Metabolic shift toward ketosis in asocial cavefish increases social-like affinity

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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 shifts 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 one 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 major behavioral shifts were found regarding sleeplessness and foraging behavior, suggesting that other evolved behaviors are not largely 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 positively associated with social affinity. Our study indicated that fish that evolved to be asocial remain capable of exhibiting social affinity under ketosis, possibly linking the seasonal food availability and sociality.

Introduction

Wild animals experience frequent fasting due to 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 to nutritional ketosis [1]. In terms of behavioral outputs, fasting also induces shifts including boldness in foraging involving risk-taking [2] and a shift from avoiding to approaching prey [3]. Interestingly, fasting also induces non-foraging-related behaviors including aggression towards cohorts [4, 5] and engagement in social dominance [6]. These non-foraging-related behaviors could be evoked by 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-related behaviors. Knowledge of such mechanism will open a path to understanding the effects of different dietary intakes according to changing environments, such as switching from glycolysis-inducing carbohydrate-rich diets to ketosis-inducing very low-carbohydrate diets or vice versa.

Recently, the ketosis-inducing ketogenic diet (KD), which contains a high amount of fat, sufficient protein, and a very low amount of carbohydrates, gained popularity among humans because of its neuroprotective and anti-inflammatory effects without impacting appetite-related hormone levels [7–9]. 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, Parkinson's disease, and autism [10–13]. Because modern humans evolved to acquire resistance to starvation [14], our body physiology and behavioral tendencies possibly evolved to accommodate drastic metabolic changes. However, the major molecular mechanisms for these possibilities are largely unknown [9, 15]. We were therefore motivated to explore the effects of metabolic shifts, particularly from glycolysis to ketosis, on behavioral outputs such as social affinity using a single species consisting of two morphotypes: typical and starvation-resistant populations.

67 A suitable model system for this purpose is the Mexican cavefish (*Astyanax mexicanus*). *A.*
68 *mexicanus* has emerged as a useful experimental platform for diverse aspects of evolution and
69 development, including those with translational relevance to human medicine, such as cataract
70 formation, diabetes, albinism-related syndrome, and insomnia [16–28]. Although there are many
71 parallels in biological phenomena, we strongly agree that the systemic and organ physiologies between
72 humans and this fish species are quite different. Therefore, we do not consider this fish system as an
73 animal model for human disorders but as a platform revealing the genetic and cellular mechanisms that
74 may be conserved among vertebrates and are otherwise difficult to hint at etiologies for similar
75 symptoms. This *A. mexicanus* consists of surface riverine epigean (surface fish) and cave-dwelling
76 hypogean (cavefish) populations. Cavefish diverged from their surface-dwelling relatives 20,000–
77 200,000 years ago [29, 30], and they have subsequently evolved many distinct morphological and
78 behavioral phenotypes in the food-sparse cave environment, including eye regression/loss, pigment
79 reduction, increased mechanosensory lateral line activity, adherence to vibration stimuli, sleeplessness,
80 hyperactivity, repetitive circling, and reduced social affinity [19, 31–33]. Compared to cavefish, surface
81 fish exhibit typical teleost phenotypes, including typical eyed and pigmented morphologies, no strong
82 adherence to vibration stimuli, nocturnal sleep patterns, and social affinity. Many cavefish traits are
83 believed to have evolved to adapt to food-sparse dark environments. Indeed, wild cavefish are estimated
84 to be exposed to approximately 6 months of food-sparse conditions annually [34], and they are likely to
85 have the ability to withstand starvation via increased fat storage, increased appetites, insulin resistance
86 for fasting [17, 24, 35, 36], slower weight loss during starvation [37], reduced energy-costing circadian
87 activities, and the lack of eyes [38, 39].
88 Regarding social behavior, cavefish exhibit no detectable schooling behavior [40–42] or
89 hierarchical dominance [43]. By contrast, surface fish school/shoal with cohorts and plastic model fish
90 [40] and exhibit group hierarchical dominance [43]. Because social behaviors in many fish (*e.g.*,
91 zebrafish) are promoted by visual stimuli, blind cavefish might not express social activities because of

92 the absence of visual acuity. However, a recent detailed study illustrated that surface fish exhibit a high
93 level of social-like nearby interactions (one-by-one affinity: social affinity) in the dark, and were
94 promoted by mechanosensory lateral line inputs [33, 41]. In contrast, blind cavefish displayed much
95 lower levels, albeit significant, of nearby interactions than surface fish [33]. Furthermore, cavefish
96 exhibited plasticity in the level of nearby interactions, wherein they increased interaction levels in a
97 familiar environment in comparison with an unfamiliar environment [33]. This observation is similar to
98 those in patients with autism [44, 45] although there is an enormous gap in the complexities of brain
99 functions between humans and fish.

100 Thus far, similarities between cavefish and patients with autism have been investigated in terms
101 of gene regulation and innate behavior profiles. First, the cavefish gene expression profile is closer to
102 that of patients with autism than to that of other mammalian model systems—a comparison between
103 cavefish and surface fish transcriptomes exhibited the same directional gene expression changes in
104 cavefish observed in the brains of patients with autism (over 58.5% of 3,152 cavefish orthologs).
105 Conversely, other proxy systems (e.g., BTBR mice [classic model for autism] and *shank3* knockout
106 mice) exhibit much less overlap (< 11%) [31, 46, 47]. Second, cavefish's evolved behaviors, including
107 asociality, repetitive behavior, sleeplessness, higher swimming activity, adherence to a particular
108 vibration stimulus, and higher anxiety-related plasma cortisol levels, are similar to those in patients with
109 autism [31]. Lastly, cavefish and human ancestors are starvation-resistant, and they share some
110 metabolic pathways [14, 17, 24, 37]. These similarities, along with the fact that the ketognic diet (KD)
111 increases socialization in patients with autism [11, 48–50], prompted us to study the effects of ketosis on
112 social affinity in asocial cavefish. Note that we do not consider *A. mexicanus* as an animal model for
113 autism due to substantial differences in their physiologies. What we expect in studies using *A. mexicanus*
114 is that the molecular and cellular responses in ketosis, where the gene expression landscape is the closest
115 to patients with autism, and whose changes are relevant to social behaviors, might provide a hint for the
116 biomedical application that would otherwise be difficult to obtain. This prediction is based on the fact

117 that we have learned so much about human molecular and cellular signaling pathways from fruit flies,
118 which are even phylogenetically more distant organisms than fish [51].

119 With both the shift in sociality under the seasonal nutrients and the genetic relevance to human
120 disorders in mind, in this study, we assessed the effects of the KD on an evolutionarily asocial cave
121 population of *A. mexicanus*. First, the two-week fasting promoted the level of nearby interaction in
122 asocial cavefish but not in surface fish. Both the two-week fasting and one month of KD feeding
123 reduced the serum glucose-ketone index, whose lower scores (less than 9) indicate ketosis in the body
124 metabolism in human [52], in both surface fish and cavefish. The one-month KD feeding also
125 significantly increased the ketone body concentration in the cavefish brain tissue. Under this feeding
126 regimen, the time-course experiment revealed that one month of KD feeding promoted and sustained the
127 juvenile level of nearby interactions, whereas cavefish fed a control diet (CD) exhibited diminished
128 nearby interactions. KD feeding also reduced repetitive turning and swimming activity. However, the
129 effects of the KD were limited. For example, sleeplessness and high adherence to a particular vibrating
130 stimulus did not show any major changes under the 1-month KD treatment. To reveal the molecular
131 basis of the effects of the KD, we provided supplementation with a major ketone body, beta-
132 hydroxybutyrate (BHB), which promoted social interactions and reduced repetitive turning, covering the
133 major effect of the KD. Finally, we interpreted the possible neural processes influenced by the KD based
134 on affected and unaffected behaviors. According to the study of shared dysregulated genes between
135 cavefish and patients with autism, GO term and KEGG pathway analyses indicated that the
136 dopaminergic system—though less likely the cholinergic, or orexinergic systems—could respond to the
137 KD. However, ketone bodies may have different effects on fish and mammalian physiologies, as
138 discrepancies have been observed in the appetite regulation [53]. Therefore, we need to be careful when
139 interpreting the results of this study in mammals. Nevertheless, *A. mexicanus* provides a unique
140 opportunity to investigate the molecular and genetic responses to ketone bodies in a genetically close

141 biological platform to a human disorder, which will be complementary in understanding deeper
142 etiologies.
143 Overall, ketosis appears to be capable of significantly shifting the asociality of evolved cavefish
144 toward the surface fish phenotype, providing new insights into the contribution of the diet to social
145 behaviors.
146

147 **Methods**

148 Fish maintenance and rearing in the lab

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

153 Fish (surface fish and Pachón cave populations) were housed in the University of Hawai‘i at
154 Mānoa *Astyanax* facility with temperatures set at 21 ± 0.5°C for rearing, 24 ± 0.5°C for behavior
155 experiments, and 25 ± 0.5°C for breeding [54, 55]. Lights were maintained on a 12-h:12-h light:dark
156 cycle [54, 55]. For rearing and behavior experiments, the light intensity was maintained at 30–100 Lux.
157 Fish husbandry was performed as previously described [19, 54, 55]. Fish were raised to adulthood and
158 maintained in standard 42-L tanks in a custom-made water-flow tank system. Adult fish were fed a
159 mixed diet to satiation twice daily, starting 3 h after the lights were turned on (Zeitgeber time 3 [ZT3]
160 and ZT9; Zeigler Adult zebrafish irradiated diet, Zeigler Bros, Inc, Gardners, PA; TetraColor Tropical
161 Fish Food Granules, Tetra, Blacksburg, VA, USA; Jumbo Mysis Shrimp, Hikari Sales USA, Inc.,
162 Hayward, CA, USA). All fish used in the behavioral experiments were between 2.5 and 5 cm in standard
163 length, fed with live *Artemia* larvae ad libitum, and occasionally supplemented with Dr. Bassleer Biofish
164 Food Fuco (Bassleer Biofish, Herselt, Belgium). They were between 3 and 12 months old. Fish ages
165 were stated for each experiment. All fish care and experimental protocols were approved under IACUC
166 (17-2560) at the University of Hawai‘i at Mānoa.

167

168 Control diet, Fasting, KD, BHB and KE treatments

169 For the Control diet (CD) feeding, the 4-month-old fish were routinely fed live *Artemia* larvae or
170 the zebrafish standard diet (adult zebrafish irradiated diet: Zeigler Bros, Inc., Gardners, PA, USA) for 3
171 weeks in the home tank of the experimental fish (Ziplock® containers, S. C. Johnson & Son, Inc.,

172 Racine, WI, USA). Fish were grouped into four fish per tank and were fed every morning (ZT 0:00–
173 3:30) and afternoon (ZT 8:00–12:00). The fish were fed ad libitum during each feeding and any
174 remaining food was removed 1 h after feeding using a pipette. Following standard operating protocols
175 (IACUC (17-2560)), water was changed twice a week, and home tanks were cleaned as usual.

176 For the fasting experiment, the fish were fasted for 2 weeks (13 full days) before recording the
177 behaviors, while the control fish were fed live *Artemia* larvae. We used two different age groups: 4-6
178 months old and 11-12 months old. The results from these two age groups were essentially the same.
179 Following standard operating protocols (IACUC (17-2560)), tank water was changed (twice a week),
180 and home tanks were cleaned as usual.

181 To prepare the ketogenic diet (KD), we used a mixture of a human KD (KetoCal3:1®—
182 nutritionally complete, ketogenic medical food; Nutricia North America, Inc. Gaithersburg, MD, USA)
183 and the zebrafish standard diet (adult zebrafish irradiated diet: Zeigler Bros, Inc., Gardners, PA, USA) in
184 a 5:1 ratio. The gross caloric amounts were 6.99 kcal/g for KetoCal3:1 and 3.89 kcal/g for the zebrafish
185 diet. Regarding the control diet (CD), we used the same KetoCal3:1 and zebrafish irradiated diet mixed
186 at a 1:5 ratio. The KetoCal3:1 powder and ground zebrafish irradiated diet were mixed in the
187 aforementioned ratios and solidified with 1% agar at a final concentration of 20% w/v (2 g of mixture in
188 10 mL of 1% agar). After solidification, both the KD and CD agar were cut into 3-mm^3 cubes, and each
189 four-fish group (3-4 months old) was given 1–2 pieces every morning (ZT 0:00–3:30) and afternoon (ZT
190 8:00–12:00). The fish were fed ad libitum in each feeding and any remaining food was removed 1 h after
191 feeding using a pipette.

192 To supplement BHB, we used a commercial fish diet (TetraColor Tropical Granules, Tetra,
193 Blacksburg, VA, USA) mixed with BHB (DL-β-Hydroxybutyric acid sodium salt, MilliporeSigma, St.
194 Louis, MO, USA) at a dosage of 10 mg per gram of body weight (10 mg/body g = 78.7 μmol/body g). In
195 detail, fish generally consume 3% of their body weight g per meal. Accordingly, the BHB supplemental
196 diet contained 0.333g/mL of BHB mixed with 0.2 g/mL of the ground Tetra fish diet (20% w/v) in 1%

197 agar. Fish with a body weight of 1 g would consume 30 mg (3%, approximatey 30 μL) of this diet,
198 which contained 10 mg of BHB. The control diet consisted of 20% w/v of the Tetra fish diet in 1% agar.
199 After solidification, both the BHB and control diet agar were cut into 3-mm^3 cubes, and each group of
200 four-fish group was given 1–2 pieces every morning (ZT 0:00–3:30) and afternoon (ZT 8:00–12:00).
201 The fish were fed ad libitum in each feeding and any remaining food was removed 1 h after feeding
202 using a pipette. Surface fish and cavefish used in this BHB study were 10-11 months old (young adult:
203 2.0-2.5 cm in the standard length) at the start of the the feeding.

204 To supplement the ketone ester (KE; D-β-hydroxybutyrate-R 1,3-Butanediol Monoester) [56],
205 we used the adult zebrafish irradiated diet (Zeigler Bros, Inc., Gardners, PA, USA) mixed with KE at a
206 dosage of 3.26 μmol per gram of body weight as used in humans [56]. In detail, similar to
207 supplementing BHB, the KE supplemental diet contained 19.1 mg/mL (=108.5 μmol/mL, MW=176
208 g/mol) mixed with 0.2 g/mL of ground Zeigler zebrafish diet (20 % w/v) in 1% agar. When a fish with
209 1g body weight eat 30 mg (3% of body weight) of this diet, it would intake 3.26 μmol of KE. The
210 control diet for KE consisted of 0.2 g/mL of ground Zeigler zebrafish diet, and adjust the calorie content
211 was adjusted with 147.3 mg of sucrose per body g (equivalent to 580.5 calories/body g, the same as 3.26
212 μmol of KE) in 1% agar. Additionally, since esters have a bitter taste, we standardized it by adding 10
213 μL/mL of a commercial bitter agent (Symrise, Holzminden, Germany). After the solidification of agar,
214 both the KE and control diet agar were cut into 3-mm^3 cubes, and each fish group was given 1–2 pieces
215 every morning (ZT 0:00–3:30) and afternoon (ZT 8:00–12:00). The fish were fed ad libitum in each
216 feeding and any remaining food was removed 1 h after feeding using a pipette. Surface fish showed
217 hesitation with the KE and its control diets during the first 2-3 days of this dietary regime by leaving
218 uneaten food, but they consumed all of it afterward. Cavefish never showed uneaten food in this study.
219 Surface fish and cavefish used in this KE study were 6-7 months old (young adult: approximately 2.0 cm
220 in the standard length) when we started the feeding regime.
221

222 Behavior assays

223 The protocol for social-like nearby interactions was described previously [33]. Briefly, four fish
224 raised in a home tray (15.6 × 15.6 × 5.7 cm^3 Ziploc containers, S. C. Johnson & Sons, Inc, Racine, WI,
225 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
226 of a custom-made infrared (IR) back-light system within a custom-built black box (75 × 50 × 155 cm,
227 assembled with a polyvinyl chloride pipe frame and covered by shading film). The IR back-light system
228 was composed of bounce lighting of IR LED strips (SMD3528 850 nm strip: LightingWill, Guang
229 Dong, China). The video was recorded at 20 frames/s using VirtualDub2 software (build 44282;
230 http://virtualdub2.com/) with the x264vfw codec for 6 min, and the last 5 min were used for the analysis.
231 After the recording, the fish were returned to the home tray. The X-Y coordinates of each fish were
232 calculated using idTracker software [57] after the video image was processed for background subtraction
233 using ImageJ [33]. These X-Y coordinates were also used for the turning bias analysis. The duration and
234 number of nearby interactions and swimming speed during and after nearby interaction events were
235 calculated using a custom-made MATLAB script (MathWorks Inc., Natick, MA, USA) [33].

