearby interactions of surface fish did not differ between CD and KD feeding (Fig. 2C and
180 D). By contrast, the nearby interactions of cavefish were significantly decreased by CD feeding
181 compared to the effects of KD feeding in weeks 4 and 5 (Fig. 2C and D), and interactions remained
182 depressed through week 9 by CD feeding. This effect of the KD diet on nearby interactions did not
183 persist. After KD deprivation and CD feeding, the nearby interactions of KD-fed cavefish were
184 indistinguishable from those of CD-fed fish (6–9 weeks, Fig. 2C, and D), suggesting that KD has a
185 promotive/supportive effect on collective behavior in genetically asocial cavefish.

186 To investigate in detail whether KD-promoted nearby interactions have social-like properties, we
187 explored swimming speed, which is an indicator of the movement of fish in the vicinity of each other.
188 Fish are more likely to have an opportunity to express affinity toward each other at a slower swimming
189 speed (Iwashita and Yoshizawa, 2021). Indeed, surface fish moved slower during nearby interactions
190 than during the non-nearby interaction period (Iwashita and Yoshizawa, 2021) (Fig. 3A). Consistently,
191 KD-fed cavefish swam slower during the nearby interaction period than during the non-nearby
192 interaction period ('5 weeks;' Fig. 3B). Overall swimming speed was also slower in the KD group than
193 in the CD group ('5 weeks;' Fig. 3B). These findings indicated that KD-fed cavefish exhibited more
194 social-like nearby interactions with a similar speed profile as surface fish. In surface fish, there was no
195 major difference in swimming speed profiles between CD and KD feeding ('5 weeks;' Fig. 3A). To
196 address whether the slower swimming speed was sufficient to increase nearby interactions, we tracked
197 the total swimming distance within 5 min from pre-treatment to week 9 of feeding (Supplementary Fig.
198 1). KD-fed cavefish exhibited a significantly shorter swimming distance (slower swimming speeds)
199 from the first week of feeding (Supplementary Fig. 1), which was much earlier than when the higher
200 level of nearby interactions emerged (weeks 4–5). This result suggests that although a slower swimming

201 speed is associated with nearby interactions (Fig. 3) and KD feeding reduced swimming speeds in
202 cavefish, a slower speed itself is not sufficient to induce nearby interactions.
203 Repetitive turning is frequently observed in an antagonistic relationship with nearby interactions
204 in cavefish (Iwashita and Yoshizawa, 2021). That is, individuals with few nearby interactions frequently
205 exhibit a high level of turning bias or 'repetitive turning.' Accordingly, CD-fed cavefish with few nearby
206 interactions (4–5 weeks) exhibited significantly higher turning bias than KD-fed cavefish (Fig. 4A, B).
207 KD-fed cavefish displayed a comparable level of balanced turning as surface fish (close to a score of '1'
208 in Fig. 4B). In summary, these results suggest that KD feeding could reduce repetitive turning while
209 maintaining longer nearby interactions.
210 Tracking behavioral changes each week (Fig. 2) may result in confounding factors such as fish
211 remembering the recording environment. To clarify whether our results captured the genuine effects of
212 KD feeding, we repeated 4–5-week dietary treatment in a new set of fish (Supplementary Fig. 2).
213 Similarly, surface fish did not exhibit a detectable change in the duration and number of nearby
214 interactions between CD and KD feeding (Supplementary Fig. 2A, B). By contrast, CD-fed cavefish
215 displayed fewer nearby interactions, whereas the level of nearby interactions was retained in KD-fed
216 cavefish, resulting in a higher level of nearby interactions in KD-fed cavefish (Supplementary Fig. 2A,
217 B). In this repeated experiment, the results for swimming distance and repetitive turning were also
218 similar to those in the previous experiment; specifically, CD-fed cavefish swam longer distances than
219 KD-fed cavefish (Supplementary Fig. 2C), whereas surface fish did not exhibit a detectable shift in
220 swimming distance between the CD and KD groups. Additionally, CD-fed cavefish displayed a high
221 level of turning bias, whereas KD-fed cavefish exhibited balanced turning (Supplementary Fig. 2D).
222
223 We then explored other changes induced by KD feeding, including changes in sleep, 24-h
224 swimming distance, and adherence to a vibrating stimulus, which are distinct between surface fish and
225 cavefish. Cavefish largely exhibit reduced sleep duration and swim almost all day, perhaps to find

226 nutrients in the food-sparse environment (Duboué et al., 2012, 2011; Jaggard et al., 2017; Yoshizawa et
227 al., 2015). After 5 weeks of dietary treatment on the 3 – 4 months old fish, both surface fish and cavefish
228 exhibited a shorter sleep duration than observed before treatment regardless of the diet (Fig. 5A,
229 particularly at night), suggesting growth between 3 – 4 and 4 – 5 months old exerted a negative effect on
230 the sleep duration. However, there was no detectable difference between CD and KD feeding.

231 Animals' sleep is usually fragmented, involving repeated sleep/awake cycles during the night
232 (diurnal animals) or day (nocturnal animals) (Campbell and Tobler, 1984). Then, the structure and
233 regulation of sleep are typically analyzed according to the number of events (bout). Our detailed sleep
234 analysis illustrated that KD-fed cavefish displayed fewer sleep bouts during the night than CD-fed
235 cavefish (Fig. 5B). However, the number of sleep bouts did not differ between CD and KD feeding (5
236 weeks; Supplementary Fig. 3). Overall, the sleep phenotype was not dramatically changed by KD
237 feeding, and cavefish exhibited a shortened sleep duration.

238 The sleep duration is negatively correlated with the 24-h swimming distance (Yoshizawa et al.,
239 2015). Cavefish displayed overall higher activities, which was consistent with previous findings
240 (Duboué et al., 2011; Yoshizawa et al., 2015) and consistent with the findings of longer swimming
241 distance in the nearby interaction assay (Fig. 5C). CD-fed cavefish swam longer distances than KD-fed
242 cavefish. Surface fish, in contrast, did not exhibit a detectable difference in swimming distance between
243 KD and CD feeding. Overall, the KD treatment induced little changes in sleep behaviors in both surface
244 and cavefish.

245 In general, the KD is assumed to induce ketosis without increasing appetite. We then checked the
246 shift of foraging behavior under KD feeding. Cavefish evolutionarily exhibit increased foraging
247 behavior, termed VAB, in which fish adhere to a particular vibration stimulus (35–40 Hz) in the dark
248 (Yoshizawa et al., 2010). VAB is advantageous for prey capture in the dark. Cavefish and surface fish
249 did not exhibit a detectable difference in VAB between CD and KD feeding, whereas VAB was
250 significantly increased during 1 month of growth (pre-treatment vs. 5 weeks; Supplementary Fig. 4A).

251 The swimming distance in 3-min VAB assays was decreased in KD-fed cavefish, consistent with the
252 nearby interaction and sleep studies (5 weeks; Supplementary Fig. 4B). In summary, the VAB analysis
253 indicated that KD feeding did not increase a foraging behavior.

254 Although the KD diet induced significant changes in some behavioral outputs, it suppressed
255 growth during treatment. The average weights of KD-fed surface fish and cavefish were 55.5 % and
256 69.9 % of those in their CD-fed counterparts, respectively (5 weeks; Fig. 6B). The standard length of
257 KD-fed surface fish was also significantly reduced (5 weeks; Fig. 6A).

258

259 Are these behavioral and growth changes induced by ketosis? The KD contains high amounts of
260 fat and other ingredients. This question motivated us to test the molecular basis of the effects of the KD
261 by adding major ketosis metabolites to the standard diet.

262 In humans, the KD induces ketosis, in which the liver releases the major ketone body, beta-
263 hydroxybutyrate (BHB), via beta-oxidation of fat (Evans et al., 2017). Instead of supplying a massive
264 amount of fat using the KD, BHB (sodium salt form of racemic BHB: 50% L-form and 50% D-form;
265 only the D-form is considered to be biologically active) might be responsible for the majority of effects
266 observed after KD feeding. With this idea, sodium salt BHB was provided to both surface fish and
267 cavefish for 4 weeks. The result indicated that the BHB supplemental diet promoted nearby interactions
268 in cavefish (Fig. 7A and B) and reduced the duration of nearby interactions in surface fish (Fig. 7A).
269 Turning bias tended to be reduced by BHB supplementation in cavefish, although significance was not
270 achieved (Fig. 7C). Swimming distance was not reduced in surface fish or cavefish (Fig. 7D). The body
271 growth of BHB-treated surface fish and cavefish was comparable to that in control fish (standard length
272 and weight; Supplementary Fig 5A and B, respectively). The night-time sleep and VAB did not exhibit
273 detectable differences between control and BHB treatment (Supplementary Fig. 6A and B, respectively)

In summary, BHB treatment covered many effects of KD treatment did, including changes in social interactions and repetitive turning. BHB had fewer negative effects on growth and swimming activities, suggesting that ketone bodies are responsible for the ‘positive’ effects of KD feeding.

Discussion

In this study, we examined the behavioral shifts induced by KD feeding and BHB supplementation. Ketosis is expected to occur frequently in wild animals because of a failure to find food (fasting) or an absence of carbohydrate inputs/synthesis (available nutrients). Certain levels of socialness can be beneficial to animal species for mating and finding food. Under KD feeding, cavefish maintained their juvenile level of nearby interactions until the treatment ended (5 weeks). Nearby interactions were then reduced to an indistinguishable level from the control levels within 1 month after stopping KD feeding. Surface fish exhibited a higher number of nearby interactions than cavefish, and no detectable difference was observed in nearby interaction levels between CD- and KD-fed surface fish. KD feeding also effectively reduced repetitive turning in cavefish, whereas CD-fed treated cavefish exhibited a high level of repetitive turning. Under KD feeding, both surface fish and cavefish significantly reduced swimming distances during development, during which the swimming distance typically becomes longer. There were no detectable changes in sleep and foraging behavior (VAB) under 1 month of KD feeding. These patterns in behaviors and growth were not changed in two replicated experiments (social affinity and repetitive turning), supporting the consistency of the observed effects under KD feeding. Finally, the major KD metabolite, BHB, could cover the KD effect, indicating the ketone body has the pivotal role on this treatment.

