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

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Author contribution:
MI: designed the experiments, performed the experiment and analyses, wrote the initial draft, and edited the manuscript.
AT: performed the experiment and analysis, edited the manuscript
MW: designed the experiments, consulted the experimental procedure, and edited the manuscript
DB: performed the experiment and analysis, edited the manuscript
RL: designed the experiments, consulted the experimental procedure, and edited the manuscript
MY: designed the experiments, performed the experiment and analyses, wrote the initial draft, and edited the manuscript
Abstract

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

Wild animals experience frequent fasting because of daily, seasonal, and yearly changes in food availability. Physiologically, fasting can increase the secretion of appetite-related hormones (e.g., ghrelin, peptide Y, orexin) and induce a metabolic shift to nutritional ketosis (McCue, 2010).

Concerning behavioral outputs, fasting also induces shifts including boldness in foraging involving risk-taking (Padilla et al., 2016) and a shift from avoiding to approaching prey (Filosa et al., 2016).

Interestingly, fasting also induces non-foraging–related behaviors including aggression toward cohorts (Fokidis et al., 2013; Solianik et al., 2016) and engagement in social dominance (Nakajo et al., 2020).

These non-foraging behaviors could be evoked by metabolic changes that occur in a state of nutritional ketosis instead of the increased production of appetite-related hormones. However, it is not fully understood whether ketosis itself in the absence of hunger drives these non-foraging behaviors. Such knowledge will open a path to understanding the effects of different dietary intakes 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 very low amount of carbohydrates, gained popularity among humans because of its neuroprotective and anti-inflammatory effects without impacting appetite-related hormone levels (Deemer et al., 2020; Ludwig, 2020; Sumithran et al., 2013). The KD is an effective treatment for refractory seizures, and there is some evidence that it may be beneficial for other nervous system-based disorders, such as Alzheimer’s disease, Parkinson’s disease, and autism (Lee et al., 2018; McDonald and Cervenka, 2018; Phillips et al., 2018; Ruskin and Masino, 2012). Because modern humans evolved to acquire resistance to starvation (Bellisari, 2008), our body physiology and behavioral tendencies possibly evolved to accommodate drastic metabolic changes. However, the major molecular mechanisms for these positive are largely unknown (Ludwig, 2020; Qin et al., 2021). We were therefore motivated to explore the effects of metabolic shifts, particularly from glycolysis to ketosis, on behavioral...
outputs such as social affinity using a single species consisting of typical and starvation-resistant populations.

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

Concerning social-like behavior, cavefish exhibit no detectable schooling behavior (Kowalko et al., 2013; Patch et al., 2020; Pierre et al., 2020) or hierarchal dominance (Elipot et al., 2013). By contrast, surface fish school/shoal with cohorts and model fish (Kowalko et al., 2013), and exhibit group hierarchical dominance (Elipot et al., 2013). Because social behaviors in many fish (e.g., zebrafish) are
promoted by visual stimuli, blind cavefish might not express social-like activities because of the absence of visual acuity. However, a recent detailed study illustrated that surface fish exhibit a high level of social-like nearby interactions (one-by-one affinity) in the dark, and these of which were promoted by mechanosensory lateral line inputs (Iwashita and Yoshizawa, 2021). Interestingly, blind cavefish displayed much lower levels, albeit significant, of nearby interactions than surface fish (Iwashita and Yoshizawa, 2021). Further, cavefish exhibited plasticity in the level of nearby interactions wherein they increased its level in a familiar environment in comparison with an unfamiliar environment (Iwashita and Yoshizawa, 2021), which is similar to the findings in patients with autism (Helt et al., 2020; Runco et al., 1986).