236 The turning bias rate was calculated as $\frac{Nl}{Ns}$, where Ns and Nl represent a smaller (Ns) or larger
237 (Nl) number of left or right turns. This turning bias rate indicates the extent to which fish turning is
238 biased to the left or right, and ranging from "1" (L-R balanced) to infinity (L or R biased). The numbers
239 of left or right turns were calculated as changes in the angles of swimming directions in every five-frame
240 window (0.25 s) as described previously [33]. An automatic calculation of the total number of the left or
241 right turns is implemented in the aforementioned homemade MATLAB script.

242 Analyses of sleep and swimming distance were described previously [31, 54]. Briefly, fish were
243 recorded in a custom-designed 10.0-L acrylic recording chamber (457.2 × 177.8 × 177.8 mm^3 and 6.4
244 mm thick) with opaque partitions that permit five individually housed fish per tank (each individual
245 chamber was 88.9 × 177.8 × 177.8 mm^3). The recording chamber was illuminated with a custom-
246 designed IR LED source in a light-controlled room on a 12-h:12-h cycle. The room light was turned on

247 at 7:00 am and turned off at 7:00 pm each day. Behavior was recorded for 24 h after overnight (18–20 h)
248 acclimation, beginning 1–2 h after turning the light on (ZT1–2). Videos were recorded at 15 frames/s
249 using a USB webcam with an IR high-pass filter. Videos were captured by VirtualDub2 software with
250 the x264vfw codec and subsequently processed using Ethovision XT (Version 16, Noldus Information
251 Technology, Wageningen, Netherlands). Water temperature was monitored throughout the recordings,
252 and no detectable differences were observed during the light and dark periods (24.0 ± 0.5°C). The
253 visible light during behavior recordings was approximately 30–100 Lux.

254 The tracking parameters for detection were as follows: the detection was set to "subject brighter
255 than background" and brightness contrast was set from 20 to 255; the current frame weight was set to
256 15; the video sample rate was set to 15 frames/s; and pixel smoothing was turned off. We monitored
257 sleep activity, and arousal thresholds via protocols previously established for *A. mexicanus* [54]. The X-
258 Y coordinates of each fish were subsequently processed using custom-written Perl (v5.23.0,
259 www.perl.org) and Python scripts (3.8) (https://zenodo.org/record/8137637).

260 We assayed vibration attraction behavior (VAB) as described previously [54, 58, 59]. Briefly,
261 fish were acclimatized for 4–5 days prior to the assay in a cylindrical assay chamber (325 mL glass dish,
262 10 cm × 5 cm, VWR, Radnor, PA, USA) filled with conditioned water (pH 6.8–7.0; conductivity 600–
263 800 μS). During the assays, vibration stimuli were created using a glass rod that vibrated at 40 Hz. The
264 number of approaches to the vibrating rod was video recorded during a 3-min period under infrared
265 illumination. The number of fish approaches within a 1.3-mm radius from the vibrating glass rod was
266 analyzed using the X-Y coordinates of each fish head detected by a trained DeepLabCut model [60])
267

268 <u>Measurement of body</u>

269 Fish were anesthetized with ice-cold conditioned water (pH: 7.0; conductivity: 700 μS), and their
270 weights were measured after taking pictures with a standard camera (Pentax K-1 DSLR with 35-70 mm

271 zoom lens, Ricoh, Tokyo, Japan). The standard body length and body depth were measured using
272 ImageJ software [61].
273
274 Blood ketone and glucose measurements
275 All blood samples were collected 2–3 h after feeding. Fish were then deeply anesthetized in ice-
276 cold water, and blood was collected from the tail artery. Blood ketone and glucose levels were measured
277 using either the Abbott Precision Xtra (Abbott Laboratories, Abbott Park, Illinois, USA) or Keto-Mojo
278 GK+ (Keto-Mojo, Napa, California, USA) blood glucose and ketone monitoring system according to the
279 manufacturers' instructions. The readings of Abbott Precision Xtra were standardized by comparing
280 them with the readings of the same blood sample with Keto-Mojo GK+. Both readings from the Abbott
281 and Keto-Mojo meters showwed a high linear correlation ($R^2 = 0.93$, $P = 0.000103$ and $R^2 = 0.74$, $P =$
282 0.00565 for glucose and ketone readings, respectively; $N = 8$). It should be noted that these blood
283 glucose and/or ketone measurements can be affected by hematocrit levels above 65% or below 20%.
284 The hematocrit level of *Astyanax* fish is approximately 30%, in which Pachón cavefish showed slightly
285 higher values (35.56 ± 0.03%, means ± standard error of means) compared to surface fish (28.51 ±
286 0.03%), as reported by Boggs et al., 2022 [62]. To test the effect of hematocrit level, we diluted the *A.*
287 *mexicanus* serum 2 times with phosphate-buffered saline (PBS, pH 7.2). The results showed no
288 significant difference in the readings of glucose and ketone bodies between surface fish and cavefish
289 (Additional data 1), suggesting that the differences in the hematocrit levels between the cave and surface
290 fish yielded no detectable effects on the readings of serum glucose and ketone bodies. For ketone
291 measurements in the brain samples, the samples were collected 2-3 h after feeding, following the deep
292 anesthesia in ice-cold water (the same as for the blood samples above). Each brain tissue was carefully
293 collected from the cranium individually and snap-frozen in a 1.5 mL microcentrifuge tube (VWR
294 International, Radnor, PA, USA) chilled with liquid nitrogen. The amount of beta-hydroxybutyrate was
295 measured by following the manufacturer's instructions of the Ketone Body Assay kit (UV)

296 (MilliporeSigma, Burlington, MA, USA). UV readings were performed by using a BioTek Epoch
297 microplate spectrophotometer (Agilent, Santa Clara, CA, USA).
298
299 Statistical analysis
300 Regarding the power analysis, we designed our experiments based on a three-way repeated-
301 measures ANOVA with a moderate effect size (f = 0.25), an alpha-error probability of 0.05, and a power
302 of 0.80. The number of groups was eight (surface fish vs. cavefish × non-treated vs. treated × pre-
303 treatment vs. post-treatment). G*Power software [63, 64] estimated that a sample size needed for this
304 experiment was nine per group. Therefore, we aimed to use at least 12 fish in each group for all
305 experiments in this study.
306 For statistical comparisons of our data, we performed tests including Student's *t*-test, Wilcoxon
307 signed-rank test, and two- or three-way generalized linear model analyses to compare surface and
308 cavefish, treatment and non-treatment, and pre-treatment and post-treatment. We applied the linear
309 model to non-processed data, the generalized linear model (Poisson family) to discrete data including the
310 blood ketone measurements (multiplied by 10 to apply the Poisson family adjustment for the blood
311 ketone measurements that showed the 0.1 step), and the generalized linear model (Gamma family) to
312 processed data (i.e., turning index). Holm's post hoc correction was used to detemine which contrasts
313 were significant [65].
314 Regarding replicates of experiments, we used different individuals for each replicate.
315 Sepcifically, we conducted two biological replicates, using different individuals in each trial. There was
316 no repeated usage of individual fish, except for the time-course experiment. For the experiments
317 measuring sleep, VAB, nearby interactions, and turning bias, we used two biological replicates and
318 confirmed that the averages of the experimental data did not differ significantly. We then merged the
319 data acquired from the two biological replicates and presented it as a single set of results.

The aforementioned calculations were performed using R version 4.0.4 software (packages *car*, *lme4*, and *lmerTest*) [66–68], and all statistical scores are available in Additional data 2 and/or the figure legends.

324 **Results**

325 From our observations in their wild habitat (the Mexican cave Pachón, Additional data 3),
326 cavefish swam slower and remained near each other more frequently than the lab population. Because
327 the cave environment has a limited diet compared to that of the surface, we predicted that cavefish
328 experience frequent ketosis induced by fasting.

329 In the 1-year-old surface and cave populations of *A. mexicanus,* 2 weeks of fasting indeed
330 reduced serum glucose levels, leading to a lower glucose ketone index (GKI = $\frac{glucose\ (mM)}{ketone\ (mM)}$; a GKI lower
331 than 9 is considered as ketosis in humans; Additional data 4A- 4D; [52, 69]). GKI is proposed as a better
332 indicator of the body ketosis instead of the sole measurement of the ketone bodies [52]. This result
333 indicates that *A. mexicanus* responded to fasting and reduced GKI in a similar manner as mammals,
334 although the serum ketone levels did not significantly increase during this fasting experiment
335 (Additional data 4B). Meanwhile the social-like nearby interactions of cavefish increased (duration and
336 event numbers, Additional data 4E and F; see below for nearby interactions). Although ketosis may be
337 primarily responsible for increasing social interactions, appetite and hormones can also be contributing
338 factors.

339 Before testing the ketosis-inducing ketogenic diet (consisting of high fat, sufficient protein
340 levels, and very low carbohydrate) to reduce appetite-related behavior, we questioned whether a dietary
341 shift from live *Artemia* larvae (Brine shrimp larvae: BS; standard rearing diet; see Methods) alters the
342 nearby interactions. Feeding Zeigler zebrafish standard diet (control diet: CD) for three weeks did not
343 show any detectable changes in the serum ketone or glucose levels in either surface or cavefish
344 compared to continuous BS-feeding (Additional data 5A and B). Nearby interaction scores were not
345 promoted by this CD feeding; instead, cavefish tended to reduce the scores in both CD and BS-feeding
346 (only detectable in BS-feeding) during 3 weeks of growth (Additional data 5C and D; see below). This
347 reduction of nearby interaction scores was consistent with the following ketogenic diet-feeding study. In

summary, the diet shift from BS to CD did not show a detectable effect on serum ketone, glucose, or nearby interaction scores.

Then, to reduce appetite-related behavior, we developed a ketogenic diet (KD) based on a human milk formula (KetoCal3:1® with Zeigler zebrafish standard irradiated diet at a 5:1 weight ratio; Table 1; Materials and Methods).

Table 1. Nutrient composition of each diet used in the study [70, 71]

%	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)

We then measured the glucose ketone index (GKI) to monitor whether our KD could induce a shift in the balance of ketone bodies and glucose levels after chronic dietary treatment. Three-month-old fish (juvenile–young adult stage) were used in this KD study because the positive effects of KD were more pronounced at the younger stage in human [11], and many adult-type behaviors of cavefish emerge in this stage, including higher adherence to a vibration stimulus (vibration attraction behavior [VAB]) [58], less social affinity, and longer swimming distances compared to surface fish. After 5 weeks of KD feeding, both ketone and glucose concentrations decreased compared to the CD-fed fish (KetoCal3:1 and Zeigler zebrafish diet at a 1:5 weight ratio; Table 1; Figure 1A–C). Surface fish exhibited a significantly higher serum ketone body level than cavefish for both diets (Figure 1B), whereas cavefish exhibited a higher serum glucose level than surface fish (Figure 1C). The GKI was lower in surface fish than in cavefish, and the value was reduced under KD feeding in both surface fish and cavefish compared to that in their CD-fed counterparts (Figure 1D). This result indicats that KD feeding more strongly reduced serum glucose levels than ketone body levels, resulting in a lower GKI in KD-fed fish than in CD-fed fish (Figure 1D). This result suggests that KD feeding could shift the metabolic state from glycolysis toward ketosis. Despite the subtle difference in the serum ketone level between KD-fed

373 and CD-fed cavefish (Figure 1B), a ketone level 8.5 times higher was detected in the brain tissue of KD-
374 fed cavefish than in CD-fed cavefish (Additional file 6; Additional file 2 for the detailed statistical
375 scores). This result implies that the brain cells may efficiently uptake ketones.

376 Regarding this dietary treatment, we first examined its ontogenic (developmental) effects on
377 collective social-like behavior [33]. Many adult behaviors emerge in the transition from juvenile to
378 young adult (adolescent) in 3–4-month-old *A. mexicanus* fish, including foraging behavior, VAB [32,
379 58], adult-type regulation of sleep (independent from catecholamine) [16, 22, 54, 72], and collective
380 behavior in young adults (under higher Reynold's number; [33]). Therefore, we therefore investigated
381 the shift in collective behavior in 3–4-month-old fish using our previously reported method [33]. Briefly,
382 it uses criteria based on the vicinity of two fish (≤5 cm) and the duration of nearby interactions (≥4 s)
383 during tracking in a four-fish group (Figure 2B), with criteria defined by permutating the four-fish-group
384 swimming trajectory data 1,000 times [33]. At 3 months old ("Pre-treatment" in Figure 2), surface fish
385 exhibited social-like nearby interactions for 17.0 ± 4.4 s (Figure 2C) and 3.1 ± 0.4 bout number of
386 nearby interactions (Figure 2D) during the 5-min assay. In contrast, cavefish exhibited an approximately
387 50% shorter interaction duration (8.3 ± 1.5 s; Figure 2C) and a smaller bout number of interactions (1.8
388 ± 0.3; Figure 2D). To track the effect of the KD treatment, fish were fed the KD for 5 weeks, followed
389 by CD feeding during weeks 6–9 to assess the persistence of the effects of the KD (Figure 2A, C, and
390 D).

391 The nearby interactions of surface fish did not differ between CD and KD feeding (Figure 2C
392 and D). In contrast, the nearby interactions of cavefish were significantly decreased by CD feeding
393 compared to the effects of KD feeding in weeks 4 and 5 (Figure 2C and D), and interactions remained
394 depressed through week 9 with CD feeding. However, the effect of the KD diet on nearby interactions
395 did not persist. After KD deprivation and CD feeding, the nearby interactions of KD-fed cavefish were
396 indistinguishable from those of CD-fed fish (6–9 weeks, Figure 2C, and D), suggesting that KD has a
397 promotive/supportive effect on collective behavior in genetically asocial cavefish.

398 To support the finding of KD-promoted nearby interactions with alternative data, we explored
399 the swimming speed profile, which is a supportive indicator of fish movement in the vicinity of each
400 other. Fish are more likely to have an opportunity to express affinity towards each other at a slower
401 swimming speed, although it may not be as obvious at a very slow speed as below 3 cm/s [33].
402 Consistent with the former report, CD-fed surface fish moved slower during nearby interactions than
403 during the non-nearby interaction period, and a similar speed profile was also observed in KD-fed
404 surface fish [33] ("5 weeks", Figure 3A). There was no detectable difference in swimming speed profiles
405 between CD and KD feeding ("5 weeks", Figure 3A). Similarly, KD-fed cavefish swam slower during
406 the nearby interaction period than during the non-nearby interaction period ("5 weeks" Figure 3B). The
407 overall swimming speed was also slower in the KD group than in the CD group ("5 weeks" Figure 3B).
408 These findings indicate that KD-fed cavefish exhibited more social-like nearby interactions with a
409 similar speed profile to surface fish. The effect of KD feeding on the ontogeny of swimming
410 speed/distance showed a somewhat surprising result. We tracked the total swimming distance within 5
411 min from pre-treatment to week 9 of feeding (Additional file 7). KD-fed cavefish exhibited a
412 significantly shorter swimming distance (indicating slower swimming speeds) from the first week of
413 feeding (Additional file 7), which was much earlier than when the higher level of nearby interactions
414 emerged (weeks 4–5). This result suggests that KD feeding induced calmer swimming in cavefish within
415 a week of the treatment, although a slower speed itself is not sufficient to induce nearby interactions.
416 Repetitive turning is frequently observed in an antagonistic relationship with nearby interactions
417 in cavefish and mammals [33, 73, 74]. That is, individuals with few nearby interactions frequently
418 exhibit a high level of turning bias or "repetitive turning." Accordingly, CD-fed cavefish with few
419 nearby interactions exhibited a significantly higher turning bias than KD-fed cavefish (Figure 4A, B).
420 KD-fed cavefish displayed a comparable level of balanced turning as surface fish (close to a score of "1"
421 in Figure 4B). In summary, these results suggest that KD feeding could reduce repetitive turning while
422 inducing longer nearby interactions in cavefish.