Effects of the KD on blood ketone levels and body growth

Under 4–5 weeks of KD feeding, blood ketone and glucose levels were reduced compared to the effects of the CD in both surface fish and cavefish, contradicting our expectation that ketone levels would be higher in the KD group. However, GKI (Meidenbauer et al., 2015) was significantly lower under KD feeding than under CD feeding. These significant changes in GKI in both surface fish and cavefish suggest that the metabolic condition was shifted toward ketosis by KD feeding. In general, cavefish had a higher GDI than surface fish under both diets, suggesting that the cavefish physiology

304 was constitutively biased toward glycolysis. For example, blood glucose levels in cavefish under KD
305 feeding were similar to those in surface fish under CD feeding, whereas cavefish had 3-fold lower
306 ketone levels than surface fish under CD feeding, resulting in a higher GKI even under KD feeding.
307 KD feeding for 4–5 weeks also resulted in slowed body growth. This growth retardation has been
308 observed in patients with epilepsy chronically fed a KD (Coppola et al., 2010; Napoli et al., 2014), and
309 these results were consistent with our observations in KD-fed fish. The detailed molecular/physiological
310 mechanisms are largely unknown, but this study identified BHB as a potential candidate mediating KD-
311 associated phenotypes (see below).
312
313 *Effects of ketones in the TCA cycle and epigenetics in the brain*
314 In mammals, KD feeding causes a 'starvation'-like state, causing the liver to release ketone
315 bodies into the bloodstream. BHB is the major ketone body produced by the liver through beta-
316 oxidation. The gut epithelia also absorb and circulate ketone bodies from the diet and/or gut microbiota.
317 Both liver- and gut-derived ketone bodies can cross the blood–brain barrier and exert two functions: (i)
318 inhibit histone deacetylase, which influences epigenetic regulation and induces gene expression in
319 neurons; and (ii) act as a general energy source that is converted into acetyl-CoA to fuel the aerobic
320 TCA cycle in neurons. Both pathways have the potential to alter brain function. The facts that cavefish
321 easily tolerate high blood glucose levels, at which surface fish was paralyzed (Riddle et al., 2018), and
322 Wnt signaling is upregulated in cavefish, potentially resulting in high glycolytic activity as humans do
323 (Vallée and Vallée, 2018; Yoshizawa et al., 2018), support the aforementioned hypothesis that cavefish
324 exhibit high blood glucose levels and generate energy via glycolysis. Also, ketone bodies can also
325 promote behavioral shifts by changing the epigenetic state by inhibiting histone deacetylase
326 (Krautkramer et al., 2017; Szyf, 2015). Histone deacetylase inhibition increases gene expression in
327 general. This possibility is supported by the fact that cavefish have more downregulated genes (2913
328 genes, $\log_2 < -1.0$) than upregulated genes (1643 genes, $\log_2 > 1.0$) in the transcriptome at 72 h post-

329 fertilization (Gross et al., 2013; Yoshizawa et al., 2018). In addition, more methylated loci are found in
330 the eye genes of cavefish than in surface fish, which could also be true for other tissues (Gore et al.,
331 2018), and most of these methylated gene loci were downregulated. The brains of patients with autism
332 are also expected to be hypermethylated, resulting in a transcription-less condition (Zhu et al., 2014).
333 Therefore, these two pathways, namely metabolism and epigenetics, were highlighted as possible targets
334 of ketone bodies during behavioral shifts under ketosis. Future research should address these
335 possibilities to clarify the metabolism-based evolution of behavior (*cf.* (Qin et al., 2021)
336
337 *Ontogeny of nearby interactions and the KD*
338 In this study, 3-4 months-old cavefish exhibited a weak but detectable level of nearby
339 interactions (social affinity), and this social affinity decayed under CD feeding. Interestingly, KD-fed
340 cavefish and surface fish fed either diet maintained a similar level of nearby interactions during 5 weeks
341 of dietary feeding. The reduction of nearby interactions in CD-treated cavefish can be explained by (1)
342 quicker exhaustion under CD feeding (aerobic ketosis produces more adenosine triphosphate than
343 anaerobic glycolysis), (2) greater anxiety in the recording environment (Iwashita and Yoshizawa, 2021),
344 and (3) less social motivation. The first explanation is unlikely because CD-fed cavefish swam longer
345 distances than KD-fed cavefish. The higher level of anxiety could explain the findings because cavefish
346 exhibited increased repetitive turning, which is related to higher anxiety in mammals (Langen et al.,
347 2011). In addition, cavefish displayed fewer nearby interactions in an anxiety-associated unfamiliar
348 environment in prior research (Iwashita and Yoshizawa, 2021). In the future, the anxiety level should be
349 monitored using plasma cortisol levels in the future (Gallo and Jeffery, 2012). Less motivation regarding
350 social affinity is also a possible cause, and this variable can be monitored by assessing activities in social
351 decision-making networks including the preoptic area, nucleus accumbens, and striatum (O'Connell and
352 Hofmann, 2012, 2011). Explanations (2) and (3) are not mutually exclusive, and co-occurrence is
353 possible. These possibilities will be assessed in our future study.

354

355 *Possible target system for ketosis*

356 Under KD feeding and BHB treatment, we observed increased social affinity and reduced
357 repetitive turning and swimming distances. However, we detected no changes in sleep and VAB. From
358 the knowledge of neurotransmitters and their associated behaviors, these behavioral phenotypes
359 indicated the possible involvement of the dopaminergic and serotonergic systems but less involvement
360 of the cholinergic, orexin/hypocretinergic, histaminergic, or adrenergic system. The mechanism by
361 which ketosis or ketone bodies more strongly affect the dopaminergic and serotonergic systems than the
362 other systems is unclear. However, the findings that the dopaminergic and serotonergic systems are
363 sensitive to ketosis in terms of evolution are extremely interesting in its mechanism and for future
364 therapy applications.

365

366 *Ketosis in the cave environment*

367 Cave-dwelling animals usually experience less temperature fluctuation and fewer dietary inputs
368 (Culver and Pipan, 2009), but these features can vary. The diets of cave-dwelling animals in the dry
369 season (approximately 6 months/year) could be organic matter in the pool bottoms, bat guano (larger
370 adults), or small crustaceans (smaller fish), whereas food is sparse in the rainy season (approximately 6
371 months/year) (Espinasa et al., 2021, 2017). These available diets contain extremely low amounts of
372 carbohydrates, and they can be high in protein and fat (e.g., crustaceans). Although some amino acids,
373 lactate, and glycerol can be used for glucose synthesis in fish (Polakof et al., 2012), cavefish are
374 expected to be exposed to carbohydrate-deprived diets or frequent fasting and therefore frequent ketosis.
375 In prior research, wild cavefish swam slower and exhibited similar social affinity as observed in KD-fed
376 cavefish in this study (Movie 1). Although these observations and dietary inputs suggested that wild
377 cavefish may be under frequent ketosis, recent multiple reports indicated that cavefish may be under
378 anaerobic glycolysis to adapt to anaerobic cave water conditions because of the approximately 20%

379 lower oxygen level in cave pools (Boggs et al., 2022; van der Weele and Jeffery, 2022). In addition,
380 cavefish tend to store lipids instead of using them through the enhanced PPARγ pathway (Xiong et al.,
381 2022). These expectations of low ketosis appear to contradict expectations in the wild—starved ketosis
382 conditions. However, it appears to fit well with the findings in cavefish, namely high blood glucose and
383 low ketone levels even under KD feeding in this study, as well as higher GKI in cavefish than in surface
384 fish in both the CD and KD groups (Fig. 1D). Cavefish appear to have evolved to maintain a high GKI
385 (high blood glucose and low ketone levels); therefore, the physiology of cavefish may allow them to
386 survive in the low-oxygen condition by using anaerobic glycolysis. KD-fed cavefish behave similarly as
387 wild cavefish because the balance between ketosis and glycolysis could reach a similar level as that in
388 the wild after KD feeding. By contrast, if cavefish are fed a typical carbohydrate-rich lab fish diet, it
389 may overactivate glycolysis and result in a higher GKI, which may lead to reduced social affinity and
390 increased repetitive circling and swimming distance. The future use of a pharmacological glycolysis
391 inhibitor (e.g., 2-deoxy-D-glucose; Yao et al., 2011) can reveal the relationship between GKI and
392 cavefish behaviors.
393
394 *Summary statement*
395 Solitary animals surprisingly share a set of dysregulated genes and behavioral outputs. In this
396 study, we demonstrated that a diet that induces ketosis shifts these behaviors toward the surface fish
397 phenotype regardless of the presence of many dysregulated genes. In addition to the gene therapy
398 approach, ketone body-based treatment may open a path for sustainable and less toxic therapy for
399 multigenic psychiatric disorders, including autism, although the target pathways remain unclear.
400 Concerning the genetics of behavior, differentially expressed metabolic genes have been largely
401 overlooked because it was difficult to interpret. Because mitochondria-based disorders are highlighted in
402 neuroscience (Chauhan et al., 2012; Rajasekaran et al., 2015), the balance between glycolysis and
403 ketosis could be the starting point for identifying a therapeutic target. The known evolved behaviors

404 should be also revisited by investigating whether the metabolic shift promotes the variations of

405 behaviors.

406

407 **Materials and Methods**

408 Fish maintenance and rearing in the lab

409 *A. mexicanus* surface fish used in this study were the laboratory-raised descendants of original
410 collections created in Balmorhea Springs State Park in Texas in 1999 by Dr. William R. Jeffery.
411 Cavefish were laboratory-raised descendants originally collected from Cueva de El Pachón (Pachón
412 cavefish) in Tamaulipas, Mexico in 2013 by Dr. Richard Borowsky.

413 Fish (surface fish and Pachón cave populations) were housed in the University of Hawai‘i at
414 Mānoa *Astyanax* facility with temperatures set at 21 ± 0.5°C for rearing, 24 ± 0.5°C for behavior
415 experiments, and 25 ± 0.5°C for breeding (Elipot et al., 2014; Yoshizawa et al., 2015). Lights were
416 maintained on a 12-h:12-h light:dark cycle (Elipot et al., 2014; Yoshizawa et al., 2015). For rearing and
417 behavior experiments, the light intensity was maintained at 30–100 Lux. Fish husbandry was performed
418 as previously described (Elipot et al., 2014; Keene et al., 2016; Yoshizawa et al., 2015). Fish were raised
419 to adulthood and maintained in standard 42-L tanks in a custom-made water-flow tank system. Adult
420 fish were fed a mixed diet to satiation twice daily starting 3 h after the lights were turned on (Zeitgeber
421 time 3 [ZT3] and ZT9; Zeigler Adult zebrafish irradiated diet, Zeigler Bros, Inc, Gardners, PA;
422 TetraColor Tropical Fish Food Granules, Tetra, Blacksburg, VA, USA; Jumbo Mysis Shrimp, Hikari
423 Sales USA, Inc., Hayward, CA, USA). All fish in the behavioral experiments were between 2.5 and 5
424 cm in standard length and between 3 and 12 months old. Fish ages were stated in each experiment. All
425 fish care and experimental protocols were approved under IACUC (17-2560) at the University of
426 Hawai‘i at Mānoa.