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

In this study, we assessed the effects of the KD on an evolutionarily asocial cave population of \textit{A. mexicanus}. The time-course experiment revealed that 1 month of KD feeding promoted and sustained
the juvenile level of nearby interactions, whereas control diet (CD)-fed cavefish exhibited diminished nearby interactions. KD feeding also reduced repetitive turning and swimming activity. These cooccurring behaviors (asociality, repetitive behavior, and increased activities) are similar to the hallmarks of the autism condition. However, the effects of the KD were limited. For example, sleeplessness and high adherence to a particular vibrating stimulus were not detectably changed under the 1-month KD treatment. To reveal the molecular basis of the effects of the KD, we provided supplementation with a major ketone body, beta-hydroxybutyrate (BHB), which promoted social interactions and reduced repetitive turning, covering the major effect of the KD. Finally, we interpreted the possible neural processes that KD influenced based on affected and unaffected behaviors. According to the study of shared dysregulated genes between cavefish and patients with autism, GO term and KEGG pathway analyses indicated that the dopaminergic system—but less likely the cholinergic, or orexinergic systems—could respond to the KD.

Overall, ketosis appears to be capable of significantly shifting the asociality of evolved cavefish toward the surface fish phenotype, providing new insights into the contribution of the diet to the evolved behaviors.
Results

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

In the 1-year-old surface and cave populations of A. mexicanus, 2 weeks of fasting indeed reduced serum glucose levels, which helped lower the glucose ketone index (GKI = $\frac{\text{glucose (mM)}}{\text{ketone (mM)}}$); GKI lower than 9 is considered as ketosis in humans; Figure 1—figure supplement 1A, 1C and 1D; Hagihara et al., 2020; Meidenbauer et al., 2015). This result indicates that A. mexicanus respond to fasting and reduced GKI in a similar manner as mammals, although the serum ketone levels did not significantly increase during this fasting experiment (Figure 1—figure supplement 1B, wherein the social-like interactions of cavefish increased (duration and event numbers, Figure 1—figure supplement 1E and F). Although ketosis may be primarily responsible for increasing social interactions, appetite and hormones may also be contributing factors.

To reduce appetite-related behavior, we developed a KD based on a human milk formula (KetoCal3:1® with Zeigler zebrafish standard irradiated diet at a 5:1 weight ratio [nutritionally complete, ketogenic medical food; Nutricia North America, Inc. Gaithersburg, MD, USA]; Table 1; Materials and Methods). We then measured GKI to monitor whether our KD could induce a shift in the balance of ketone body and glucose levels after chronic dietary treatment. Three-month-old fish (juvenile–young adult stage) were used in this study because many adult-type behaviors of cavefish emerge in this stage, including higher adherence to a vibration stimulus (vibration attraction behavior [VAB]) (Yoshizawa et al., 2010), less social affinity, and longer swimming distances compared to the findings in surface fish. After 5 weeks of KD feeding, both ketone and glucose concentrations were decreased compared to the findings in CD-fed fish (KetoCal3:1 and Zeigler zebrafish diet at a 1:5
weight ratio; Table 1; Figure 1A–C). For both diets, surface fish exhibited a significantly higher ketone body level than cavefish (Figure 1B), whereas cavefish exhibited a higher 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 indicated that KD feeding more strongly reduced glucose levels than ketone body levels, resulting in a lower GKI in KD-fed fish than in CD-fed fish (Figure 1D) and suggesting that KD feeding could shift the metabolic state from glycolysis toward ketosis.

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

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

(Paffenhöfer, 1967; Panda, 2016)