423 Recording behavior each week (Figure 2) may yield a confounding factor, such as fish
424 remembering the recording environment and behaving differently than naive fish. To clarify whether our
425 results captured the genuine effects of KD feeding, we repeated the 4–5-week dietary treatment in a new
426 set of fish (Additional file 8). As observed in Figure 2, surface fish did not exhibit a detectable change in
427 the duration and number of nearby interactions between CD and KD feeding (Additional file 8A, B). In
428 contrast, CD-fed cavefish displayed fewer nearby interactions, whereas the level of nearby interactions
429 was retained in KD-fed cavefish, resulting in a higher level of nearby interactions in KD-fed cavefish
430 (Additional file 8A, B). In this repeated experiment, the results for repetitive turning were also similar to
431 those in the previous experiment; specifically, CD-fed cavefish displayed a high level of turning bias,
432 whereas KD-fed cavefish exhibited balanced turning (Additional file 8D).
433
434 We then explored other changes induced by KD feeding, including changes in sleep, 24-h
435 swimming distance, and adherence to a vibrating stimulus, which are distinct between surface fish and
436 cavefish. Cavefish exhibit reduced sleep duration and swim almost all day, perhaps to find nutrients in
437 the food-sparse environment [16, 20, 22, 54]. After 5 weeks of dietary treatment on the 3–4-month-old
438 fish, both surface fish and cavefish exhibited shorter sleep duration than observed before treatment,
439 regardless of the diet (Figure 5A, particularly at night), suggesting that growth between 3–4 and 4–5
440 months old exerted a negative effect on the sleep duration. However, there was no detectable difference
441 between CD and KD feeding.
442 Animals' sleep is usually fragmented, involving repeated sleep/awake cycles during the night
443 (diurnal animals) or day (nocturnal animals) [75]. Then, the structure and regulation of sleep are
444 typically analyzed according to the average duration and the number of events (bouts). Our detailed
445 sleep analysis illustrated that KD-fed cavefish displayed shorter sleep bout duration during the night
446 than CD-fed cavefish (Figure 5B). However, the number of sleep bouts did not differ between CD and

447 KD feeding (5 weeks; Additional file 9). Overall, the sleep phenotype showed a subtle change by KD
448 feeding, as cavefish exhibited shortened sleep duration.

449 Sleep duration is negatively correlated with the 24-h swimming distance [54]. Cavefish displayed
450 overall higher activity, which was consistent with previous findings [20, 54], and consistent with the
451 findings of longer swimming distances in the nearby interaction assay (Figure 5C). CD-fed cavefish
452 swam longer distances after the 5-week treatment, but KD-fed cavefish did not show a detectable change
453 in it after the treatment (during the day period, Figure 5C). Surface fish, in contrast, did not exhibit a
454 detectable difference in swimming distance between KD and CD feeding. Overall, the KD treatment
455 induced subtle changes in sleep-associated behaviors in both surface and cavefish.

456 In general, the KD is assumed to induce ketosis without increasing appetite. We then checked the
457 shift in foraging behavior under KD feeding. Cavefish evolutionarily exhibit increased foraging
458 behavior, which can be quantified with vibration attraction behavior (VAB), in which fish adhere to a
459 particular vibration stimulus (35–40 Hz) in the dark [58]. VAB is advantageous for prey capture in the
460 dark. Cavefish and surface fish did not exhibit a detectable difference in VAB between CD and KD
461 feeding, whereas VAB was significantly increased during 1 month of growth (pre-treatment vs. 5 weeks;
462 Additional file 10A). In summary, the VAB analysis indicated that KD feeding did not significantly
463 increase or decrease foraging behavior.

464 Although the KD diet induced significant changes in some behavioral outputs, it suppressed
465 growth during treatment. The average weights of KD-fed surface fish and cavefish were 55.5 % and
466 69.9 % of those in their CD-fed counterparts, respectively (5 weeks; Figure 6B). The standard length of
467 KD-fed surface fish was also significantly reduced (5 weeks; Figure 6A).

468

469 Are these behavioral and growth changes induced by ketosis? The KD contains high amounts of
470 fat, sufficient levels of proteins, and a minimum amount of carbohydrates. This question motivated us to

471 test the molecular basis of the effects of KD feeding by supplementing major ketosis metabolites, ketone
472 bodies, to the standard diet.

473 In humans, KD feeding induces ketosis, in which the liver releases beta-hydroxybutyrate (BHB)
474 and acetoacetate via beta-oxidation of fat [76]. Instead of supplying a massive amount of fat using the
475 KD, BHB might be responsible for the majority of effects observed after KD feeding. With this idea, the
476 ketone ester (D-β-hydroxybutyrate-R 1,3-Butanediol Monoester; delta-G® [56]) was provided as a
477 supplement to both surface fish and cavefish for 5 weeks. The ketone ester (KE) was expected to
478 undergo complete hydrolysis by the gut esterases, resulting in two BHB molecules (and
479 acetoacetate)[56]. It does not contain any salt ions, unlike the sodium or potassium salt forms of BHB,
480 nor does it has the racemic L-form; where only the D-form is considered to be biologically active [77].
481 Since we were unsure whether gut esterases were available in juvenile-adolescent fish at 3 months old,
482 we used 6-7 months old fish that have a mature gut system but are in the young adult stage. The results
483 indicated that the KE supplementation significantly reduced the serum GKI (Additional file 11), while
484 promoting nearby interactions in cavefish (Figure 7A and B). Swimming distance was slightly reduced
485 in cavefish (Figure 7C). Turning bias was not reduced by KE supplementation in cavefish (Figure 7D).
486 There was no detectable difference between CD and KE supplemental diets in sleep duration or VAB
487 (Additional file 12A and 12B, respectively).

488 Interestingly, the body growth of KE-treated surface and cavefish was not significantly different
489 from that of the control diet (CD), suggesting that the KE molecule (and therefore, the BHB molecule)
490 did not have a detectable negative effect on growth (Additional file 12C and D) (cf. Figure 6 in KD).

491 We also tested the supplemental feeding of the BHB salt form (sodium salt form of racemic
492 BHB: 50% L-form and 50% D-form). We used 11-12 months-old fish in this study since the younger
493 fish seemed to suffer from the high-salt-containing diet. The 4-week feeding result was essentially the
494 same as the KE supplemented diet feeding: the BHB salt supplemental diet significantly reduced GKI in
495 the serum of surface and cavefish (Additional file 13), while promoting nearby interactions in cavefish

496 (Additional file 14A and B) but reduced the duration of nearby interactions in surface fish (Additional
497 file 14A). No major change in response to the BHB feeding was detected in, swimming distance
498 (Additional file 14C), turning bias (Additional file 14D), sleep (Additional file 15A) and VAB
499 (Additional file 15B) in cavefish, while the BHB salt reduced growth (standard length and weight) in
500 surface fish (Additional file 15C and D). In contrast, cavefish did not show any detectable negative
501 effects on growth under the BHB salt supplemental feeding (Additional file 15C and D).
502 In summary, BHB (KE and BHB salt) treatment encompassed the effect of the KD treatment—
503 promoting social interactions. BHB, particularly KE, had a no-detectable negative effect on growth.
504 These facts suggest that ketone bodies can be responsible factors for the positive effects on social
505 behaviors of KD feeding. BHB treatment also indicated that older-age cavefish (6-7 months or 11-12
506 months old) were still capable of responding to ketone bodies, not only younger age groups (3-4 months
507 old).
508
509

510 **Discussion**

511 In this study, we examined the behavioral shifts induced by KD feeding and supplementation
512 with ketone bodies. Ketosis (high metabolic usage of ketone bodies comapring with glucose) is expected
513 to occur frequently in wild animals due to a failure to find food (fasting) or a reduced carbohydrate
514 inputs/synthesis (available nutrients). Even in asocial species that have evolutionarily reduced sociality
515 levels, certain levels of social interaction can still be crucial for mating. Under KD feeding, cavefish
516 maintained their juvenile level of nearby interactions until the treatment ended (5 weeks). Subsequently,
517 within 1 month after stopping KD feeding, nearby interactions were reduced to an indistinguishable
518 level from the control group. Surface fish exhibited a higher number of nearby interactions than
519 cavefish, and no detectable difference was observed in nearby interaction levels between CD- and KD-
520 fed surface fish. KD feeding also effectively reduced repetitive turning in cavefish, whereas CD-fed
521 cavefish exhibited a high level of repetitive turning. There were no major changes in sleep duration and
522 foraging behavior (VAB) after 1 month of KD feeding. These patterns in behaviors and growth were
523 consistent across two replicated experiments (social affinity and repetitive turning), supporting a
524 scientific rigor of the observed effects under KD feeding. Additionally, the diet shift from live brine
525 shrimp (the standard diet for juveniles) to the standard zebrafish pellet diet (used as the control diet in
526 this study) did not yield any detectable changes in behaviors, serum glucose, and ketone levels, further
527 supporting the effect of KD feeding. Finally, the major KD metabolite, BHB, could account for KD's
528 positive effect on nearby interactions, indicating that the ketone bodies play a pivotal role in this
529 treatment.

530

531 *Effects of the KD on blood ketone levels and body growth*

532 During 4–5 weeks of KD feeding, blood ketone and glucose levels were reduced compared to the
533 effects of the CD in both surface fish and cavefish, contradicting our expectation of higher serum ketone
534 levels in the KD group. However, the GKI [52] was significantly lower under KD feeding than under

535 CD feeding. These significant changes in GKI in both surface fish and cavefish suggest that the
536 metabolic balance shifted toward the ketosis side due to KD feeding. Indeed we detected the higher
537 ketone concentration in cavefish brain tissues under KD feeding compared to CD feeding. In general,
538 cavefish had a higher GKI than surface fish under both diets, indicating that the cavefish physiology was
539 constitutively biased toward glycolysis. For example, blood glucose levels in cavefish under KD feeding
540 were similar to those in surface fish under CD feeding, while cavefish had ~2.5-fold lower ketone levels
541 than surface fish under CD feeding, resulting in a higher GKI even under KD feeding (Fig. 1).
542 KD feeding for 4–5 weeks also resulted in slowed body growth. This growth retardation has been
543 observed in patients with epilepsy who were chronically fed a KD [49, 78], and these results were
544 consistent with our observations in KD-fed fish. This study found that a ketone body (ketone ester) did
545 not suppress body growth while increased the social-like activities. The detailed molecular/physiological
546 mechanisms by which ketosis affects behaviors are at the early stage of investigations (see murine
547 resaerches [15, 79, 80]). Our studies using ketone ester and BHB sodium salt provided an alternative
548 starting point to unravel the mechanisms underlying KD-associated phenotypes (see below).
549
550 *Effects of ketones in the TCA cycle and epigenetics in the brain*
551 In mammals, KD feeding induces a "starvation"-like state, causing the liver to release ketone
552 bodies into the bloodstream. BHB is the major ketone body produced by the liver through beta-
553 oxidation. The gut epithelia also absorb and circulate ketone bodies from the diet and/or gut microbiota.
554 Both liver-derived and gut-derived ketone bodies can cross the blood–brain barrier and serve two
555 functions: (i) inhibiting histone deacetylase, which influences epigenetic regulation and induces gene
556 expression in neurons; and (ii) acting as a general energy source that is converted into acetyl-CoA to fuel
557 the aerobic TCA cycle in neurons. Both pathways have the potential to alter brain function. The fact that
558 cavefish can easily tolerate high blood glucose levels that would paralyze surface fish [24], and the
559 upregulated Wnt signaling in cavefish, potentially resulting in high glycolytic activity as observed in

560 humans [31, 81], support the aforementioned hypothesis that cavefish exhibit high blood glucose levels
561 and mainly generate energy via glycolysis. Additionally, ketone bodies can promote behavioral shifts by
562 changing the epigenetic state through histone deacetylase (HDAC) inhibition [82, 83]. Inhibition of
563 HDAC increases gene expression in general. This possibility may lead to a positive effect in cavefish
564 neurons due to the fact that cavefish have more downregulated genes (2,913 genes, $\log_2 < -1.0$) than
565 upregulated genes (1,643 genes, $\log_2 > 1.0$) in the transcriptome at 72 h postfertilization [31, 84].
566 Furthermore, more methylated loci are found in the eye genes of cavefish than in surface fish, which
567 could also occur in other tissues including the brain regions [85], and most of these methylated gene loci
568 were downregulated. The brains of patients with autism are also expected to be hypermethylated,
569 resulting in a transcription-less condition [86]. Therefore, these two pathways, namely metabolism and
570 epigenetics, are highlighted as possible targets of ketone bodies during behavioral shifts under ketosis.
571 Future research should address these possibilities to clarify the metabolism-based evolution of behavior
572 (*cf.* [15])
573
574 *Ontogeny of nearby interactions and the KD*
575 In this study, 3–4-months-old cavefish exhibited a detectable level of nearby interactions (social
576 affinity), which decayed under CD feeding. Interestingly, KD-fed cavefish and surface fish fed either
577 diet maintained a similar level of nearby interactions during the 5 weeks of dietary feeding. The
578 reduction of nearby interactions in CD-treated cavefish can be explained by (1) quicker exhaustion
579 under CD feeding (aerobic ketosis produces more adenosine triphosphate than anaerobic glycolysis), (2)
580 greater anxiety in the recording environment [33], and (3) less social motivation. The first explanation is
581 unlikely because CD-fed cavefish swam at comparable or longer distances than KD-fed cavefish (e.g.,
582 Additional file 7 and 8C). The higher level of anxiety could explain the findings because cavefish
583 exhibited increased repetitive turning, which is related to higher anxiety in mammals [74]. In addition,
584 prior rerearch has shown that cavefish displayed fewer nearby interactions in an anxiety-associated

585 unfamiliar environment [33]. In the future, the anxiety level should be monitored using plasma cortisol
586 levels [87]. Less motivation regarding social affinity is also a possible cause, and this possibility can be
587 assessed by examining neural activities in social decision-making networks, including the preoptic area,
588 nucleus accumbens, and striatum [88, 89]. Explanations (2) and (3) are not mutually exclusive, and co-
589 occurrence is possible. These possibilities will be assessed in our future study.
590
591 *Possible target system for ketosis*
592 Under KD feeding and BHB treatment, we observed an increased in social affinity and a
593 reduction in repetitive turning. However, we did not detect major changes in sleep and VAB.
594 Studies regarding neurotransmitters and their associated behaviors have revealed tight
595 associations between them, such as the dopaminergic system being associated with social and repetitive
596 behavior, and the cholinergic/orexinergic/histaminergic systems playing a major role in sleep regulation
597 (Additional file 16). The behavioral phenotypes in this study highlighted the possible involvement of the
598 dopaminergic system but less involvement of others (i.e., the serotonergic, cholinergic,
599 orexin/hypocretinergic, histaminergic, or adrenergic system). The dopaminergic and other
600 social/repetitive behavior-associated pathways were also highlighted in the GO term/KEGG pathway
601 analysis using genes showing the same directional expression changes (upregulation or downregulation)
602 in patients with autism versus neurotypical individuals [90] and cavefish versus surface fish at 72 hrs
603 postfertilization [31, 91, 92] (Additional file 17). The shared same directional expression genes are
604 enriched in the synaptic vesicle cycle, long-term depression/potentiation, dopaminergic/serotonergic
605 synapses, and oxytocin-signaling pathway (Additional file 18). The underlying reason why ketosis or
606 ketone bodies have a stronger effect on the dopaminergic system than on the other systems is
607 undetermined, calling for further investigation. However, the possibility that the neurons and other cells
608 involved in the dopaminergic system can be sensitive to ketosis in a vertebrate with genes dysregulated
609 in a similar manner (upregulation or downregulation) to patients with autism is extremely interesting,

610 and it indicates that ketosis-induced adjustment of dysregulated genes may not be sufficient to mitigate
611 cellular rhythms associated with insomnia.
612 Although this prediction, we do not consider our finding is directly applicable to human
613 disorders without careful interpretation because systemic and organ physiologies are vastly different
614 between fish and mammals. The knowledge gained from this unique fish system hinted a good starting
615 point to investigate ketosis-induced behaviors in other asocial fish species and mammals.
616 This study, including the results of ketone bodies treatment, sheds light on the candidate genetic
617 and molecular mechanisms associated with ketosis, deepening our knowledge of animal behaviors in
618 response to metabolic states.
619
620 *The BHB supplement and body growth*
621 In this study, ketone ester supplementation did not yield detectable negative impacts on growth,
622 while the BHB sodium salt retarded body growth in surface fish. In contrast, the BHB salt treatment did
623 not show a negative effect on cavefish growth (Additional file 12C, D and 15C, D). We suspect that the
624 high level of sodium salt ingestion in the BHB salt treatment has a negative effect on growth, and the
625 tolerance levels for high sodium ions (from BHB salt) may be different between surface fish and
626 cavefish. Additionally, we also suspect that the reduction of nearby interaction in BHB-fed surface fish
627 was caused by the high sodium ion levels (Additional file 14A). However, future physiological studies
628 on the rhinal function in surface fish and cavefish are needed to provide answers.
629
630 *Ketosis in the cave environment*
631 Cave-dwelling animals usually experience less temperature fluctuation and fewer dietary inputs
632 [93], although these features can vary according to caves. The diets of cave-dwelling animals in the dry
633 season (approximately 6 months/year) could consist of organic matter in pool bottoms, bat guano (for
634 larger adults), or small crustaceans (for smaller fish), whereas food is sparse in the rainy season