427

428 Ketogenic diet

429 To prepare the KD, we used a mixture of a human KD (KetoCal3:1) and zebrafish standard diet
430 (adult zebrafish irradiated diet) in a 5:1 ratio. The gross caloric amounts were 6.99 kcal/g for KetoCal3:1
431 and 3.89 kcal/g for the zebrafish diet. Regarding the CD, we used the same KetoCal3:1 and zebrafish

irradiated diet but mixed at a 1:5 ratio. The KetoCal3:1 powder and ground zebrafish irradiated diet were mixed in the aforementioned ratios and solidified with 1% agar at a final concentration of 20% w/v (2 g of mixture in 10 mL of 1% agar). After solidification, both KD and CD agar was cut into 3-mm^3 cubes, and each four-fish group was given 1–2 pieces every morning (ZT 0:00–3:30) and afternoon (ZT 8:00–12:00). The fish were fed ad libitum in each feeding and the remaining amount was removed 1 h after feeding using a pipette.

Behavior assays

The protocol for social-like nearby interactions was described previously (Iwashita and Yoshizawa, 2021). Briefly, four fish raised in a home tray (15.6 × 15.6 × 5.7 cm^3 Ziploc containers, S. C. Johnson & Sons, Inc, Racine, WI, USA) were released in a recording arena (49.5 × 24.2 × 6.5 cm^3) with a water depth of 3 cm on the stage of a custom-made infrared (IR) back-light system within a custom-built black box (75 × 50 × 155 cm, assembled with polyvinyl chloride pipe frame and covered by shading film). The IR back-light system was composed of bounce lighting of IR LED strips (SMD3528 850 nm strip: LightingWill, Guang Dong, China). The video was recorded at 20 frame/s using VirtualDub2 software (build 44282; http://virtualdub2.com/) with the x264vfw codec for 6 min, and the last 5 min were used for the analysis. After the recording, the fish were returned to the home tray. The X-Y coordinates of each fish were calculated using idTracker software (Pérez-Escudero et al., 2014) after the video image was processed for background subtraction using ImageJ (Iwashita and Yoshizawa, 2021). This X-Y coordinate was also used for the turning bias analysis. The duration and number of nearby interactions and swimming speed during and after nearby interaction events were calculated using custom-made MATLAB script (MathWorks Inc., Natick, MA, USA) (Iwashita and Yoshizawa, 2021).

The turning bias rate was calculated as $\frac{Nl}{Ns}$, where Ns and Nl represent a smaller (Ns) or larger (Nl) number of left or right turns. This turning bias rate indicates the extent to which fish turning is

457 biased to the left or right, and ranging from ‘1’ (L-R balanced) to exponential (L or R biased). The
458 numbers of left or right turns were calculated as changes in the angles of swimming directions in every
459 five frame window (0.25 s) as described previously (Iwashita and Yoshizawa, 2021). An automatic
460 calculation of the total number of the left or right turns is implemented in the aforementioned homemade
461 MATLAB script.

462 Analyses of sleep and swimming distance were described previously (Yoshizawa et al., 2018,
463 2015). Briefly, fish were recorded in a custom-designed 10.0-L acrylic recording chamber (457.2 ×
464 177.8 × 177.8 mm^3 and 6.4 mm thick) with opaque partitions that permit five individually housed fish
465 per tank (each individual chamber was 88.9 × 177.8 × 177.8 mm^3). The recording chamber was
466 illuminated with a custom-designed IR LED source the light-controlled room on a 12-h:12-h cycle. The
467 room light was turned on at 7:00 am and turned off at 7:00 pm each day. Behavior was recorded for 24 h
468 after overnight (18–20 h) acclimation beginning 1–2 h after turning the light on (ZT1–2). Videos were
469 recorded at 15 frames/s using a USB webcam with an IR high-pass filter. Videos were captured by
470 VirtualDub2 software with the x264vfw codec and subsequently processed using Ethovision XT
471 (Version 16, Noldus Information Technology, Wageningen, Netherlands). Water temperature was
472 monitored throughout the recordings, and no detectable differences were observed during the light and
473 dark periods (24.0 ± 0.5°C). The visible light during behavior recordings was approximately 30–100
474 Lux.

475 The tracking parameters for detection were as follows: the detection was set to ‘subject brighter
476 than background’ and brightness contrast was set from 20 to 255; the current frame weight was set to 15;
477 the video sample rate was set to 15 frames/s; and pixel smoothing was turned off. We monitored sleep,
478 activity, and arousal thresholds via protocols previously established for *A. mexicanus* (Yoshizawa et al.,
479 2015). The X-Y coordinates of each fish were subsequently processed using custom-written Perl
480 (v5.23.0, www.perl.org) and Python scripts (3.8).

481 We assayed VAB as described previously (Yoshizawa et al., 2015, 2012, 2010). Briefly, fish
482 were permitted to acclimate for 4–5 days prior to the assay in a cylindrical assay chamber (325-mL glass
483 dish, 10 cm in diameter and 5 cm in height, VWR, Radnor, PA, USA) filled with conditioned water (pH
484 6.8–7.0; conductivity 600–800 μS). During the assays, vibration stimuli were created using a glass rod
485 that vibrated at 40 Hz. The number of approaches to the vibrating rod was video recorded during a 3-min
486 period under infrared illumination. The number of fish approaches in a 1.3-mm radius from the vibrating
487 glass rod were analyzed using the X-Y coordinate of each fish head detected by the trained DeepLabCut
488 model (Mathis et al., 2018; Fernandez et al., *submitted*)
489
490 Blood ketone and glucose measurements
491 The fish used to measure ketone and glucose levels were the siblings of the fish used for the
492 behavioral assay. Eight fish were used for each treatment group (CD vs. KD, surface fish vs. cavefish;
493 four groups in total). All blood samples were collected 2 h after feeding. Fish were then deeply
494 anesthetized in ice-cold water, and blood was collected from the tail artery. Blood ketone and glucose
495 levels were measured using an Abbott Precision Xtra blood glucose & ketone monitoring system
496 (Abbott Laboratories, Abbott Park, IL, USA)
497
498 Measurement of tissue structure
499 Fish were anesthetized with 0.2 mg/mL ethyl 3-aminobenzoate methanesulfonate (MS-222:
500 MilliporeSigma, Burlington, MA, USA) in ice-cold conditioned water (pH: 7.0; conductivity: 700 μS),
501 and weight was measured after taking pictures with a standard camera (Pentax K-1 DSLR with 35-70
502 mm zoom lens, Ricoh, Tokyo, Japan). The standard body length and body depth were measured using
503 ImageJ.
504
505 Statistical analysis

506 Regarding the power analysis, we designed our experiments based on three-way repeated-
507 measures ANOVA with a moderate effect size (f = 0.25), alpha-error probability of 0.05, and power of
508 0.80, and the number of groups was eight (surface fish vs. cavefish × non-treated vs. treated × pre-
509 treatment vs. post-treatment). G*Power software (Erdfelder et al., 2009; Faul et al., 2007) estimated that
510 the sample size needed for this experiment was nine per group. We thus aimed to use at least 12 fish in
511 each group for all experiments in this study.

512 For statistical comparisons of our data, we performed tests including Student's *t*-test and two- or
513 three-way generalized linear model analyses to compare surface and cavefish, treatment and non-
514 treatment, and pre-treatment and post-treatment. We calculated Akaike's information criterion (AIC) for
515 each linear and generalized linear model and chose the model with the lowest AIC. Holm's post hoc
516 correction was used to understand which contrasts were significant (Holm, 1979).

517 Regarding replicates of experiments, we used different individuals for the replicates, namely two
518 biological replicates, using different individuals in each trial (*e.g.*, Fig. 2 and Supplementary Fig. 2).
519 There was no repeated usage of individual fish excluding the time-course experiment (Fig. 2). For the
520 experiments measuring sleep and VAB in addition to nearby interactions and turning bias (Figs. 5–6,
521 Supplementary Figs. 2–4), we used two biological replicates and confirmed that the averages of
522 experimental data did not largely differ from each other. We then merged the data acquired in two
523 biological replicates and presented the data as a single set of results.

524 The aforementioned calculations were performed using R version 4.0.4 software (packages of
525 car, lme4, and lmerTest) (Bates et al., 2015; Fox and Weisberg, 2019; Kuznetsova et al., 2017), and all
526 statistical scores are available in Supplementary Table 1, the figure legends, or the text.

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528
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746

Figure Legends

Figure 1. Blood glucose and ketone levels under the control diet (CD) or ketogenic diet (KD).
(A) Experimental procedure. After fish were raised for 3–4 months on a brine shrimp larva diet, fish were fed the CD or KD for 5 weeks. After 5 weeks, blood glucose and ketone levels were measured. (B) Blood ketone level (mmol/L). Ketone levels were significantly reduced by KD feeding in both surface fish (SF) and cavefish (CF). Data are presented as the mean ± standard error of the mean. Dots indicate individual data. (C) Blood glucose level (mg/dL). Glucose levels were significantly reduced by KD feeding in both SF and CF. (D) The glucose ketone index (GKI) indicated that the ratio of glucose to ketone was lowered by KD feeding in both SF and CF, suggesting that this diet altered the balance between glucose and ketone.
SF: N = 13 for CD feeding, N = 8 for KD feeding. CF: N = 13 for CD feeding, N = 11 for KD feeding. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. All detailed statistical data are available in Supplementary Table 1.

Figure 2. Time course of nearby interaction changes during 9 weeks of control diet (CD) or ketogenic diet (KD) feeding.
(A) Experimental procedure. After rearing fish for 3–4 months on a brine shrimp larva diet, the pre-treatment recording was performed, followed by CD or KD feeding for 5 weeks. Nearby interactions were recorded every week until week 6 of feeding. Subsequently, all groups including KD-fed fish were given the CD until week 9. (B) An example of nearby interaction events among surface fish (SF). The left panel presents an example frame of the video. The colored lines indicate the trajectories of individual fish. A red-labeled fish was followed by a blue-labeled fish. Each nearby event that met the detection criteria, namely a distance of ≤ 5 cm between two fish that was maintained for more than 4 s, was counted as a nearby interaction event. The right panel presents an example of the detected events presented in a raster plot (each yellow bar indicates a nearby interaction event). Each pair of fish (six pairs among four fish) is presented in the rows. (C) Duration of nearby interactions. Although SF did not exhibit any differences in the duration of nearby interactions (s) between CD (green) and KD (blue) feeding, differences were detected among cavefish (CF) in week 5. However, the nearby interaction duration was indistinguishable from that of the CD group starting in week 6 when the KD was withdrawn from the experimental group. (D) Number of nearby interactions. Whereas SF exhibited no differences between CD and KD feeding, differences were observed in CF in weeks 4–6. After the KD was withdrawn in week 6, the number of events decreased to the level observed with CD feeding.
Data are presented as the mean ± standard error of the mean. Dots indicate individual data. N = 20 for each group. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. All detailed statistical data are available in Supplementary Table 1.