Regarding this dietary treatment, we first examined its ontogenic (developmental) effects on collective social-like behavior (Iwashita and Yoshizawa, 2021). Many adult behaviors emerge in the transition from juvenile to young adult (adolescent) in 3–4-month-old *A. mexicanus* fish, including foraging behavior, VAB (Yoshizawa, 2015; Yoshizawa et al., 2010), adult-type regulation of sleep (independent from catecholamine) (Duboué et al., 2012; Jaggard et al., 2020, 2017; Yoshizawa et al., 2015), and the collective behavior in young adults (under higher Reynold’s number; Iwashita and Yoshizawa, 2021). We therefore investigated the shift of collective behavior in 3–4-month-old fish using criteria based on the vicinity of two fish (≤5 cm) and duration of nearby interactions (≥4 s) during tracking in a four-fish group (Iwashita and Yoshizawa, 2021) (Figure 2B). At 3 months old, (“Pre-treatment” in Figure 2), surface fish exhibited social-like nearby interactions for 17.0 ± 4.4 s (Figure 2C) and 3.1 ± 0.4 bout number of nearby interactions (Figure 2D) during the 5-min assay. Conversely,
cavefish exhibited an approximately 50% shorter interaction duration (8.3 ± 1.5 s; Figure 2C) and a smaller bout number of interactions (1.8 ± 0.3; Figure 2D). To track the effect of the KD treatment, fish were fed the KD for 5 weeks, followed by CD feeding during weeks 6–9 to assess the persistence of the effects of the KD (Figure 2A, C, and D).

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

To investigate in detail whether KD-promoted nearby interactions have social-like properties, we explored swimming speed, which is an indicator of the movement of fish in the vicinity of each other. Fish are more likely to have an opportunity to express affinity toward each other at a slower swimming speed (Iwashita and Yoshizawa, 2021). Indeed, surface fish moved slower during nearby interactions than during the non-nearby interaction period (Iwashita and Yoshizawa, 2021) (Figure 3A). Consistently, KD-fed cavefish swam slower during the nearby interaction period than during the non-nearby interaction period (“5 weeks;” Figure 3B). Overall swimming speed was also slower in the KD group than in the CD group (“5 weeks;” Figure 3B). These findings indicated that KD-fed cavefish exhibited more social-like nearby interactions with a similar speed profile as surface fish. In surface fish, there was no detectable difference in swimming speed profiles between CD and KD feeding (“5 weeks;” Figure 3A). To address whether the slower swimming speed was sufficient to increase nearby interactions, we tracked the total swimming distance within 5 min from pre-treatment to week 9 of feeding (Figure 2—figure supplement 1). KD-fed cavefish exhibited a significantly shorter swimming distance (slower swimming speeds) from the first week of feeding (Figure 2—figure supplement 1),
which was much earlier than when the higher level of nearby interactions emerged (weeks 4–5). This result suggests that although a slower swimming speed is associated with nearby interactions (Figure 3) and KD feeding reduced swimming speeds in cavefish, a slower speed itself is not sufficient to induce nearby interactions.

Repetitive turning is frequently observed in an antagonistic relationship with nearby interactions in cavefish and mammals (Iwashita and Yoshizawa, 2021; Langen et al., 2011b, 2011a). That is, individuals with few nearby interactions frequently exhibit a high level of turning bias or “repetitive turning.” Accordingly, CD-fed cavefish with few nearby interactions exhibited significantly higher turning bias than KD-fed cavefish (Figure 4A, B). KD-fed cavefish displayed a comparable level of balanced turning as surface fish (close to a score of “1” in Figure 4B). In summary, these results suggest that KD feeding could reduce repetitive turning while maintaining longer nearby interactions.

Tracking behavioral changes each week (Figure 2) may result in confounding factors such as fish remembering the recording environment. To clarify whether our results captured the genuine effects of KD feeding, we repeated 4–5-week dietary treatment in a new set of fish (Figure 4—figure supplement 1). Similarly, surface fish did not exhibit a detectable change in the duration and number of nearby interactions between CD and KD feeding (Figure 4—figure supplement 1A, B). By contrast, CD-fed cavefish displayed fewer nearby interactions, whereas the level of nearby interactions was retained in KD-fed cavefish, resulting in a higher level of nearby interactions in KD-fed cavefish (Figure 4—figure supplement 1A, B). In this repeated experiment, the results for repetitive turning were also similar to those in the previous experiment; specifically, CD-fed cavefish displayed a high level of turning bias, whereas KD-fed cavefish exhibited balanced turning (Figure 4—figure supplement 1D).