635 (approximately 6 months/year) [34, 94]. These available diets would contain extremely low amounts of
636 carbohydrates and could be high in protein and fat (e.g., crustaceans). Although some amino acids,
637 including lactate, and glycerol can be used for glucose synthesis in fish [95], cavefish are expected to be
638 exposed to carbohydrate-deprived diets or frequent fasting and therefore experience frequent ketosis. In
639 our observations, wild cavefish exhibited similar social affinity as observed in KD-fed cavefish in this
640 study (Movie 1). Although these observations and dietary inputs suggest that wild cavefish may undergo
641 frequent ketosis, recent multiple reports have indicated that cavefish may undergo anaerobic glycolysis
642 to adapt to the cave water, which has approximately 20% lower oxygen levels (sevral cave pools [62, 96,
643 97]). Additionally, cavefish tend to store lipids instead of using them (via beta-oxidation) through the
644 enhanced PPARγ pathway [36]. These expectations of low ketosis appear to contradict expectations in
645 the wild—starved ketosis/aerobic conditions. However, they appears to align well with the findings in
646 cavefish, namely higher blood glucose levels compared to surface fish in many feeding conditions
647 (including KD feeding) in this study (Fig. 1, Additional files 4, 5 and 11). Cavefish appear to have
648 evolved to maintain a high GKI (high blood glucose and low ketone levels); therefore, the physiology of
649 cavefish may allow them to survive in low-oxygen conditions by utilizing anaerobic glycolysis. KD-fed
650 cavefish behave similarly to wild cavefish because the balance between ketosis and glycolysis could
651 reach a similar level as that in the wild after KD feeding. In contrast, if cavefish are fed a typical
652 carbohydrate-rich lab fish diet, it may overactivate glycolysis and result in a higher GKI, which may
653 lead to reduced social affinity and increased repetitive circling. The future use of a pharmacological
654 glycolysis inhibitor (e.g., 2-deoxy-D-glucose; [98]) can reveal the relationship between GKI and
655 cavefish behaviors.
656
657 *Summary statement*
658 Surprisingly, solitary animals share a set of dysregulated genes and behavioral outputs (e.g.,
659 bees, and caveefish). In this study, we demonstrated that a diet inducing ketosis shifts these behaviors

660 towards the surface fish phenotype, regardless of the presence of over a thousand dysregulated genes.
661 There is a possibility that ketone body-based tratment, alongside ongoing gene therapy approaches, may
662 open a path for sustainable and less toxic therapy for multigenic psychiatric disorders, including autism,
663 although the affected gene pathways under ketosis remain unclear. Additionally, differentially
664 expressed metabolic genes, which have been largely overlooked due to interpretational difficiulties,
665 reemerged with their importance in understanding the genetics of behaivors. Given the highlighted role
666 of mitochondria-based disorders in neuroscience [99, 100], investigating the balance between glycolysis
667 and ketosis could serve as a starting point for identifying molecular mechanisms associated with
668 neuronal states and behavioral shifts. Furthermore, reinvestigating the genetic factors for known evolved
669 behaviors in the context of the metabolic shifts, in addition to the neural genes, may uncover the
670 evolution of behaviors based on the evolution of metabolisms.

Declarations

Ethics approval and Consent to participate:

The experimental protocols used in this study and fish care were approved by the institutional animal care and use committee (IACUC) at the University of Hawai‘i (17-2560).

Consent to publish:

All authors agreed on publishing these data and this manuscript.

Availability of data and materials:

The video datasets generated and/or analyzed during the current study are available at the university's shared server and will be deposited to Zenodo (https://zenodo.org/). All program scripts used in this study are available at https://zenodo.org/record/5122894#.YvM4dPHMLsM

Competing interests:

The authors declare no competing financial interests.

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Author contribution:

MI: designed the experiments, performed the experiment and analyses, interpreted the data wrote the initial draft, and edited the manuscript.

AT: performed the BHB salt experiment and analysis, edited the manuscript

MG: performed the KE experiment and analysis, edited the manuscript

JC: performed the KE experiment and analysis, eidthed the manuscirpt

DB: performed the fasting experiment and analysis, edited the manuscript

VS: performed the BS vs CD experiment and analysis, edited the manuscript

MH: performed the fasting experiment and analysis, edited the manuscript

RBU: performed KD deprived exxperiment and analysis, edited the manuscript

KP: performed the blood measurements of the KE and BHB treatments analysis, edited the manuscript

MW: designed the experiments, consulted the experimental procedure, and edited the manuscript

RL: designed the experiments, consulted the experimental procedure, and edited the manuscript

MY: designed the experiments, performed the experiment and analyses, interpreted the data and wrote the initial draft, and edited the manuscript

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Figures

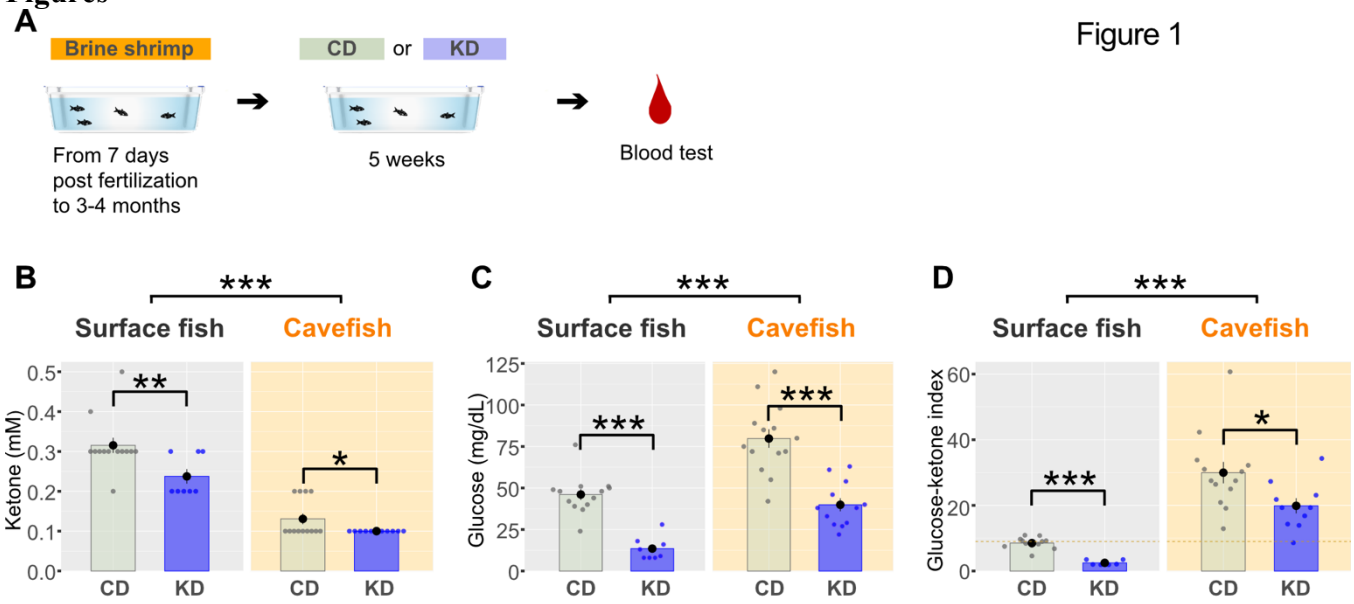

Figure 1. Blood glucose and ketone levels under the control diet (CD) or ketogenic diet (KD). (A) Experimental procedure. After fish were raised for 3–4 months on a brine shrimp larva diet, fish were fed the CD or KD for 5 weeks. Blood glucose and ketone levels were measured after the 5-week period. (B) Blood ketone level (mmol/L). Ketone levels were significantly reduced by KD feeding in both surface fish (SF) and cavefish (CF). Bars represent the data mean and whiskers represent ± standard error of the mean. Dots indicate individual data. The generalized linear model (family = Poisson) followed by post-hoc Holm's correction was applied for the statistical tests (see Methods and Additional file 2). (C) Blood glucose level (mg/dL). Glucose levels were significantly reduced by KD feeding in both SF and CF. The linear model (family = Gaussian) followed by post-hoc Holm's correction was applied for the statistical tests. (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. The generalized linear model (family = Gamma) followed by post-hoc Holm's correction was applied for the statistical tests.
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 Additional file 2.

Figure 2

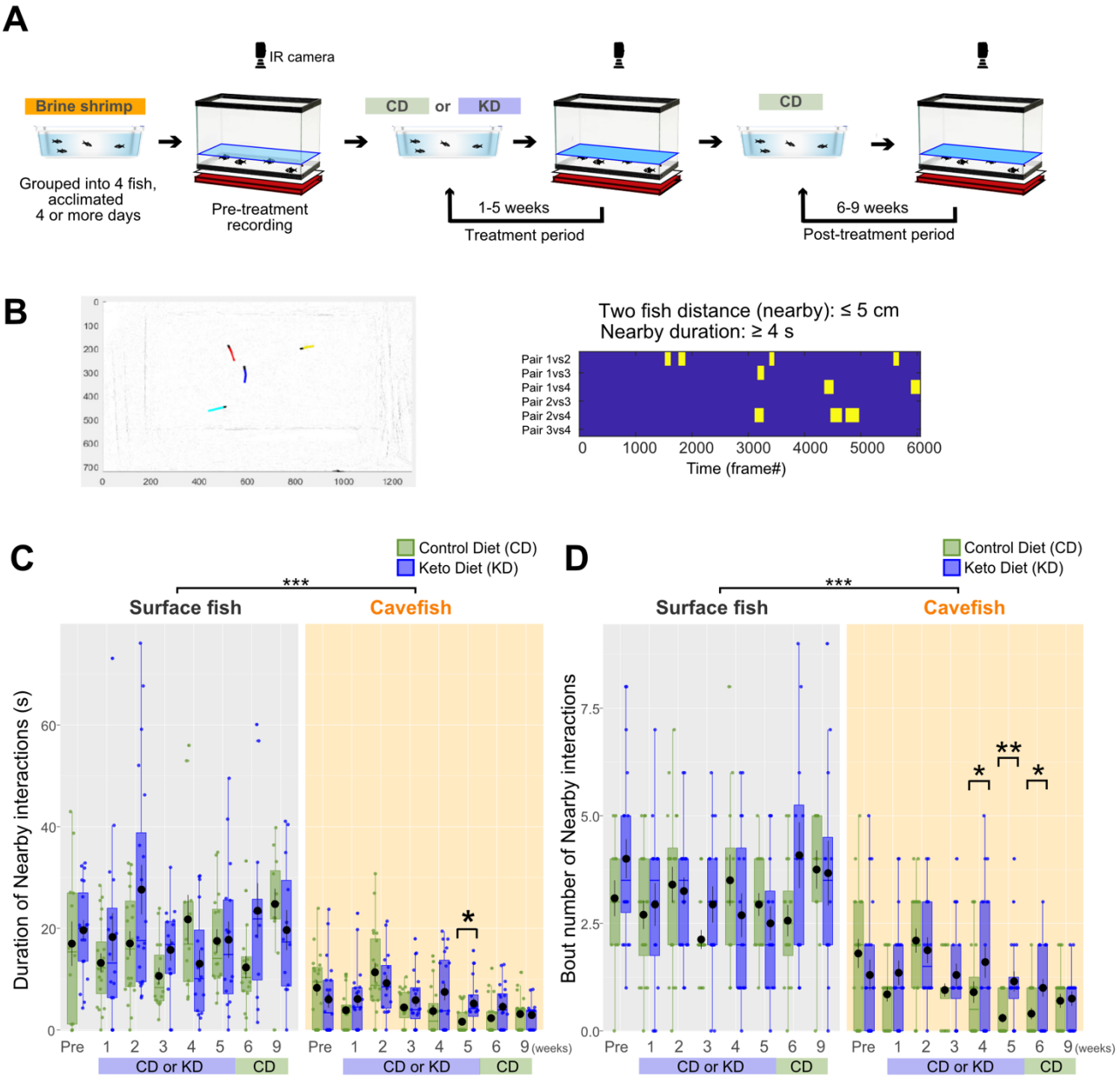

Figure 2. Time-course of nearby interaction changes during 9 weeks of control diet (CD) or ketogenic diet (KD) feeding.

(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 5 of feeding. Subsequently, all groups, including KD-fed fish, were given the CD until week 9. (B) Example of nearby interaction events among surface fish (SF). The left panel presents an example frame of the video, with colored lines indicating 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 in a raster plot, where 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. Data are shown in boxplots indicating the 25th, 50th and 75th percentiles in the boxes. The linear mixed-effect model followed by post-hoc Holm's correction was applied for the statistical tests. (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 boxplots indicating the 25th, 50th, and 75th percentiles. The generalized linear model (family = Poisson) followed by post-hoc Holm's correction was applied for the statistical tests. 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 Additional file 2.

Figure 3

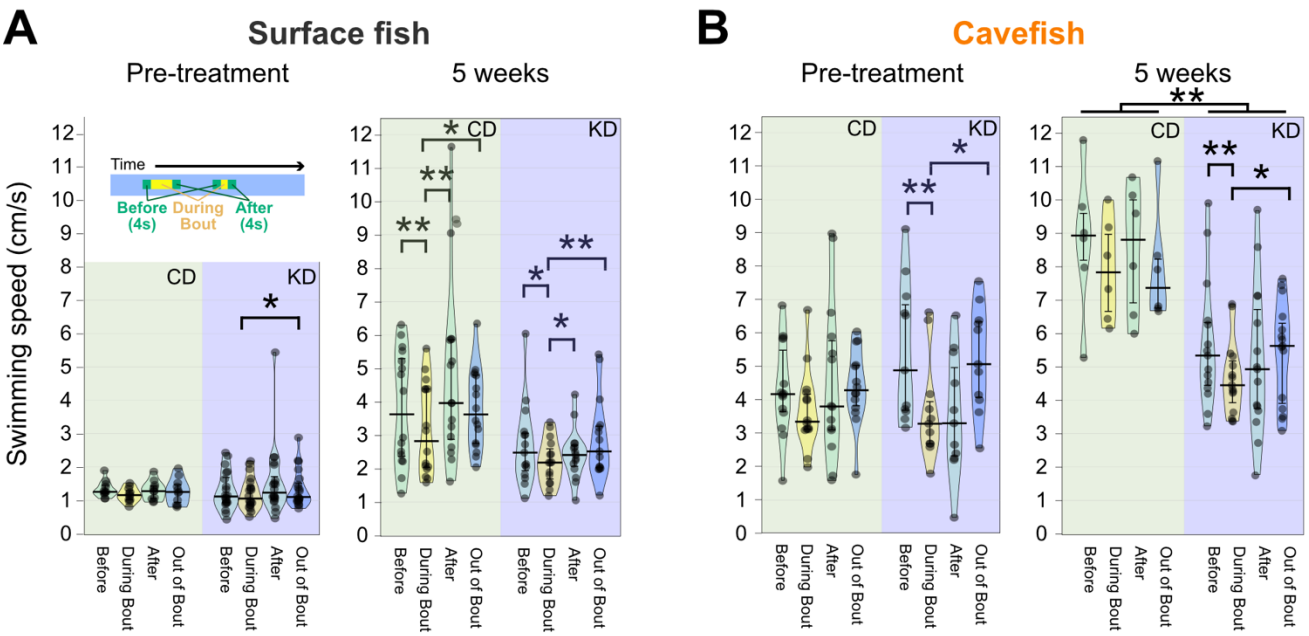

Figure 3. Ketogenic diet (KD) feeding induced surface fish (SF)-like speed profiles during nearby interactions in cavefish (CF).

Changes in swimming speed before, during, and after nearby interaction events in SF (A) and CF (B). The mean swimming speeds were plotted for: (i) 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 (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). The linear mixed-effect model followed by post-hoc Holm's correction was applied for the statistical tests. (B) Swimming speed was reduced during nearby interactions only in the KD group in week 5 (right panel). The bars indicate the 25th, medians, and 75th percentiles of the data points. The different speed profiles between the CD and KD groups in the Pre-treatment are due to the naturalistic standing variation in *A. mexicanus* system. The linear mixed-effect model followed by post-hoc Holm's correction was applied for the statistical tests.
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 Additional file 2.