Figure 3. Ketogenic diet (KD) feeding induced surface fish (SF)-like speed profiles during nearby interactions in cavefish (CF).
Swimming speed changes before, during, and after nearby interaction events in SF (A) and CF (B). The mean swimming speeds (i) for 4 s before the nearby interaction event, (ii) during the event, (iii) during 4 s after the event, and (iv) during the out-of-event period were plotted (see the top-left inset of A). (A) Swimming speed was reduced during nearby interactions in SF in both the CD and KD groups. This profile was clearer in the fifth week (right panel). (B) Swimming speed was reduced during nearby interactions only in the KD group in week 5 (right panel). The bars indicate the 25th percentiles, medians, and 75th of the data points. Dots indicate individual data. SF: N = 11 for CD, N = 20 for KD. CF: N = 16 for CD, N = 15 for KD. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. All detailed statistical data are available in Supplementary Table 1.

Supplementary Figure 1. Swimming distance of cavefish was suppressed by ketogenic diet (KD) feeding.
Surface fish exhibited an increase in the swimming distance over time under both the control diet (CD) and KD. By contrast, in cavefish, the swimming distance and activity were suppressed by KD feeding starting in week 1, and these values remained smaller than those in CD-fed fish until week 6 when the KD was withdrawn. KD-fed cavefish subsequently exhibited an increased swimming distance (week 0). Data are presented as the mean ± standard error of the mean. Dots indicate individual data. N = 20 in all groups. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. All detailed statistical data are available in Supplementary Table 1.

Figure 4. Biased turning was attenuated by the ketogenic diet (KD).
(A) Diagram and the calculation formula for the turning bias index. The changes in the left or right traveling directions were calculated every five frames (every 0.25 s) across all trajectories and expressed as radians. Positive radian values represent left (anticlockwise) turning, and negative values indicate right turning. The ratio between the numbers of clockwise and anticlockwise turns was used as the turning rate (1 – infinity, positive value). (B) Turning biases of surface fish (left) and cavefish (right). There was no difference between CD and KD feeding in surface fish, whereas the turning index in CD-fed cavefish than in KD-fed cavefish (see week 6).
Data are presented as the mean ± standard error of the mean. Dots indicate individual data. N = 20 for all groups. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. All detailed statistical data are available in Supplementary Table 1.

Supplementary Figure 2. Consistent results were obtained in the repeated experiment for the duration and number of nearby interactions, swimming distance, and turning bias under control diet (CD) or ketogenic diet (KD) feeding.
(A) The duration of nearby interactions, (B) the number of nearby interactions, (C) swimming distance, and (D) turning bias are presented. The overall tendencies were the same as those observed in the original experiments (Figs. 2 and 4, Supplementary Fig. 1). Surface fish did not exhibit any significant differences regarding the duration (A) or number of nearby interactions (B), the swimming distance (C) or the turning bias (D). The duration (A) and number of nearby interactions were maintained in KD-fed cavefish, (B) whereas they were reduced in CD-fed cavefish, which also exhibited a higher level of turning bias (D). The swimming distance was not significantly reduced in KD-fed cavefish compared to that in CD-fed controls in this repeated experiment (C).
Data are presented as the mean ± standard error of the mean. Dots indicate individual data. Surface fish: N = 28 for CD, N = 32 for KD. Cavefish: N = 28 for CD, N = 32 for KD. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. All detailed statistical data are available in Supplementary Table 1.

Figure 5. Day and night sleeping durations and swimming distances were not altered by ketogenic diet (KD) feeding.
(A) Sleep duration (min/h) during the day (left) and night (right). During 5 weeks of growth, the sleep duration decreased in surface fish and cavefish regardless of the diet. (B) Number of events (min/10 min) during the day (left) and night (right). During 5 weeks of growth, the number of sleep events was lower in surface fish under both dietary conditions and in KD-fed cavefish. (C) Swimming distance during the day (left) and night (right). Control diet (CD)-fed cavefish exhibited a longer swimming distance during the day and night. Conversely, surface fish fed either diet and cavefish fed the KD exhibited a significantly increased swimming distance only at night.

Data are presented as the mean ± standard error of the mean. Dots indicate individual data. Surface fish: N = 28 for CD, N = 32 for KD. Cavefish: N = 28 for CD, N = 32 for KD. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. All detailed statistical data are available in Supplementary Table 1.

Supplementary Figure 3. Daytime and nighttime number of sleeping events under control diet (CD) or ketogenic diet (KD) feeding.

After the 5 weeks of growth, surface fish exhibited a reduced number of sleeping events during the day under both diets. CD-fed cavefish exhibited reduced numbers of sleeping events during the day and night. However, the number of sleeping events did not differ according to the diet in cavefish or surface fish.

Data are presented as the mean ± standard error of the mean. Dots indicate individual data. Surface fish: N = 28 for CD, N = 32 for KD. Cavefish: N = 28 for CD, N = 32 for KD. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. All detailed statistical data are available in Supplementary Table 1.

Supplementary Figure 4. Vibration attraction behavior (VAB) and swimming distance during VAB under control diet (CD) or ketogenic diet (KD) feeding.

(A) Number of approaches to the vibration rod in the 3-min assays. After 5 weeks of growth, the number of approaches was increased in CD- and KD-fed cavefish, but no difference according to the diet was detected in either surface fish or cavefish. (B) Swimming distance during VAB. KD-fed cavefish swam significantly shorter distances than CD-fed cavefish.

Data are presented as the mean ± standard error of the mean. Dots indicate individual data. Surface fish: N = 28 for CD, N = 32 for KD. Cavefish: N = 28 for CD, N = 32 for KD. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. All detailed statistical data are available in Supplementary Table 1.

Figure 6. Body length and weight under control diet (CD) or ketogenic diet (KD) feeding.

(A) Standard length (cm). KD-fed surface fish and cavefish were significantly smaller than their CD-fed counterparts. (B) Body weight (g). KD-fed surface fish and cavefish weighed less than their CD-fed counterparts.

Data are presented as the mean ± standard error of the mean. Dots indicate individual data. Surface fish: N = 28 for CD, N = 32 for KD. Cavefish: N = 28 for CD, N = 32 for KD. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. All detailed statistical data are available in Supplementary Table 1.

Figure 7. Nearby interactions and other behaviors under control diet (CD) or beta-hydroxybutyrate–supplemented diet (BHB) feeding.

(A) Duration of nearby interactions (s). After 4 weeks, the duration of nearby interactions was decreased in BHB-treated surface fish and increased in BHB-treated cavefish. (B) Number of nearby interactions. The number of nearby interactions was increased in BHB-treated cavefish. (C) Turning bias ratio. BHB-treated cavefish tend to exhibit decreased biased turning, although this reduction was not significant. (D) Swimming distance. No difference was detected between the CD and BHB groups.

Data are presented as the mean ± standard error of the mean. Dots indicate individual data. N = 20 for all groups. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. All detailed statistical data are available in Supplementary Table 1.

Supplementary Figure 5. Body size and weight under control diet (CD) or beta-hydroxybutyrate-supplemented diet (BHB) feeding.

(A) Standard length (cm). No statistical difference was found for either diet. (B) Body weight (g). BHB-treated surface fish exhibited significantly reduced weight, whereas the weight of cavefish did not differ according to the diet.

890 Data are presented as the mean ± standard error of the mean. Dots indicate individual data. N = 20 for all
891 groups. *: P < 0.05, **: P < 0.01, ***: P < 0.001. All detailed statistical data are available in
892 Supplementary Table 1.
893
894 **Supplementary Figure 6. Day and night sleeping durations and vibration attraction behavior**
895 **(VAB) were not drastically changed by beta-hydroxybutyrate-supplemented diet (BHB) feeding.**
896 (A) Sleep duration (min/h) during the day (left) and night (right). During 4 weeks of treatment, the
897 daytime sleep duration in surface fish and cavefish increased in BHB feeding but not in the nighttime.
898 (B) Number of approaches per the 3 min assay (VAB level). During 4 weeks of treatment, the VAB
899 level did not shift in surface fish or cavefish regardless of diets. Data are presented as the mean ±
900 standard error of the mean. Dots indicate individual data. N = 20 for all groups. *: P < 0.05, **: P <
901 0.01, ***: P < 0.001. All detailed statistical data are available in Supplementary Table 1.

Figure 1

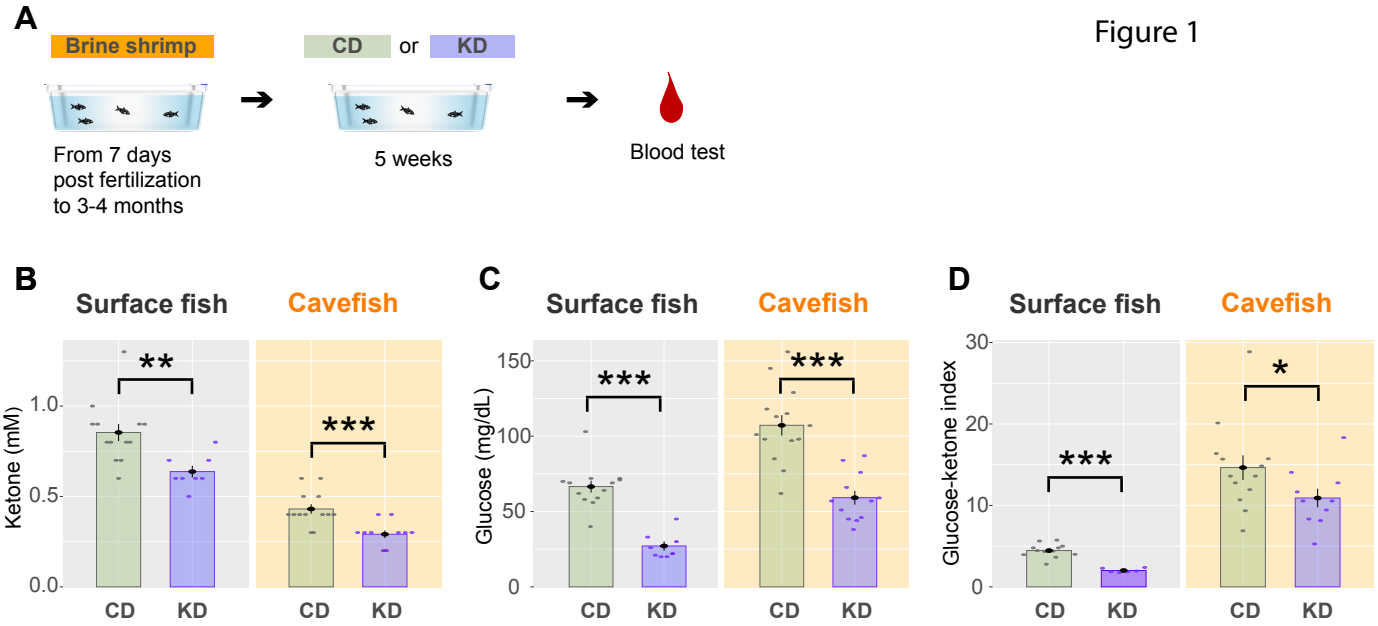

A
Brine shrimp
CD
or
KD
From 7 days
post fertilization
to 3-4 months
5 weeks
Blood test
B
Surface fish
Cavefish
Ketone (mM)
1.0
0.5
0.0
**
***
CD
KD
C
Surface fish
Cavefish
Glucose (mg/dL)
150
100
50
0
***
***
CD
KD
D
Surface fish
Cavefish
Glucose-ketone index
30
20
10
0
***
*
CD
KD