We then explored other changes induced by KD feeding, including changes in sleep, 24-h swimming distance, and adherence to a vibrating stimulus, which are distinct between surface fish and cavefish. Cavefish largely exhibit reduced sleep duration and swim almost all day, perhaps to find...
nutrients in the food-sparse environment (Duboué et al., 2012, 2011; Jaggard et al., 2017; Yoshizawa et al., 2015). After 5 weeks of dietary treatment on the 3–4-month-old fish, both surface fish and cavefish exhibited shorter sleep duration than observed before treatment regardless of the diet (Figure 5A, particularly at night), suggesting growth between 3–4 and 4–5 months old exerted a negative effect on the sleep duration. However, there was no detectable difference between CD and KD feeding.

Animals’ sleep is usually fragmented, involving repeated sleep/awake cycles during the night (diurnal animals) or day (nocturnal animals) (Campbell and Tobler, 1984). Then, the structure and regulation of sleep are typically analyzed according to the number of events (bout). Our detailed sleep analysis illustrated that KD-fed cavefish displayed fewer sleep bouts during the night than CD-fed cavefish (Figure 5B). However, the number of sleep bouts did not differ between CD and KD feeding (5 weeks; Figure 5—figure supplement 1). Overall, the sleep phenotype was not dramatically changed by KD feeding, and cavefish exhibited shortened sleep duration.

The sleep duration is negatively correlated with the 24-h swimming distance (Yoshizawa et al., 2015). Cavefish displayed overall higher activities, which was consistent with previous findings (Duboué et al., 2011; Yoshizawa et al., 2015) and consistent with the findings of longer swimming distance in the nearby interaction assay (Figure 5C). CD-fed cavefish swam longer distances after the 5-week treatment but KD-fed cavefish did not show a detectable change in it after the treatment (in the day period, Figure 5C). Surface fish, in contrast, did not exhibit a detectable difference in swimming distance between KD and CD feeding. Overall, the KD treatment induced little changes in sleep-associated behaviors in both surface and cavefish.

In general, the KD is assumed to induce ketosis without increasing appetite. We then checked the shift of foraging behavior under KD feeding. Cavefish evolutionarily exhibit increased foraging behavior, termed VAB, in which fish adhere to a particular vibration stimulus (35–40 Hz) in the dark (Yoshizawa et al., 2010). VAB is advantageous for prey capture in the dark. Cavefish and surface fish did not exhibit a detectable difference in VAB between CD and KD feeding, whereas VAB was
significantly increased during 1 month of growth (pre-treatment vs. 5 weeks; Figure 5—figure supplement 2A). In summary, the VAB analysis indicated that KD feeding did not increase a foraging behavior.