Figure 4

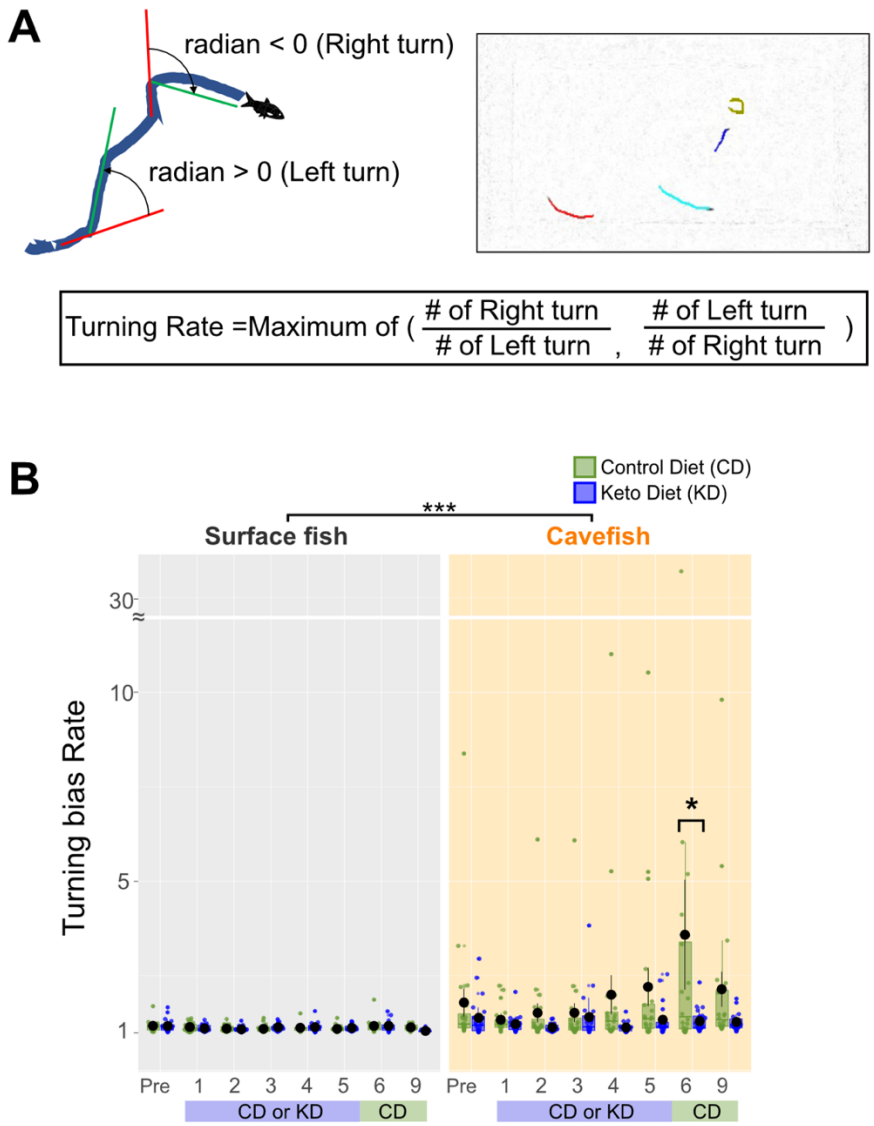

Figure 4. Biased turning was attenuated by the ketogenic diet (KD).
(A) Diagram and the calculation formula for the turning bias index. The changes in the left or right traveling directions were calculated every five frames (every 0.25 s) across all trajectories and expressed as radians. Positive radian 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 was larger than in KD-fed cavefish (see week 6). The generalized linear model followed by post-hoc Holm's correction was applied for the statistical tests.
Bars represent the data mean and whiskers represent ± 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 Additional file 2.

Figure 5

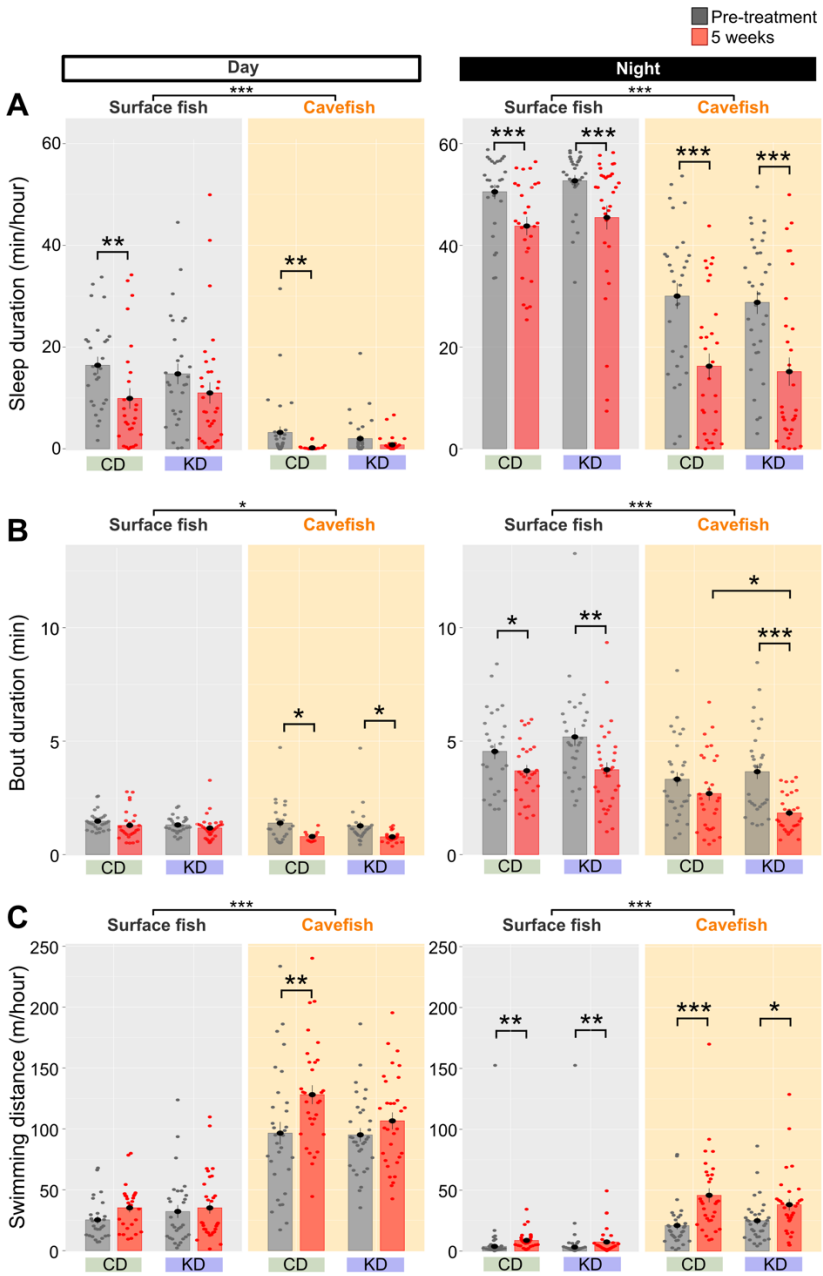

Figure 5. Day and night sleeping durations and swimming distances were not altered by ketogenic diet (KD) feeding.

(A) Sleep duration (min/h) during the day (left) and night (right). During 5 weeks of growth, the sleep duration decreased in both surface fish and cavefish regardless of the diet (particularly during night). The linear mixed-effect model followed by post-hoc Holm's correction was applied for the statistical tests. (B) Average sleep-bout duration (min/10 min bin) during the day (left) and night (right). During 5 weeks of growth, the sleep bout duration was lower in surface fish under both dietary conditions and in KD-fed cavefish (night). The linear mixed-effect model followed by post-hoc Holm's correction was applied for the statistical tests. (C) Swimming distance during the day (left) and night (right). Cavefish fed the control diet (CD) exhibited a longer swimming distance during the day and night. Conversely, both surface fish fed either diet and cavefish fed the KD exhibited a significantly increased swimming

distance only at night. The linear mixed-effect model followed by post-hoc Holm's correction was applied for the statistical tests.
Bars represent the data mean and whiskers represent ± 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 Additional file 2.

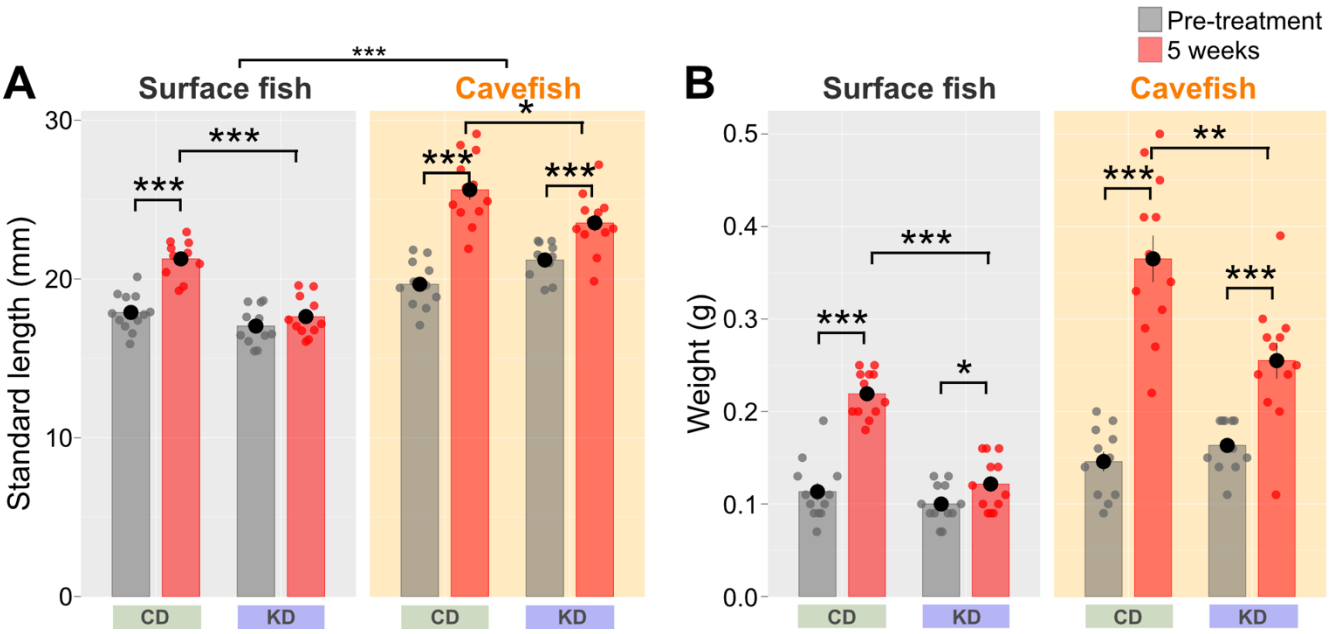

Figure 6. Body length and weight under control diet (CD) or ketogenic diet (KD) feeding. (A) Standard length (cm). KD-fed surface fish and cavefish were significantly smaller than their CD-fed counterparts. The linear mixed-effect model followed by post-hoc Holm's correction was applied for the statistical tests. (B) Body weight (g). KD-fed surface fish and cavefish weighed less than their CD-fed counterparts. The linear mixed-effect model followed by post-hoc Holm's correction was applied for the statistical tests.

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 Additional file 2.

Figure 7

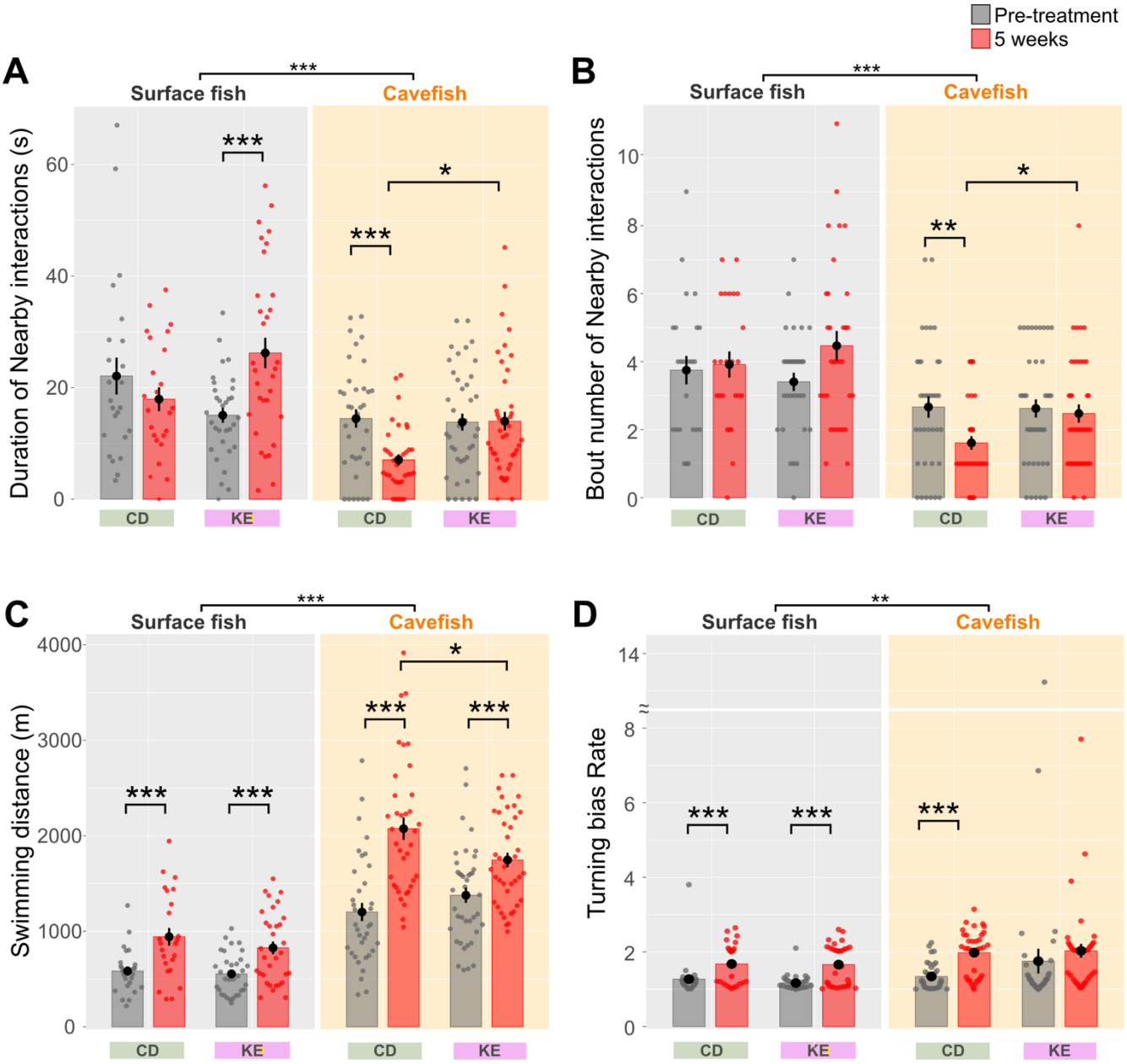

Figure 7. Nearby interactions and other behaviors under control diet (CD) or ketone ester–supplemented diet (KE) feeding.

(A) Duration of nearby interactions (s). After 5 weeks, the duration of nearby interactions increased in KE-treated surface fish and cavefish. The linear mixed-effect model followed by post-hoc Holm's correction was applied for the statistical tests. (B) Number of nearby interaction events. The number of nearby interactions was promoted in KE-treated cavefish. The generalized linear model (family = Poisson) followed by post-hoc Holm's correction was applied for the statistical tests. (C) Swimming distance. KE-treated cavefish exhibited a slight but significant decrease in swimming distance compared to CD-treated cavefish in a 5-min assay. The linear mixed-effect model followed by post-hoc Holm's correction was applied for the statistical tests. (D) Turning bias ratio. No significant difference was detected between the CD and KE groups. The generalized linear model (family = Gamma) followed by post-hoc Holm's correction was applied for the statistical tests.