Figure 2

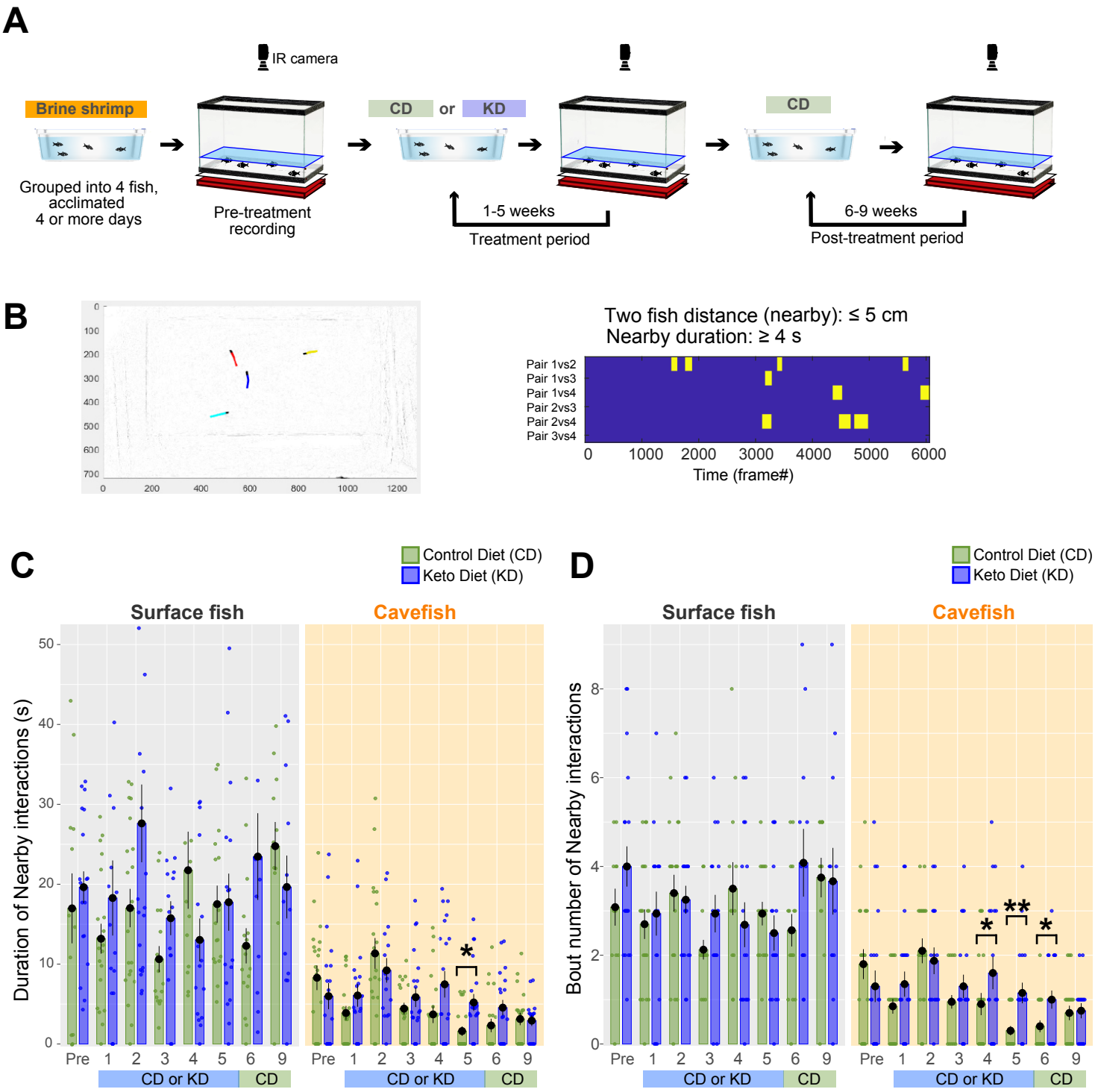
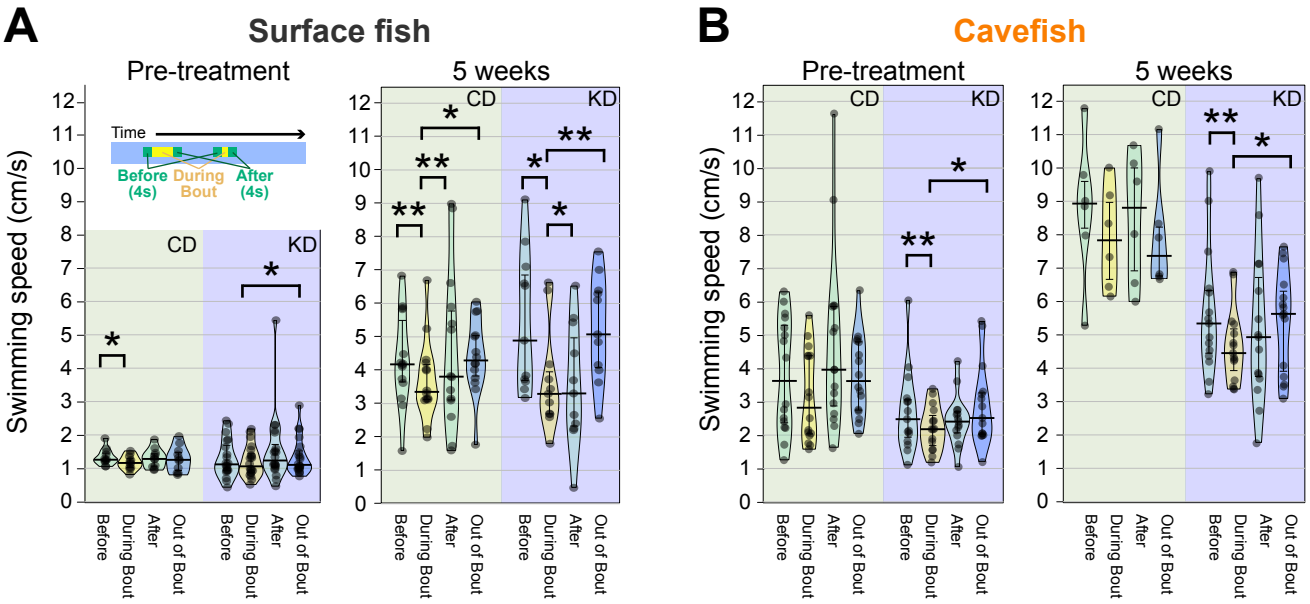

A
IR camera
Brine shrimp
Grouped into 4 fish, acclimated 4 or more days
Pre-treatment recording
CD or KD
1-5 weeks
Treatment period
CD
6-9 weeks
Post-treatment period
B
Two fish distance (nearby): ≤ 5 cm
Nearby duration: ≥ 4 s
Pair 1vs2
Pair 1vs3
Pair 1vs4
Pair 2vs3
Pair 2vs4
Pair 3vs4
Time (frame#)
C
Control Diet (CD)
Keto Diet (KD)
Surface fish
Cavefish
Duration of Nearby interactions (s)
Pre
CD or KD
CD
D
Bout number of Nearby interactions

Figure 3


A
Surface fish
Pre-treatment
5 weeks
Time
Before (4s)
During Bout
After (4s)
CD
KD
Swimming speed (cm/s)
Before
During Bout
After
Out of Bout
B
Cavefish
Pre-treatment
5 weeks