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

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

In humans, the KD induces ketosis, in which the liver releases the major ketone BHB, via beta-oxidation of fat (Evans et al., 2017). Instead, of supplying a massive amount of fat using the KD, BHB (sodium salt form of racemic BHB: 50% L-form and 50% D-form; only the D-form is considered to be biologically active) might be responsible for the majority of effects observed after KD feeding. With this idea, sodium salt BHB was provided to both surface fish and cavefish for 4 weeks. The result indicated that the BHB supplemental diet promoted nearby interactions in cavefish (Figure 7A and B) and reduced the duration of nearby interactions in surface fish (Figure 7A). Swimming distance was not reduced in surface fish or cavefish (Figure 7C). Turning bias tended to be reduced by BHB supplementation in cavefish, although significance was not achieved (Figure 7D). Interestingly, the body growth of BHB-treated surface fish was reduced whereas the one of BHB-treated cavefish was increased compared with that in control fish (standard length and weight; Figure 7—figure supplement 1A and B). The nighttime sleep and VAB did not exhibit detectable differences between control and BHB treatment (Figure 7—figure supplement 2A and B, respectively)
In summary, BHB treatment covered many effects of the KD treatment, including changes in social interactions and repetitive turning. BHB had fewer negative effects on growth and swimming activities, suggesting that ketone bodies are responsible for the positive effects on social and repetitive behaviors of KD feeding.
In this study, we examined the behavioral shifts induced by KD feeding and BHB supplementation. Ketosis is expected to occur frequently in wild animals because of a failure to find food (fasting) or an absence of carbohydrate inputs/synthesis (available nutrients). Certain levels of socialness can be beneficial to animal species for mating and finding food. Under KD feeding, cavefish maintained their juvenile level of nearby interactions until the treatment ended (5 weeks). Nearby interactions were then reduced to an indistinguishable level from the control levels within 1 month after stopping KD feeding. Surface fish exhibited a higher number of nearby interactions than cavefish, and no detectable difference was observed in nearby interaction levels between CD- and KD-fed surface fish. KD feeding also effectively reduced repetitive turning in cavefish, whereas CD-fed treated cavefish exhibited a high level of repetitive turning. There were no detectable changes in sleep and foraging behavior (VAB) under 1 month of KD feeding. These patterns in behaviors and growth were not changed in two replicated experiments (social affinity and repetitive turning), supporting the consistency of the observed effects under KD feeding. Finally, the major KD metabolite, BHB, could cover the KD effect, indicating the ketone body plays a pivotal role in this treatment.

Effects of the KD on blood ketone levels and body growth

Under 4–5 weeks of KD feeding, blood ketone and glucose levels were reduced compared to the effects of the CD in both surface fish and cavefish, contradicting our expectation that ketone levels would be higher in the KD group. However, GKI (Meidenbauer et al., 2015) was significantly lower under KD feeding than under CD feeding. These significant changes in GKI in both surface fish and cavefish suggest that the metabolic condition was shifted toward ketosis by KD feeding. In general, cavefish had a higher GKI than surface fish under both diets, suggesting that the cavefish physiology was constitutively biased toward glycolysis. For example, blood glucose levels in cavefish under KD
feeding were similar to those in surface fish under CD feeding, whereas cavefish had 3-fold lower
ketone levels than surface fish under CD feeding, resulting in a higher GKI even under KD feeding.

KD feeding for 4–5 weeks also resulted in slowed body growth. This growth retardation has been
observed in patients with epilepsy chronically fed a KD (Coppola et al., 2010; Napoli et al., 2014), and
these results were consistent with our observations in KD-fed fish. This study found that BHB did not
suppress body growth yet increased the social-like activities and reduced repetitive behavior. The
detailed molecular/physiological mechanisms how ketosis affects behaviors are largely unknown, but
BHB provided a good starting point to resolve the mechanism for KD-associated phenotypes (see
below).

Effects of ketones in the TCA cycle and epigenetics in the brain

In mammals, KD feeding causes a “starvation”-like state, causing the liver to release ketone
bodies into the bloodstream. BHB is the major ketone body produced by the liver through beta-
oxidation. The gut epithelia also absorb and circulate ketone bodies from the diet and/or gut microbiota.