1067 Data are presented as the mean ± standard error of the mean. Dots indicate individual data. N = 20 for all
1068 groups. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. All detailed statistical data are available in Additional
1069 file 2.
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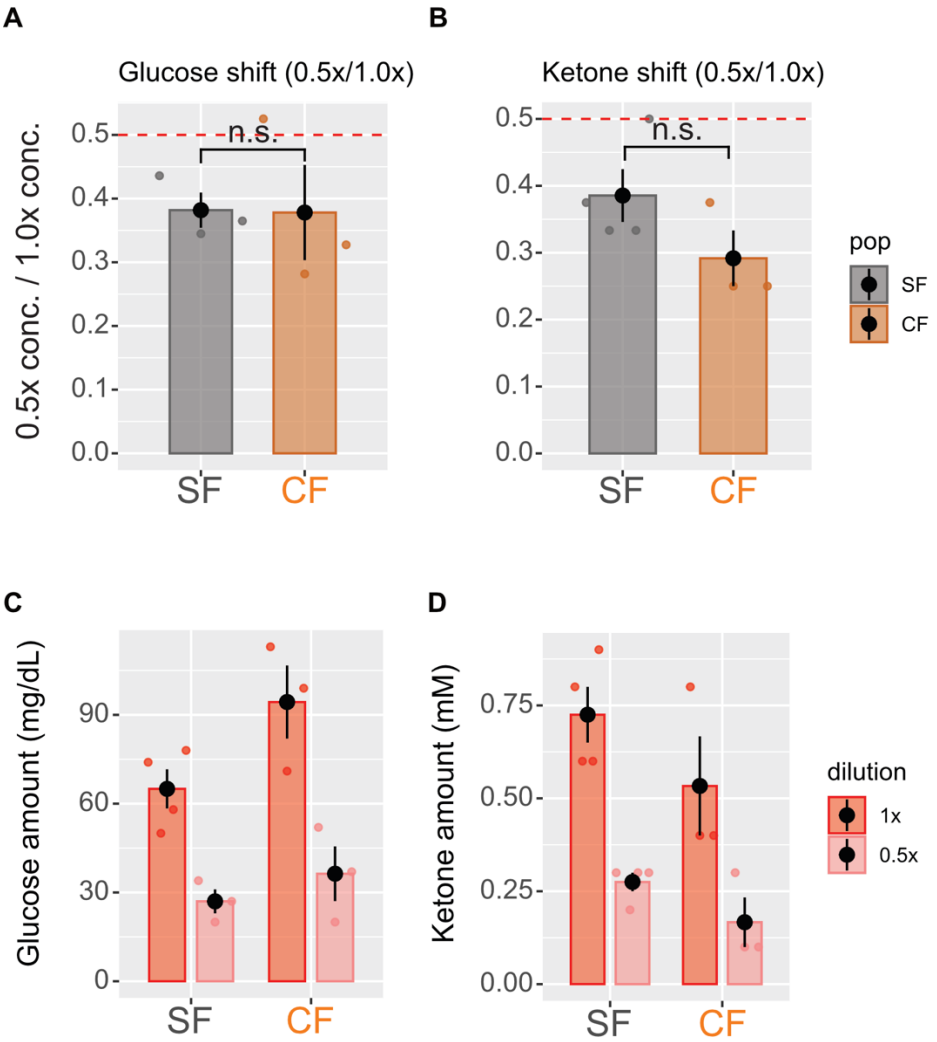
1071

1072 Additional file, figure legends

Additional file1



1073
1074 Additional file 1: **No detectable effect of the hematocrit levels on serum glucose or ketone body**
1075 **measurements.** The blood of three surface fish and three cavefish was individually collected. Each
1076 blood sample was divided into non-diluted (1.0x) and diluted (0.5x) portions diluted with phosphate
1077 buffered saline (PBS). The blood samples were then measured using by Abbott Precision Xtra. If the
1078 difference of the hematocrit levels between surface and cavefish (28.51 ± 0.03% and 35.56± 0.03%,
1079 respectively) affected the readings of glucose or ketone bodies, the diluted/undiluted ratios would show
1080 a significant difference between surface fish and cavefish. (A) Ratio of glucose concentration in diluted
1081 (0.5x) and non-diluted (1.0x) blood. The expected ratio is 0.5, but it was shifted towards a lower
1082 concentration, possibly due to the low hematocrit level (~15%) in 0.5x. However, the ratio of glucose
1083 concentrations showed no detectable difference between surface fish and cavefish, suggesting that the
1084 different hematocrit levels did not have a significant effect on the serum glucose readings. (B) Ratio of
1085 ketone bodies concentration in diluted (0.5x) and non-diluted (1.0x) blood. Similar to (A), the ratio
1086 showed no significant difference between surface fish and cavefish, indicating that the different
1087 hematocrit levels between the two populations had little effect on the ketone body concentration
1088 readings. (C) Raw measurements of the glucose concentrations used in (A). (D) Raw measurements of
1089 the ketone body concentrations used in (B). Detailed statistical scores are available in Additional file 2.
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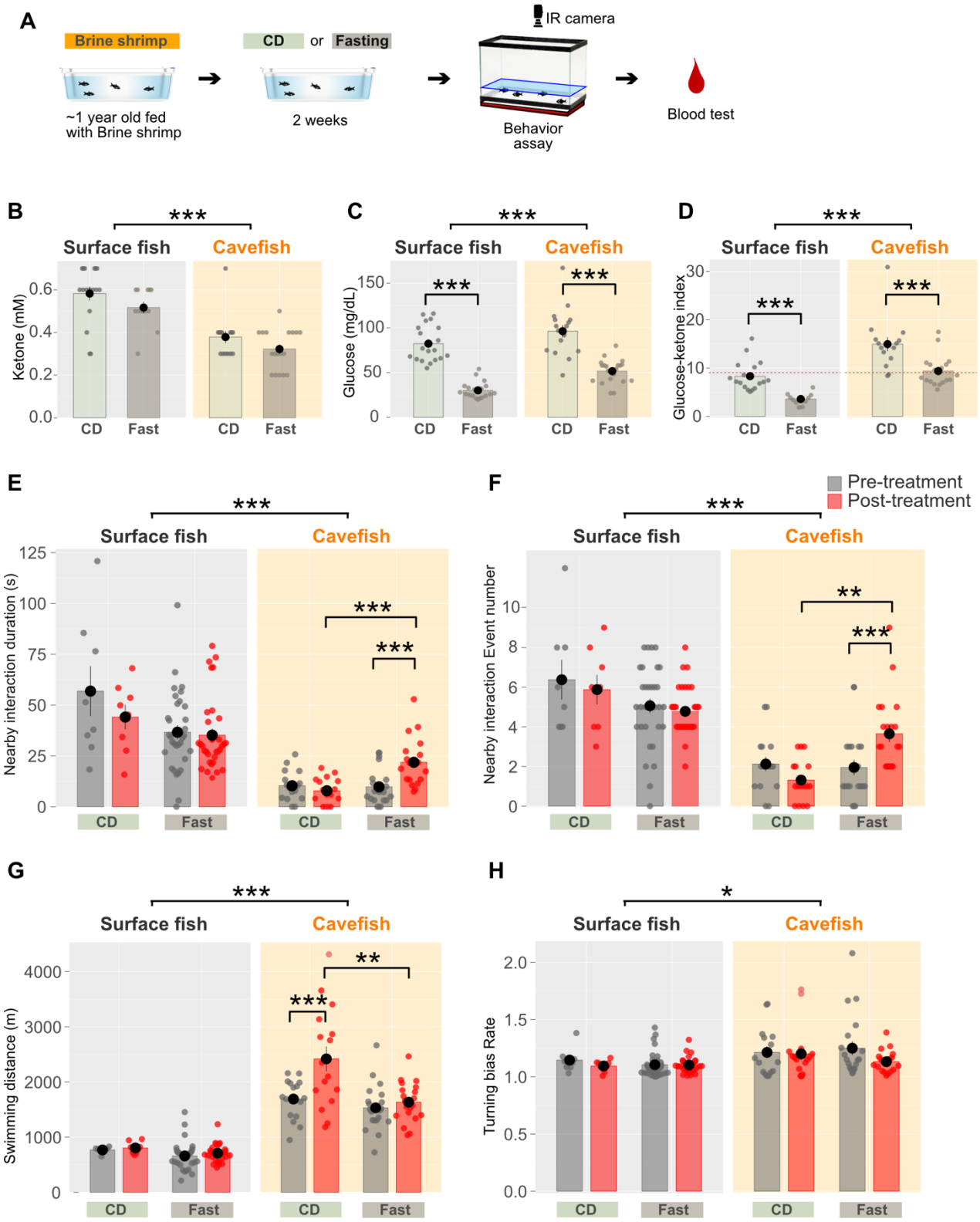
Additional file 2: **Statistical scores** for Figure 1-7; Additional file 1,4-15
https://docs.google.com/spreadsheets/d/1CFnKjQj2kQ1cvwpxl2N-Z6pEIOq69LtH/edit?usp=sharing&ouid=102214478617384425040&rtpof=true&sd=true

Additional file 3: **Movie file recorded in the Pachón cave pool in 2019**. Approximately 20 fish were transferred from the original cave pool to a foldable 2.44 x 2.44 m round pool (Play Day Round Kiddie Pool, Walmart Inc., Bentonville, Arkansas, USA) within a distance of approximately 10 m from the original pool in the same Pachón cave. These wild Pachón cavefish were acclimated for 1 day (24 hrs). Fish swimming behavior was then recorded using an infrared camcorder (DCR-SR200C, Sony, Tokyo, Japan)
https://drive.google.com/file/d/15iu09o_Jkl6vs1lwL0g52b5St8lZ5-LO/view?usp=sharing



Additional file 4: **Two weeks of fasting reduced the glucose ketone index (GKI) in both surface fish and cavefish, and increased nearby interactions in cavefish.**
(A) Experimental procedure. Fish were either fasted (Fasting) or fed with brine shrimp (CD) for 2 weeks, after which blood glucose and ketone levels were measured. (B) Blood ketone level (mmol/L).

1110 The 2-week fasting did not result in detectable changes in ketone levels in either surface fish (SF) or
1111 cavefish (CF). Data are presented as the mean ± standard error of the mean. Dots indicate individual
1112 data points. (C) Blood glucose level (mg/dL). Fasting significantly reduced glucose levels in both SF
1113 and CF. (D) The glucose ketone index (GKI) indicated that the ratio of glucose to ketone was lowered
1114 by fasting in both SF and CF, suggesting that this diet altered the balance between glucose and ketone.
1115 (E) Duration of nearby interactions in the 5-min assays. The time periods when a fish was within
1116 proximity (≤5 cm) and spent more than 4 s in a 5-min assay were summed for each fish. Fasted
1117 cavefish showed longer nearby interactions than the fed control. (F) Number of nearby interaction
1118 events. The number of nearby events (≤5 cm and ≥4 s) in a 5-min assay was counted. Fasted cavefish
1119 showed a higher number of nearby interactions than the fed control. (G) Swimming distance in a 5-min
1120 assay. Cavefish swam a longer distance than surface fish, and the fasted cavefish swam a shorter than
1121 the CD-fed cavefish after the 2 weeks treatment. (H) Turning biases of surface fish (left) and cavefish
1122 (right). Cavefish exhibited a higher turning index than surface fish, but none of the treatments induced
1123 further turning bias. See also Figure 4.
1124 SF: N = 8 for CD feeding, N = 5 for fasting. CF: N = 8 for CD feeding, N = 8 for fasting. *: $P < 0.05$, **: P
1125 < 0.01, ***: $P < 0.001$. All detailed statistical data are available in Additional file 2.
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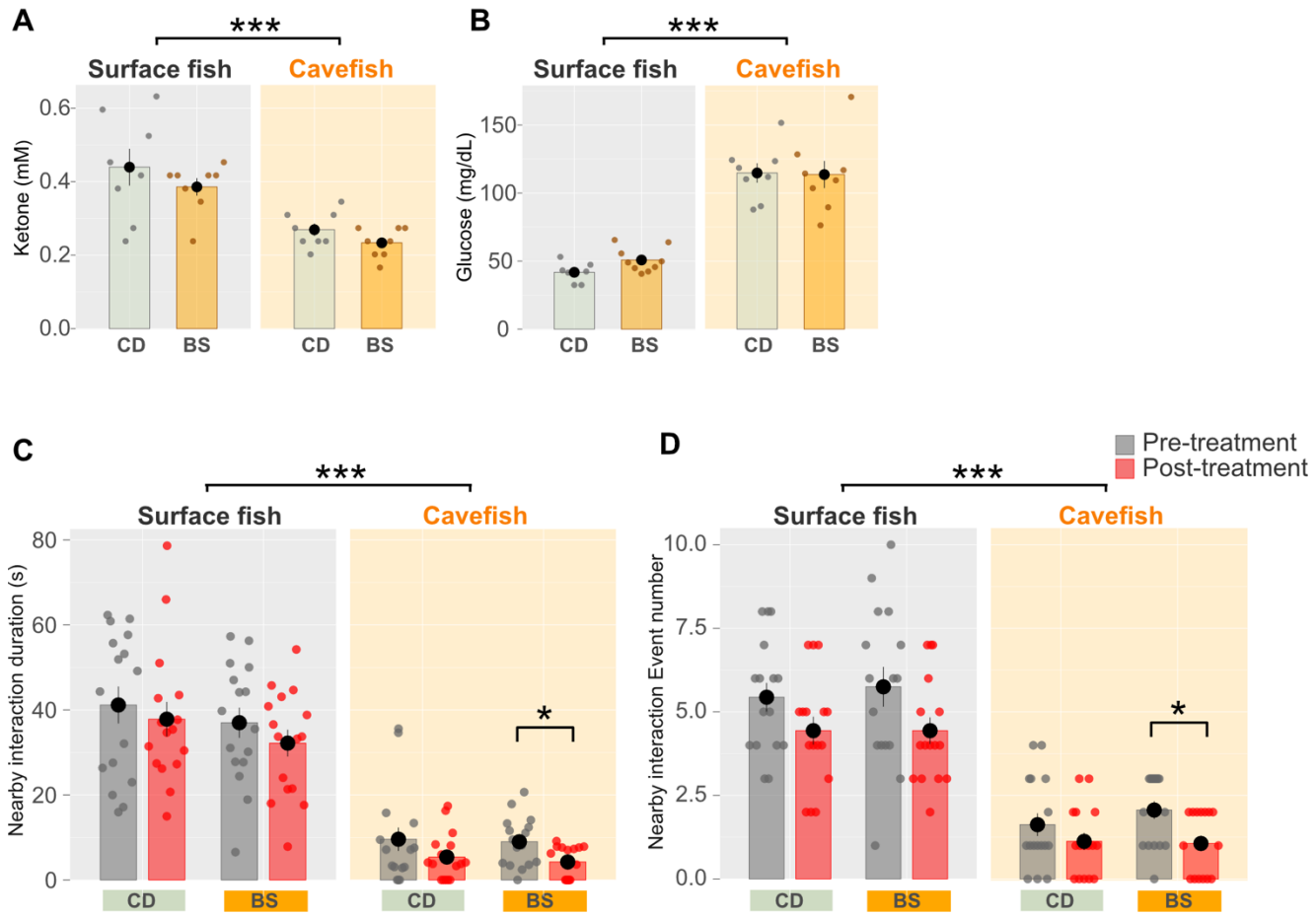
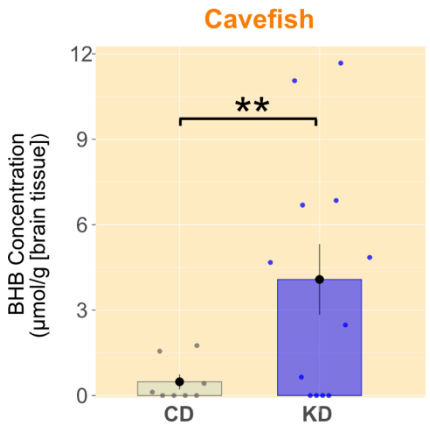


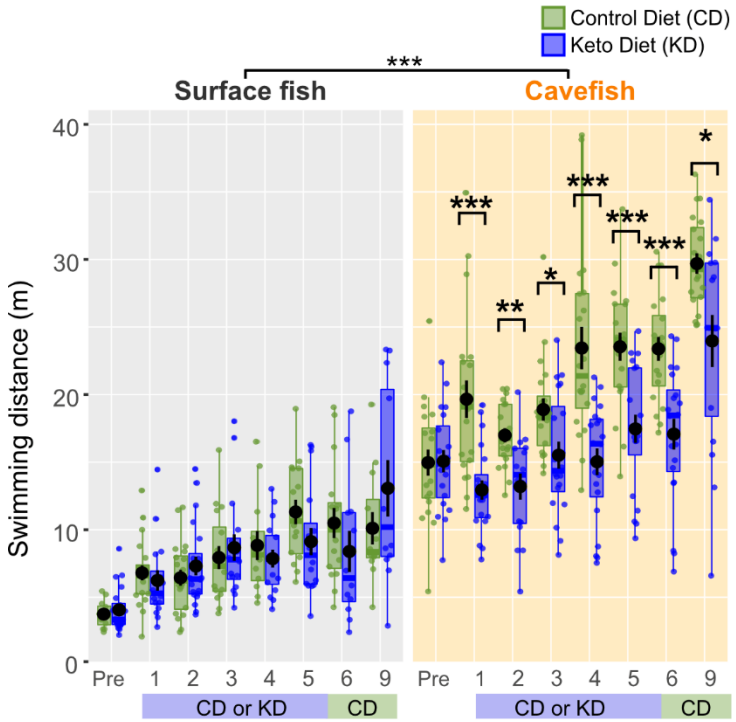
Additional file 5: **No detectable effect of the shift from live Artemia larvae (brine shrimp: BS) to the zebrafish standard control diet (CD) on the levels of serum glucose or ketone bodies, or nearby interactions.**
(A) Blood ketone level (mmol/L). After three weeks of treatment, ketone levels did not show detectable changes between CD and BS feeding in either surface fish or cavefish. Data are presented as the mean ± standard error of the mean. Dots indicate individual data points. (B) Blood glucose level (mg/dL). Glucose levels were not significantly changed by CD feeding in either surface or cavefish. (C) Duration of nearby interactions (s). During the 3 weeks of development, the average of the duration of nearby interactions was slightly reduced, but this was only detectable in BS-fed cavefish. (D) Number of nearby interaction events. The number of nearby interactions showed a similar result to (C): a slight reduction in interactions was detected in BS-fed cavefish. N = 20 for all groups. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. All detailed statistical data are available in Additional file 2. For (A) and (B), N = 8 for each group, For (C) and (D), N= 16 for each group. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.
All detailed statistical data are available in Additional file 2.