Supplementary Figure 1

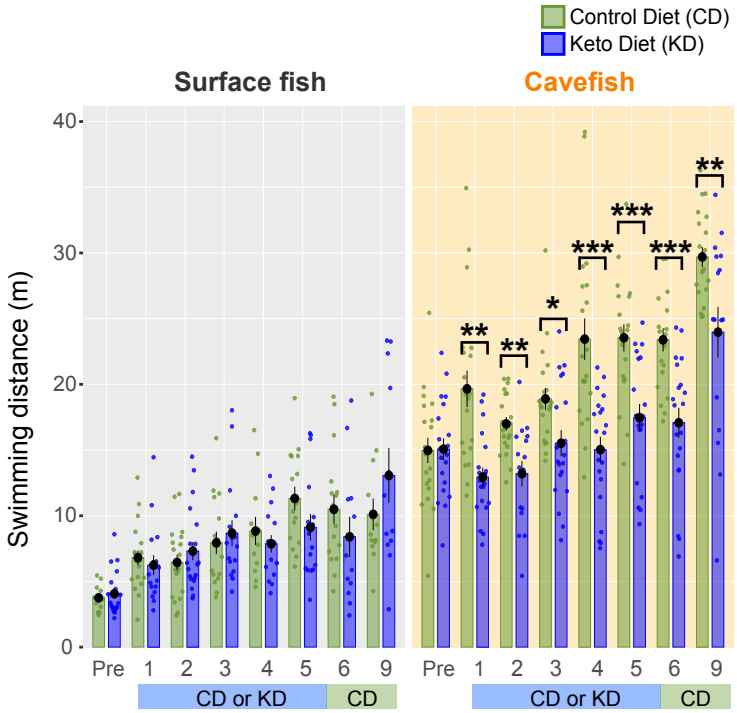
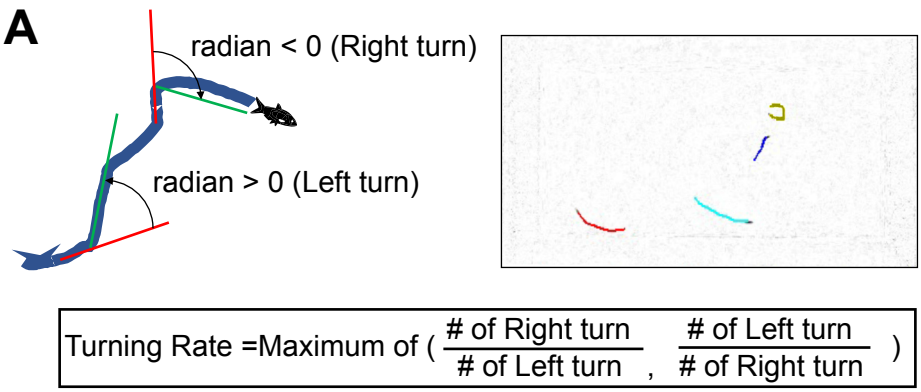
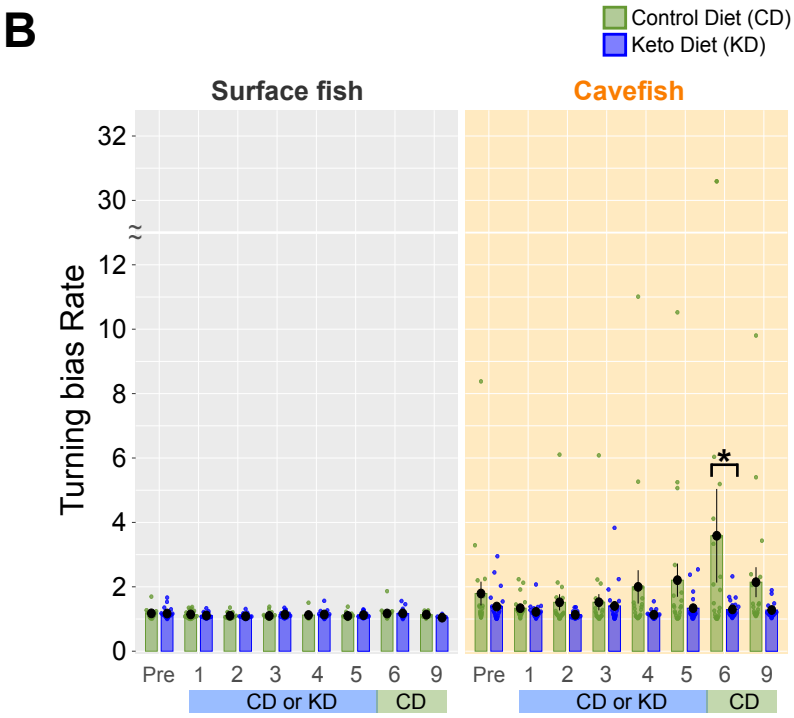


Figure 4



$$\text{Turning Rate} = \text{Maximum of}\left(\frac{\#\text{ of Right turn}}{\#\text{ of Left turn}}, \frac{\#\text{ of Left turn}}{\#\text{ of Right turn}}\right)$$



Supplementary Figure 2

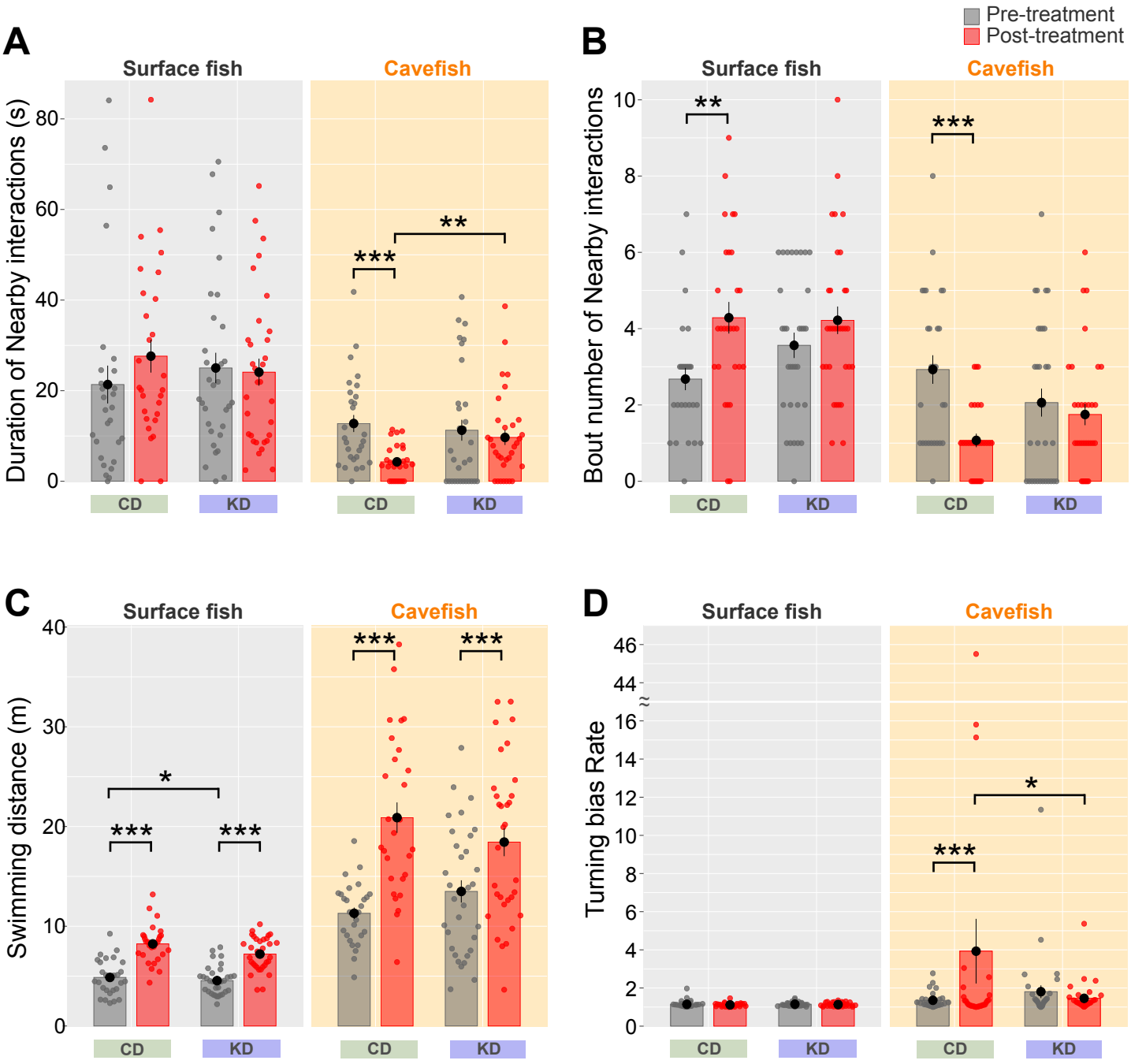
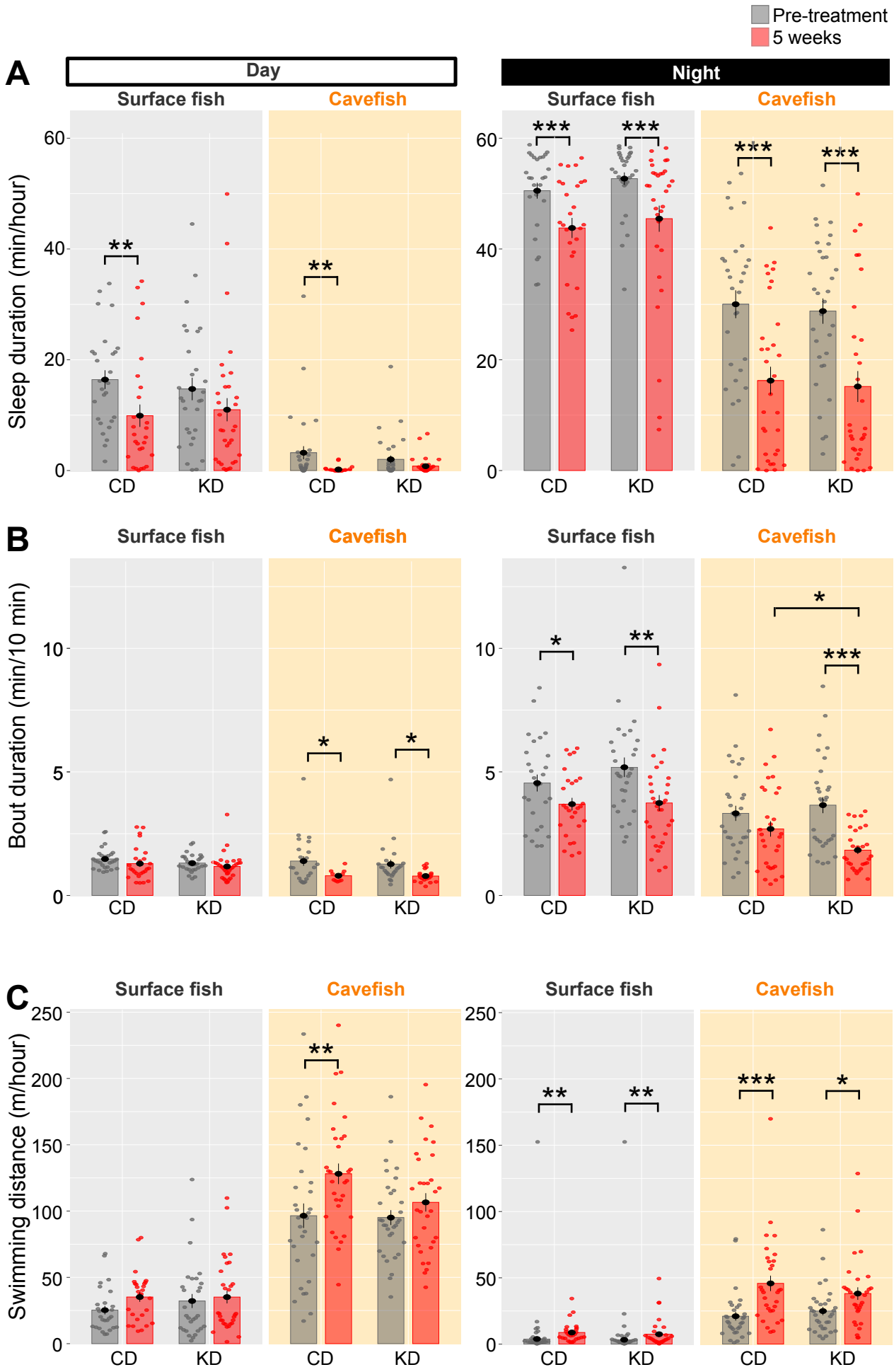
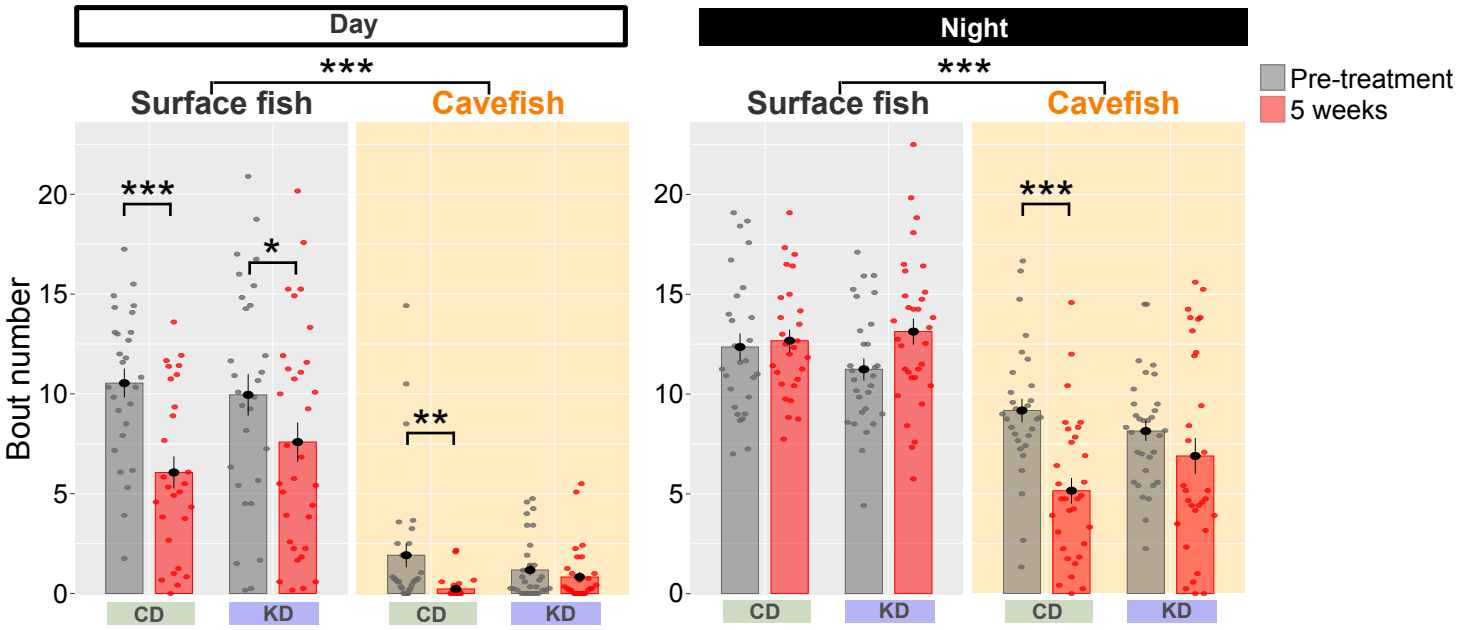