Both liver- and gut-derived ketone bodies can cross the blood–brain barrier and exert two functions: (i)
inhibit histone deacetylase, which influences epigenetic regulation and induces gene expression in
neurons; and (ii) act as a general energy source that is converted into acetyl-CoA to fuel the aerobic
TCA cycle in neurons. Both pathways have the potential to alter brain function. The facts that cavefish
easily tolerate high blood glucose levels, at which surface fish was paralyzed (Riddle et al., 2018), and
Wnt signaling is upregulated in cavefish, potentially resulting in high glycolytic activity as humans do
(Vallée and Vallée, 2018; Yoshizawa et al., 2018), support the aforementioned hypothesis that cavefish
exhibit high blood glucose levels and generate energy via glycolysis. Also, ketone bodies can also
promote behavioral shifts by changing the epigenetic state by inhibiting histone deacetylase
(Krautkramer et al., 2017; Szyf, 2015). Histone deacetylase inhibition increases gene expression in
general. This possibility is supported by the fact that cavefish have more downregulated genes (2913
genes, \( \log_2 < -1.0 \) than upregulated genes (1643 genes, \( \log_2 > 1.0 \)) in the transcriptome at 72 h postfertilization (Gross et al., 2013; Yoshizawa et al., 2018). In addition, more methylated loci are found in the eye genes of cavefish than in surface fish, which could also be true for other tissues (Gore et al., 2018), and most of these methylated gene loci were downregulated. The brains of patients with autism are also expected to be hypermethylated, resulting in a transcription-less condition (Zhu et al., 2014). Therefore, these two pathways, namely metabolism and epigenetics, were highlighted as possible targets of ketone bodies during behavioral shifts under ketosis. Future research should address these possibilities to clarify the metabolism-based evolution of behavior (cf. (Qin et al., 2021).

**Ontogeny of nearby interactions and the KD**

In this study, 3–4-months-old cavefish exhibited a detectable level of nearby interactions (social affinity), and this social affinity decayed under CD feeding. Interestingly, KD-fed cavefish and surface fish fed either diet maintained a similar level of nearby interactions during 5 weeks of dietary feeding. The reduction of nearby interactions in CD-treated cavefish can be explained by (1) quicker exhaustion under CD feeding (aerobic ketosis produces more adenosine triphosphate than anaerobic glycolysis), (2) greater anxiety in the recording environment (Iwashita and Yoshizawa, 2021), and (3) less social motivation. The first explanation is unlikely because CD-fed cavefish swam at comparable or longer distances to KD-fed cavefish (e.g., Figure 2—figure supplement1, Figure 5—figuresupplement 2). The higher level of anxiety could explain the findings because cavefish exhibited increased repetitive turning, which is related to higher anxiety in mammals (Langen et al., 2011a). In addition, cavefish displayed fewer nearby interactions in an anxiety-associated unfamiliar environment in prior research (Iwashita and Yoshizawa, 2021). In the future, the anxiety level should be monitored using plasma cortisol levels (Gallo and Jeffery, 2012). Less motivation regarding social affinity is also a possible cause, and this variable can be monitored by assessing activities in social decision-making networks including the preoptic area, nucleus accumbens, and striatum (O’Connell and Hofmann, 2012, 2011).
Explanations (2) and (3) are not mutually exclusive, and co-occurrence is possible. These possibilities will be assessed in our future study.

Possible target system for ketosis

Under KD feeding and BHB treatment, we observed increased social affinity and reduced repetitive turning. However, we did not detect changes in sleep and VAB.

Table 2. Possible biological processes in each behavior tested in this study.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Results in this study</th>
<th>Known biological pathway or processes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Social behavior (Churchland and Winkielman, 2012; Kiser et al., 2012)</td>
<td>Promoted</td>
<td>Dopaminergic, Serotonergic, Oxytocinergic, Learning and memory processes</td>
</tr>
<tr>
<td>Repetitive behavior (Langen et al., 2011b)</td>
<td>Promoted</td>
<td>Dopaminergic, Serotonergic, GABAergic, Glutamatergic, Synaptic plasticity processes</td>
</tr>
<tr>
<td>Vibration attraction behavior (foraging, and adherence) (Barson, 2020; Penney and Volkoff, 2014; Wall and Volkoff, 2013)</td>
<td>No detectable change</td>
<td>Orexinergic (?), Ghrelin (?), Peptide Y (?), cholecystokinin (?)</td>
</tr>
<tr>
<td>Sleep (Duboué et al., 2011; Jaggard et al., 2020, 2017; Siegel, 2004)</td>
<td>No detectable change</td>
<td>Histaminergic, Cholinergic, Serotonergic, Orexinergic/Hypocretinergic Glutamatergic, Glycinegic, GABAergic, Noradrenergic</td>
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</tbody>
</table>

(?): implied by assuming VAB as a foraging behavior. Underlined pathways appeared in GO term/KEGG analysis too (see in the Discussion).