Additional file 6



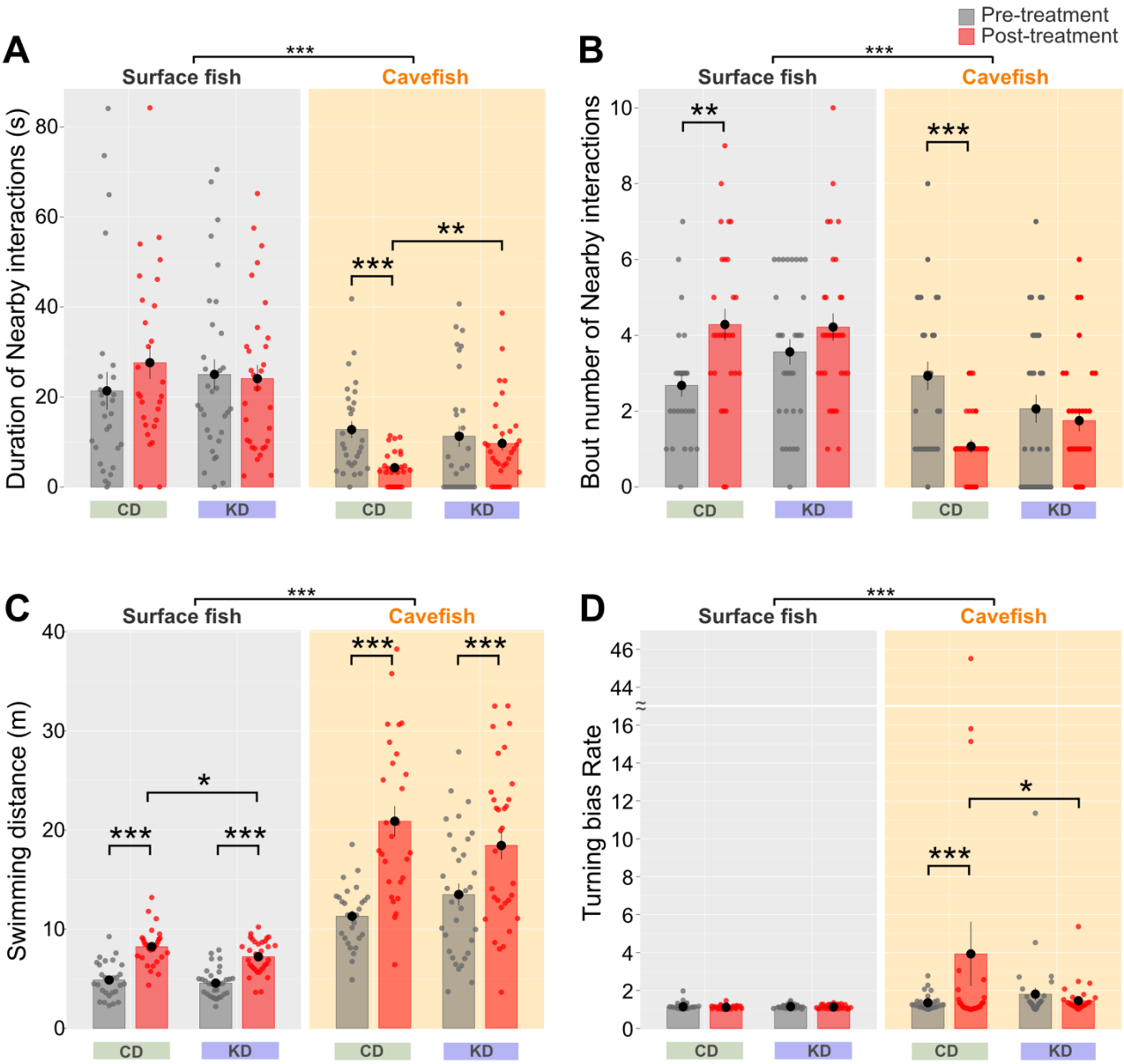
Additional file 6: **Brain ketone levels (beta-hydroxybutyrate) in cavefish under the CD or KD treatments.**
The concentrations of beta-hydroxybutyrate in the cavefish brain tissues were measured in 8 individuals fed the control diet (CD) and 12 individuals fed the ketogenic diet (KD) after 1 month of the diet treatment. The concentration was adjusted by the brain weight (g). Generalized linear model (family = Gamma) resulted in $X^2(1) = 10.5$, $P = 0.0012$ **. N = 8 for the CD-fed cavefish, N = 12 for the KD-fed cavefish.

Additional file 7

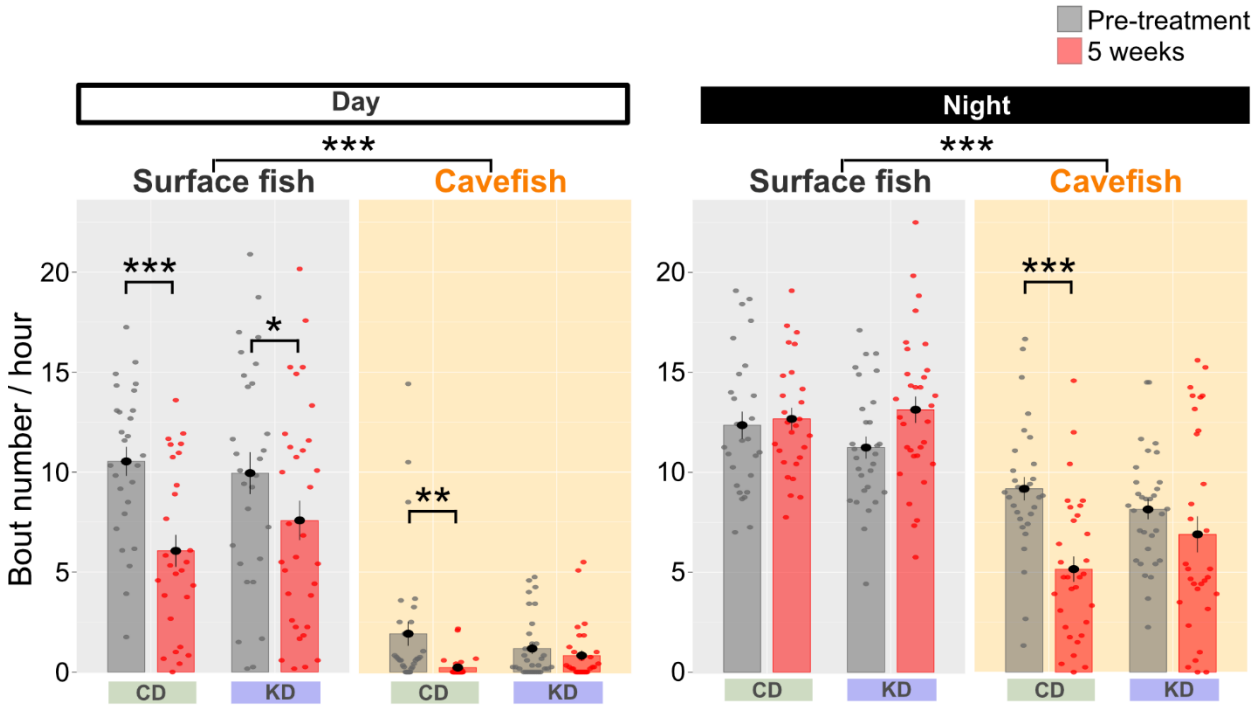


Additional file 7: **Ontogeny of swimming distance under ketogenic diet (KD) feeding.** Surface fish exhibited an increase in the swimming distance over time under both the control diet (CD) and KD. In contrast, cavefish fed KD showed a suppression of the swimming distance and activity, starting in week 1, and these values remained smaller than those in CD-fed fish until week 9. However, KD-fed cavefish subsequently exhibited an increased swimming distance at week 9 after depriving KD feeding from week 6.

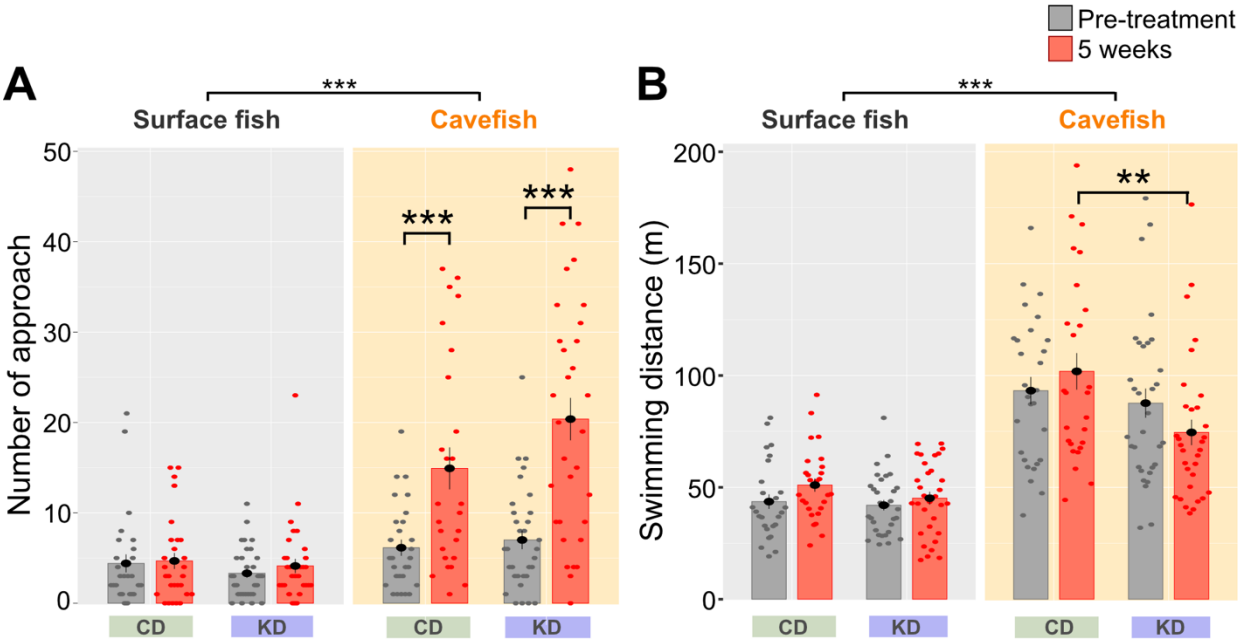
Data are presented as box plots. Dots indicate individual data. N = 20 in all groups. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. All detailed statistical data are available in Additional file 2.



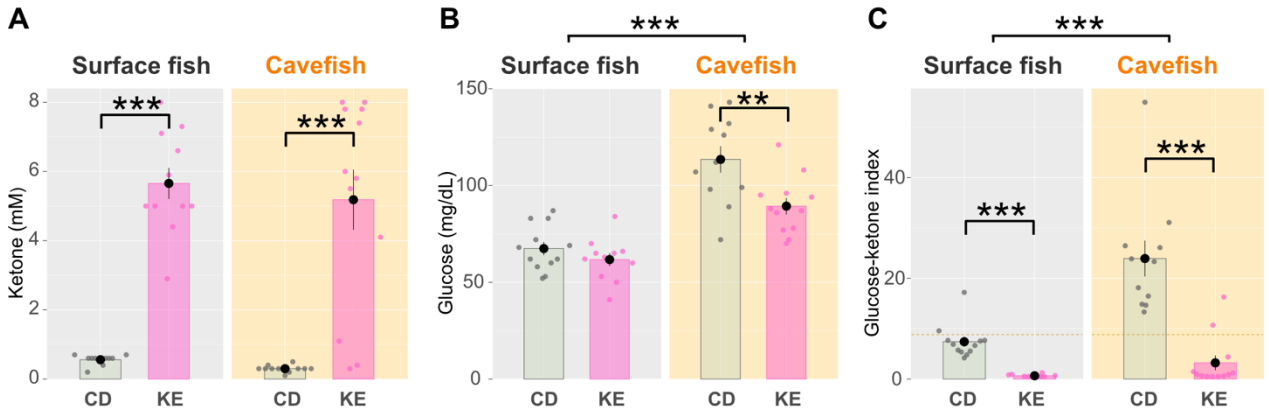
Additional file 8: **Consistent results were obtained in the repeated experiment for the duration and number of nearby interactions, and turning bias under control diet (CD) or ketogenic diet (KD) feeding.**
(A) Duration of nearby interactions, (B) Number of nearby interactions, (C) Swimming distance, and (D) Turning bias are presented. The overall tendencies were consistent with those observed in the original experiments, except for a shift in swimming distance (Figures 2 and 4). Surface fish did not exhibit any significant differences regarding the duration (A) or number of nearby interactions (B), the swimming distance (C), or turning bias (D). The duration (A) and number of nearby interactions (B) were maintained in KD-fed cavefish, while 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 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 Additional file 2.



Additional file 9: **Daytime and nighttime number of sleeping events per hour under control diet (CD) or ketogenic diet (KD) feeding.**
After 5 weeks of growth, surface fish exhibited a reduced number of sleeping events during the day under both diets. CD-fed cavefish also exhibited a reduced number 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 points. 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 Additional file 2.

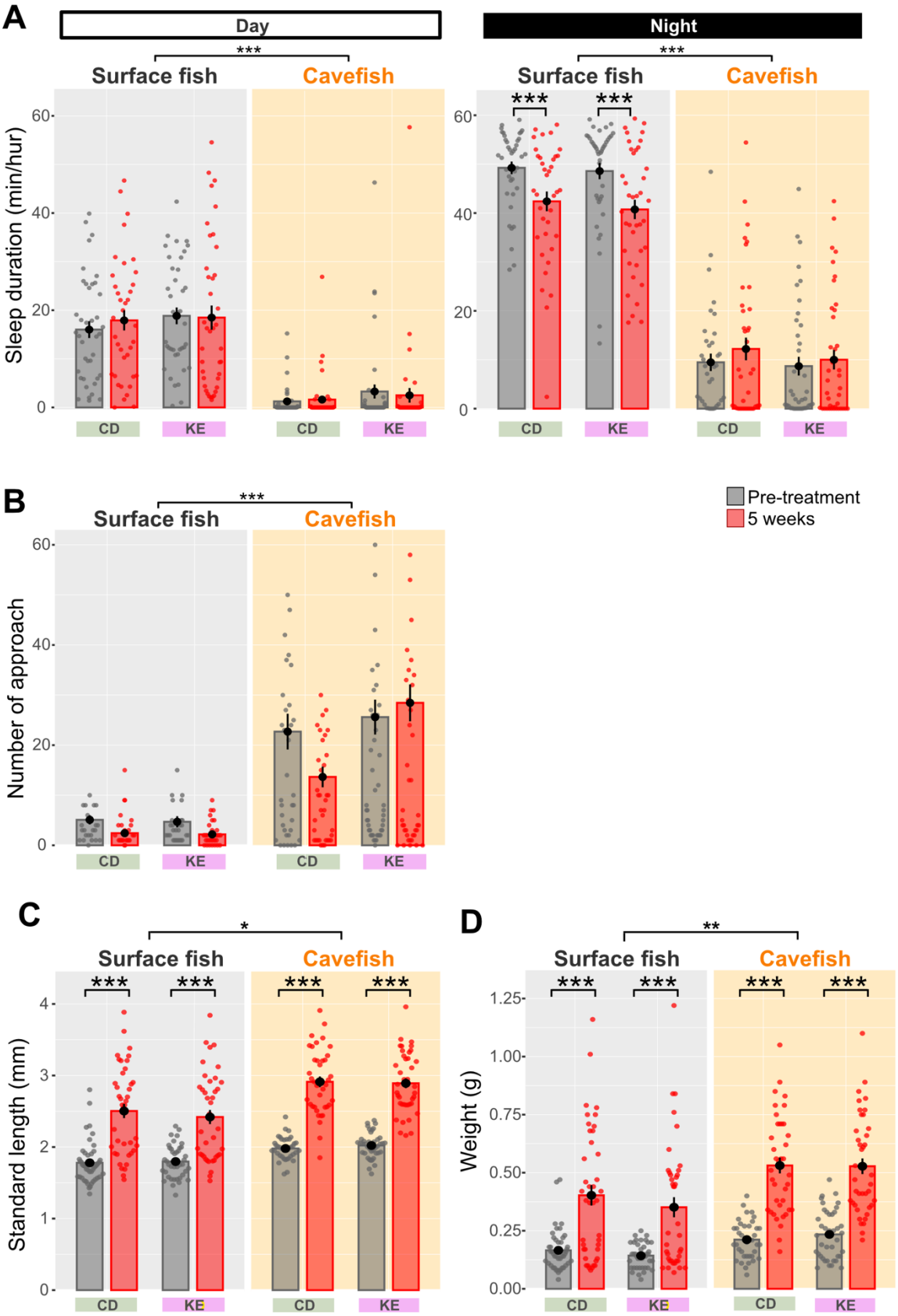


Additional file 10: **Vibration attraction behavior (VAB) and swimming distance during VAB under control diet (CD) or ketogenic diet (KD) feeding.**
(A) Number of approaches to the vibration rod in the 3-min assays. After 5 weeks of growth, the number of approaches increased in CD- and KD-fed cavefish, 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, consistent with the nearby interaction and sleep studies.
Data are presented as the mean ± standard error of the mean. Dots indicate individual data points. 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 Additional file 2.