Figure 5


Pre-treatment
5 weeks
A
Day
Night
Surface fish
Cavefish
Sleep duration (min/hour)
CD
KD
B
Bout duration (min/10 min)
C
Swimming distance (m/hour)

Supplementary Figure 3



Supplementary Figure 4

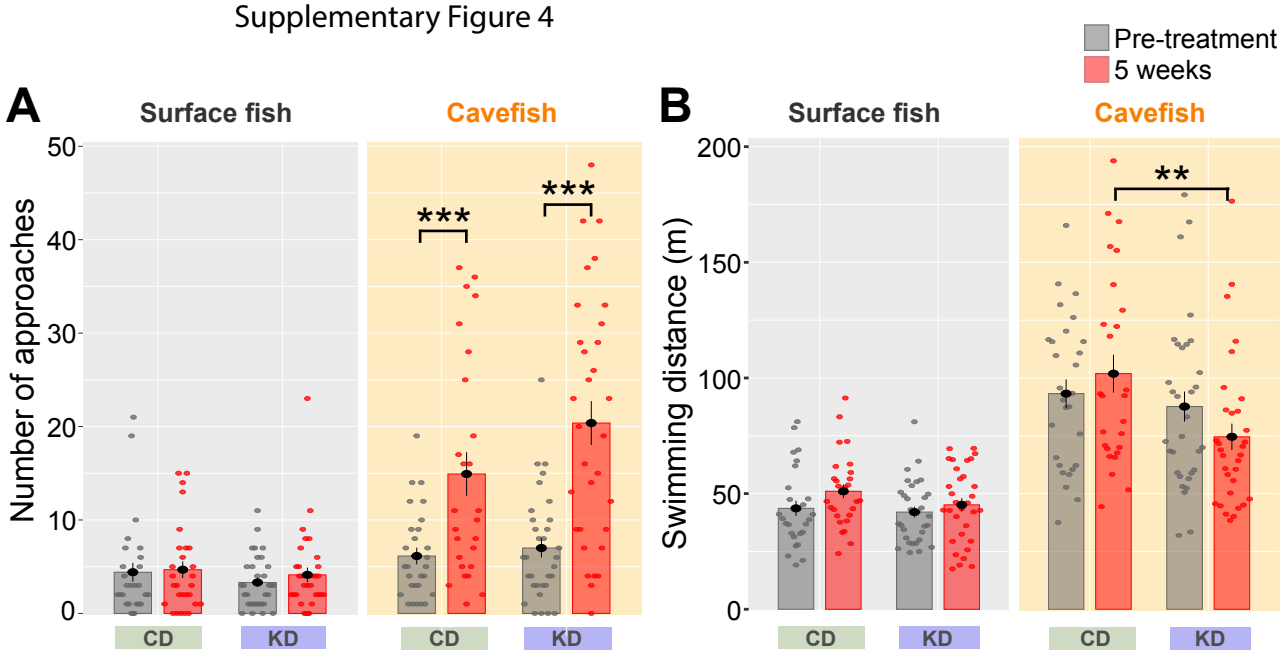


Figure 6

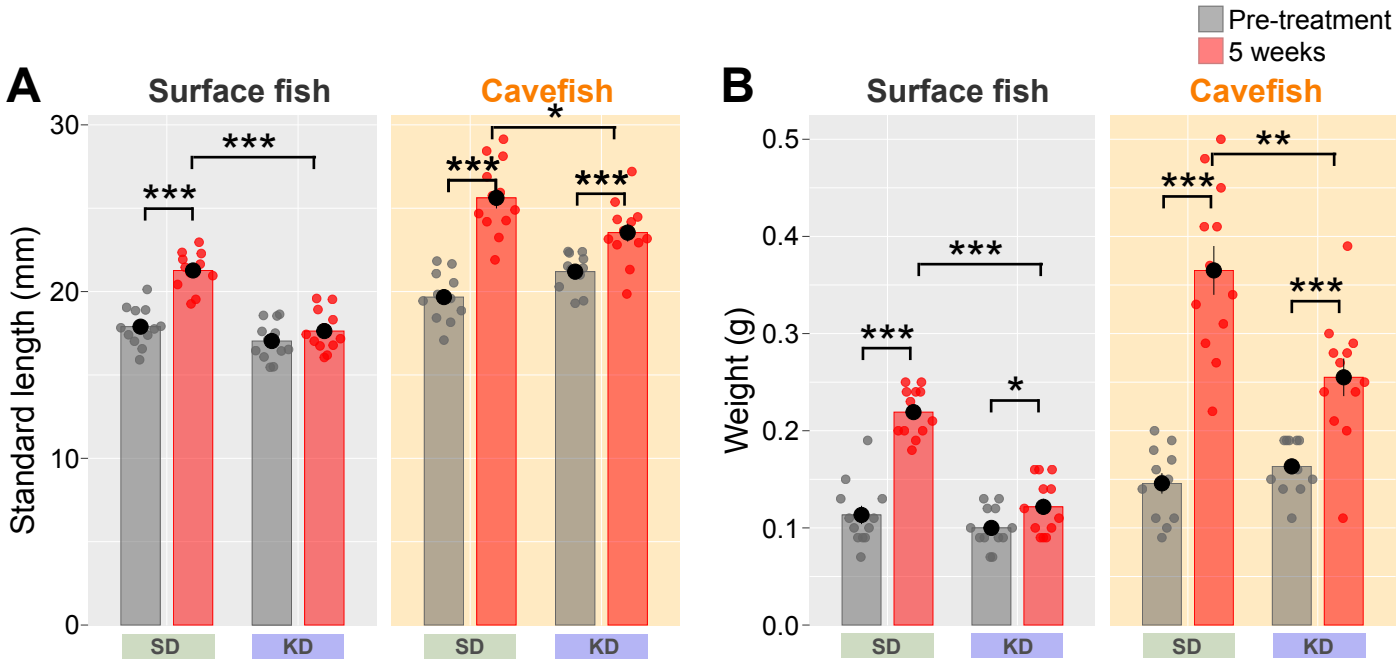
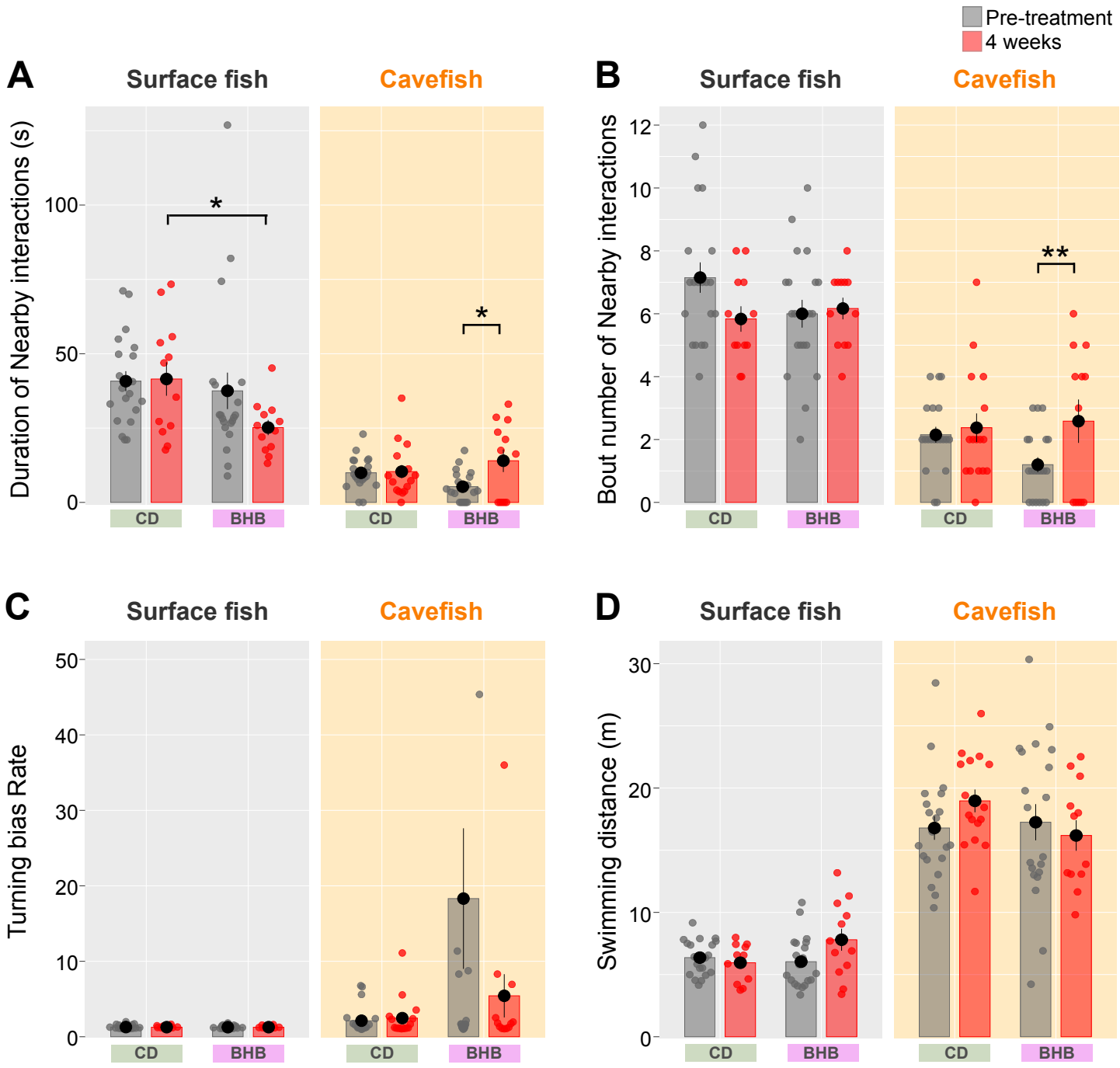
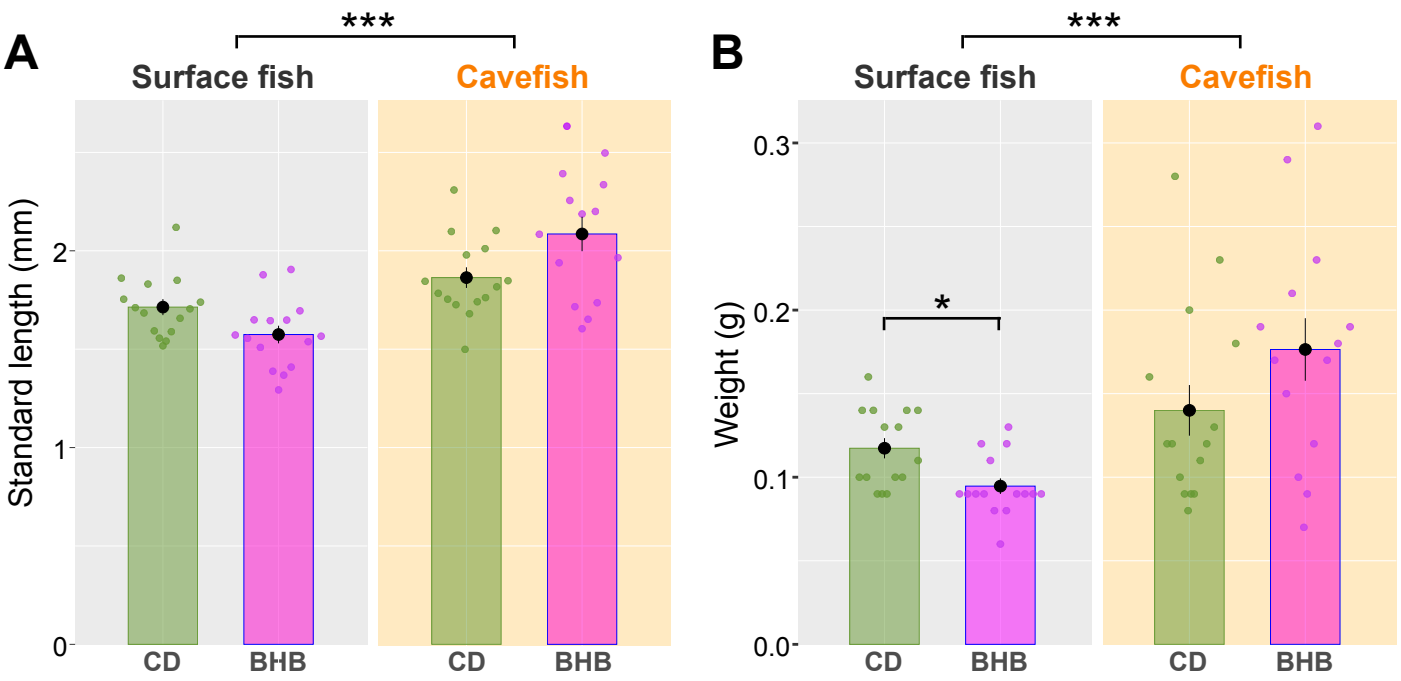
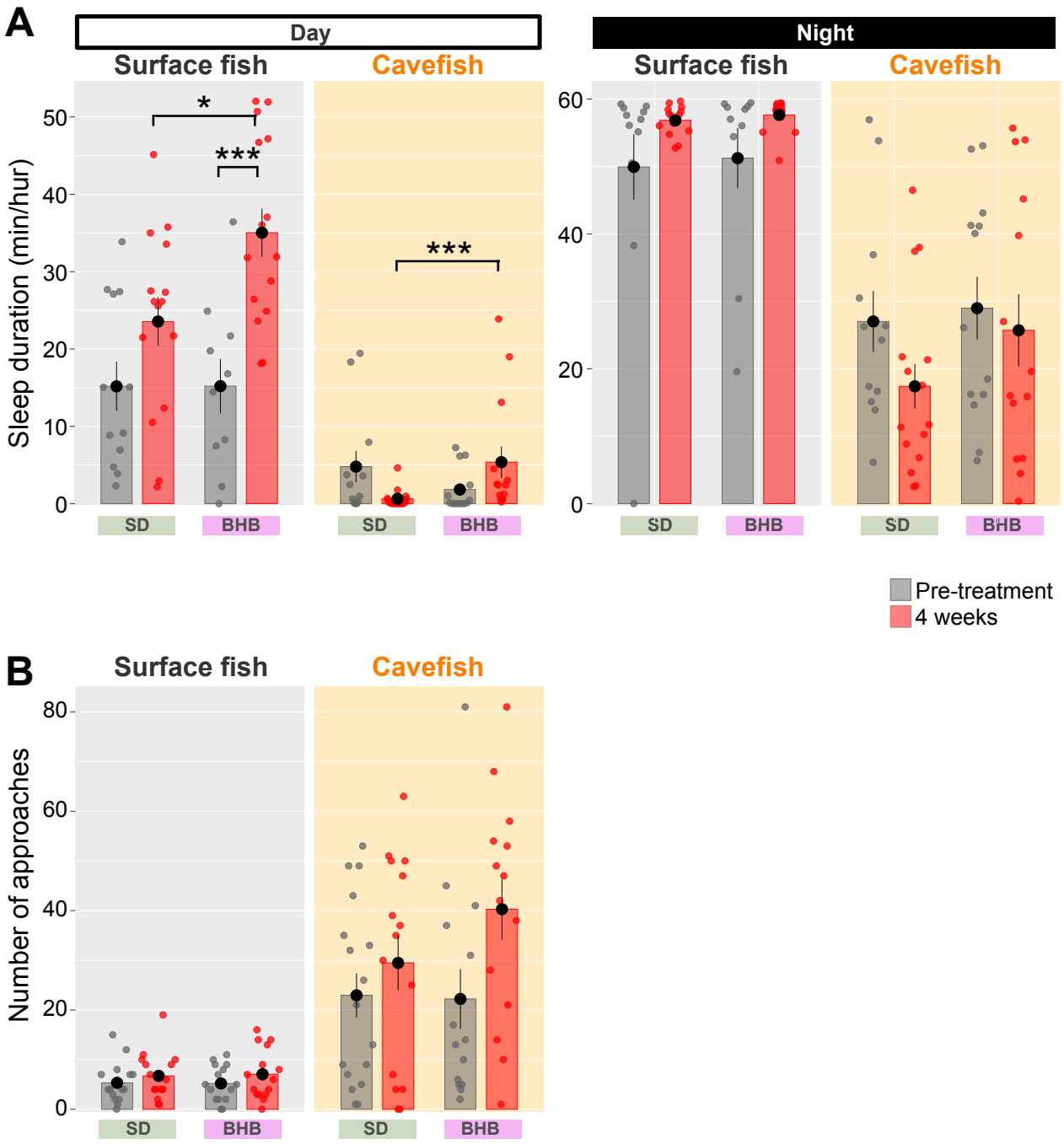

Pre-treatment
5 weeks
A
Surface fish
Cavefish
Standard length (mm)
30
20
10
0
***
***
*
***
***
SD
KD
SD
KD
B
Surface fish
Cavefish
Weight (g)
0.5
0.4
0.3
0.2
0.1
0.0
***
***
*
**
***
***
SD
KD
SD
KD

Figure 7


Pre-treatment
4 weeks
A
Surface fish
Cavefish
Duration of Nearby interactions (s)
100
50
0
*
*
CD
BHB
CD
BHB
B
Surface fish
Cavefish
Bout number of Nearby interactions
12
10
8
6
4
2
0
**
CD
BHB
CD
BHB
C
Surface fish
Cavefish
Turning bias Rate
50
40
30
20
10
0
CD
BHB
CD
BHB
D
Surface fish
Cavefish
Swimming distance (m)
30
20
10
0
CD
BHB
CD
BHB

Supplementary Figure 5



Supplementary Fig. 6