Additional file 11: **Five weeks of the ketone ester (KE)-supplemented diet feeding reduced glucose ketone index (GKI) in both surface fish and cavefish.**
(A) Blood ketone level (mmol/L). Ketone levels significantly changed after the 5-week feeding in both surface fish and cavefish. Data are presented as the mean ± standard error of the mean. Dots indicate individual data points. (B) Blood glucose level (mg/dL). Glucose levels were significantly reduced in cavefish (CF). (C) The glucose ketone index (GKI) indicated that the ratio of glucose to ketone was lowered by the 5-week KE supplemental feeding in both surface fish (SF) and CF, suggesting that this diet altered the balance between glucose and ketone.
SF: N = 12 for CD feeding, N = 10 for KE feeding. CF: N = 11 for CD feeding, N = 12 for KE feeding. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. All detailed statistical data are available in Additional file 2.

Additional file 12



Additional file 12: **Day and night sleeping durations, vibration attraction behavior (VAB) and growth were not drastically changed by ketone ester-supplemented diet (KE) feeding.**

1218 (A) Sleep duration (min/h) during the day (left) and night (right). During the 5 weeks of treatment,
1219 neither surface fish nor cavefish exhibited any detectable changes in KE feeding, although the night-
1220 time sleep in surface fish was reduced after 5 weeks of growth. (B) Number of approaches per the 3-
1221 min assay (VAB level). During the 5 weeks of treatment, the VAB level did not detectably shift in
1222 surface fish or cavefish regardless of diets. (C) Standard length (cm). KE-fed surface fish and cavefish
1223 were comparable to their CD-fed counterparts. (D) Body weight (g). KE-fed surface fish and cavefish
1224 weighed comparable to their CD-fed counterparts.
1225 Data are presented as the mean ± standard error of the mean. Dots indicate individual data points. For
1226 (A) and (B), Surface fish: N = 37 for CD, N = 39 for KE. Cavefish: N = 39 for CD, N = 40 for KE. For (C)
1227 and (D), Surface fish: N = 40 for CD, N = 39 for KE. Cavefish: N = 39 for CD, N = 40 for KE. *: $P < 0.05$,
1228 **: $P < 0.01$, ***: $P < 0.001$. All detailed statistical data are available in Additional file 2.
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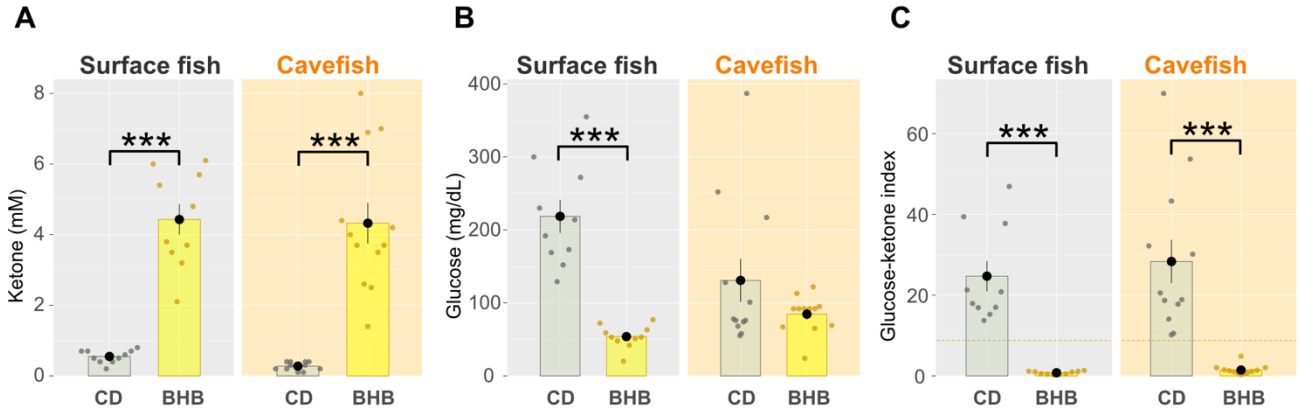
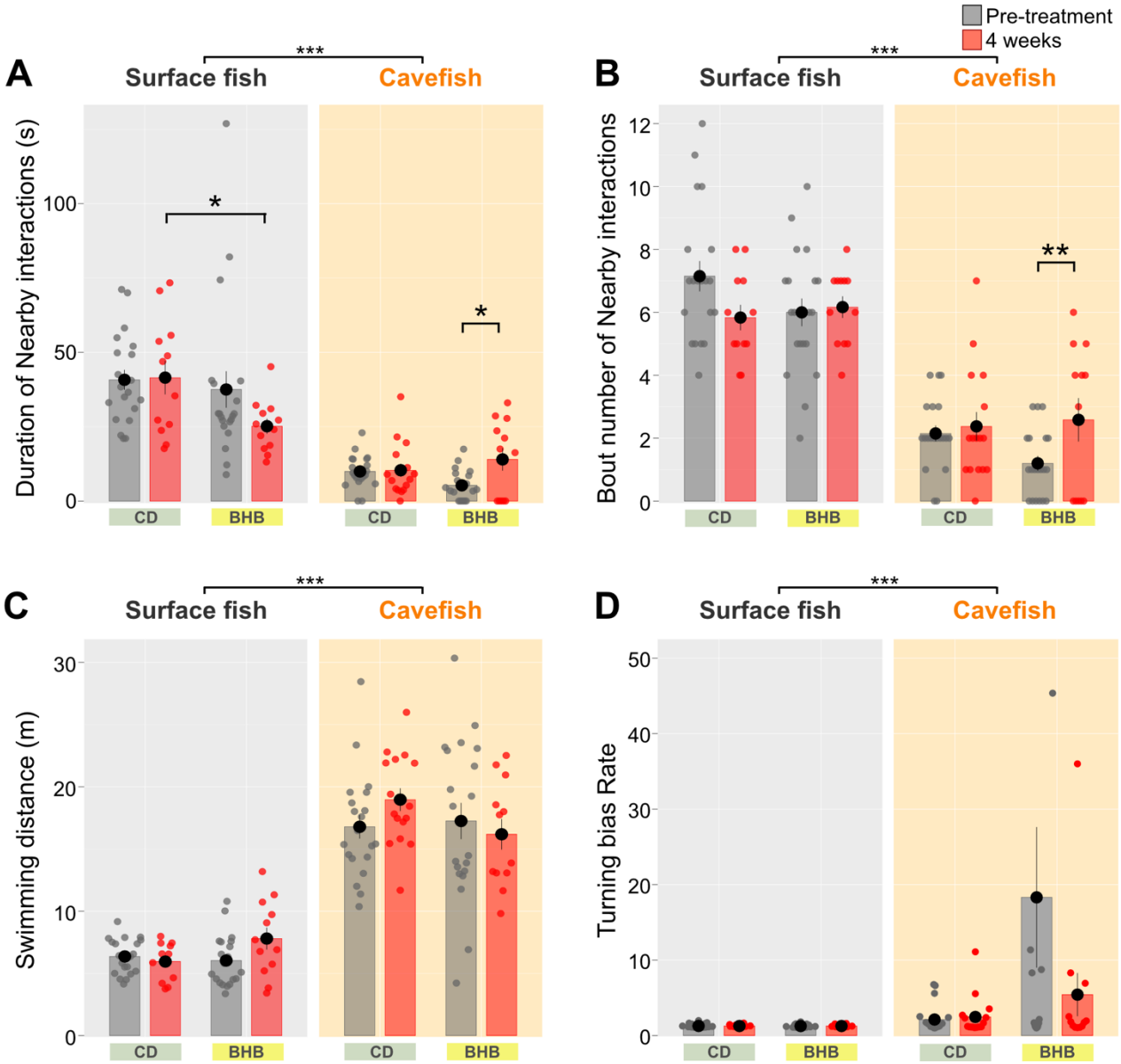
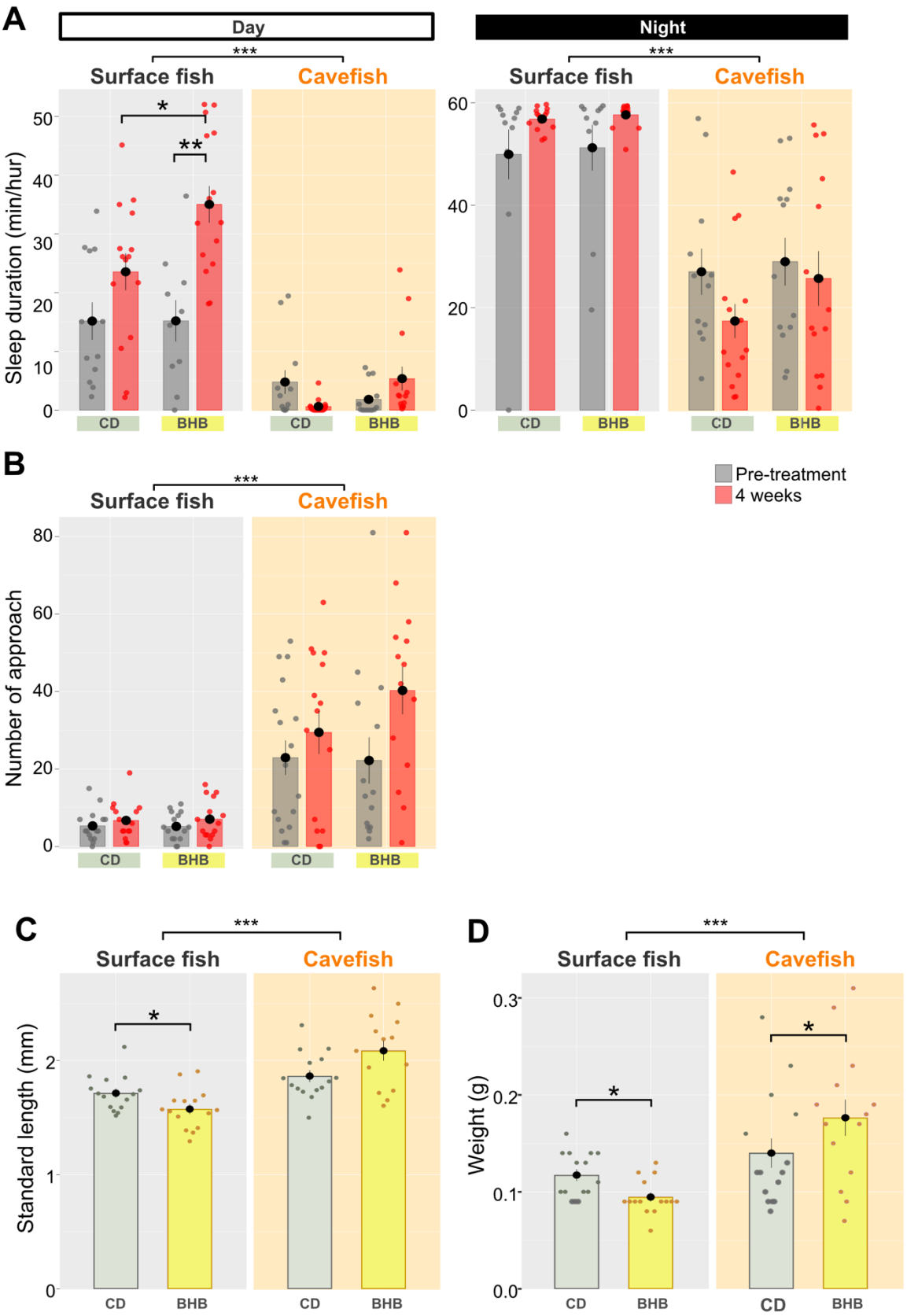


Additional file 13: **Four weeks of the BHB salt-supplemented diet feeding reduced glucose ketone index (GKI) in both surface fish and cavefish.**
(A) Blood ketone level (mmol/L). Ketone levels significantly changed by the 4-week feeding in both surface fish (SF) and cavefish (CF). Data are presented as the mean ± standard error of the mean. Dots indicate individual data. (B) Blood glucose level (mg/dL). Glucose levels were significantly reduced in SF. (C) The glucose ketone index (GKI) indicated that the ratio of glucose to ketone was lowered by the 4-week BHB salt-supplemented feeding in both SF and CF, suggesting that this diet altered the balance between glucose and ketone.
SF: N = 10 for CD feeding, N = 10 for BHB feeding. CF: N = 10 for CD feeding, N = 10 for BHB feeding.
*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. All detailed statistical data are available in Additional file 2.



Additional file 14: **Nearby interactions and other behaviors under control diet (CD) or BHB sodium salt–supplemented diet (BHB) feeding.**
(A) Duration of nearby interactions (s). After 4 weeks, the duration of nearby interactions decreased in BHB-treated surface fish and increased in BHB-treated cavefish. (B) Number of nearby interactions. The number of nearby interactions increased in BHB-treated cavefish. (C) Swimming distance. No difference was detected between the CD and BHB groups. (D) Turning bias ratio. BHB-treated cavefish tended to exhibit decreased biased turning, although this reduction was not significant.
Data are presented as the mean ± standard error of the mean. Dots indicate individual data points. N = 20 for all groups. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. All detailed statistical data are available in Additional file 2.



Additional file 15: **Day and night sleeping durations and vibration attraction behavior (VAB) were not drastically changed by beta-hydroxybutyrate-supplemented diet (BHB) feeding**.

(A) Sleep duration (min/h) during the day (left) and night (right). During 4 weeks of treatment, the daytime sleep duration increased in surface fish under BHB feeding but not during nighttime. Cavefish did not exhibit any detectable changes. (B) Number of approaches per the 3-min assay (VAB level). During 4 weeks of treatment, the VAB level did not shift in surface fish or cavefish regardless of the diets. Data are presented as the mean ± standard error of the mean. Dots indicate individual data points. N = 20 for all groups. (C) Standard length (cm). The BHB-treated surface fish grew slower than the control diet-treated ones. Cavefish showed no detectable change between the control and BHB supplementation. (D) Body weight (g). BHB-treated surface fish exhibited significantly reduced weight, whereas the weight of BHB-fed cavefish did not show any negative effect on the growth.
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 Additional file 2.

Additional file 16: **Table of possible biological processes in each behavior tested in this study**
https://drive.google.com/file/d/1LdXTn68vlqU7I-bxra6k5TCNj4-NcGdz/view?usp=sharing

Additional file 17: **GO term re-analysis of the 72-hour post-fertilization transcriptome**
For the RNAseq transcriptome analysis, variation in gene expression was analyzed using previously published RNAseq data (GenBank Sequence Read Archive (SRA), accession code: PRJNA258661) [26, 92, 101]. The data were analyzed by following previously published protocols [31, 102]. Gene ontology terms (GO terms) were analyzed using the AmiGO 2 platform [103].
https://docs.google.com/spreadsheets/d/1Lh2legpa-kikYvU8BCS9ZMM95oLwReOp/edit?usp=sharing&ouid=102214478617384425040&rtpof=true&sd=true

Additional file 18: **KEGG pathway analysis used the data in Yoshizawa et al., 2018**
https://drive.google.com/file/d/1LgI201j4d2R7FIwOC5Z7BlMQLVVwgkNU/view?usp=sharing