Studies regarding neurotransmitters and their associated behaviors have revealed tight associations between them, such as the dopaminergic system being associated with social and repetitive behavior, and the cholinergic/orexinergic/histaminergic systems playing a major role in sleep regulation (Table 2). The behavioral phenotypes in this study highlighted the possible involvement of the dopaminergic system but less involvement of others (i.e., the serotonergic, cholinergic, orexin/hypocretinergic, histaminergic, or adrenergic system). The dopaminergic and other social/repetitive behavior-associated pathways were also highlighted in the GO term/KEGG pathway analysis using genes showing the same directional expression changes (upregulation or downregulation) in patients with autism versus neurotypical individuals (Parikshak et al., 2016) and cavefish versus...
surface fish 72 hours postfertilization (Gross et al., 2016; Stahl and Gross, 2017; Yoshizawa et al., 2018) (Supplementary file 2). The shared same directional expression genes are enriched in the synaptic vesicle cycle, long-term depression/potentiation, dopaminergic/serotonergic synapses, and oxytocin-signaling pathway (Supplementary file 3). The underlying reason why ketosis or ketone bodies have a stronger effect on the dopaminergic system than on the other systems is undetermined, calling for further investigation. However, the possibility that the dopaminergic system can be sensitive to ketosis in a vertebrate with genes dysregulated in a similar manner (upregulation or downregulation) to patients with autism is extremely interesting, and it indicates that ketosis-inducing therapy may not increase sleep duration in these patients. This study together with the BHB result indicated that the physiological and molecular mechanisms bridging the gap between ketosis and behavioral outputs are addressable in the A. mexicanus system.

The BHB supplement and body growth

In this study, the BHB supplementation retarded the body growth in surface fish. In contrast, the BHB treatment exhibited a rather encouraging effect on cavefish growth (Figure 7—figure supplement 1). We suspect that the tolerance levels for high sodium and potassium ions (from BHB salt) may be different between surface fish and cavefish. This estimate is based on our preliminary result using the ketone ester supplement (ketone bodies but no accompanying ion), which did not have a negative impact on body growth in surface fish. However, future physiological studies on the rhinal function in surface fish and cavefish are needed to answer.

Ketosis in the cave environment

Cave-dwelling animals usually experience less temperature fluctuation and fewer dietary inputs (Culver and Pipan, 2009), but these features can vary. The diets of cave-dwelling animals in the dry season (approximately 6 months/year) could be organic matter in the pool bottoms, bat guano (larger...
adults), or small crustaceans (smaller fish), whereas food is sparse in the rainy season (approximately 6 months/year) (Espinasa et al., 2021, 2017). These available diets contain extremely low amounts of carbohydrates, and they can be high in protein and fat (e.g., crustaceans). Although some amino acids, lactate, and glycerol can be used for glucose synthesis in fish (Polakof et al., 2012), cavefish are expected to be exposed to carbohydrate-deprived diets or frequent fasting and therefore frequent ketosis. In prior research, wild cavefish exhibited similar social affinity as observed in KD-fed cavefish in this study (Movie 1). Although these observations and dietary inputs suggested that wild cavefish may be under frequent ketosis, recent multiple reports indicated that cavefish may be under anaerobic glycolysis to adapt to anaerobic cave water conditions because of the approximately 20% lower oxygen level in cave pools (Boggs et al., 2022; van der Weele and Jeffery, 2022). In addition, cavefish tend to store lipids instead of using them (beta-oxidation) through the enhanced PPARγ pathway (Xiong et al., 2022).

These expectations of low ketosis appear to contradict expectations in the wild—starved ketosis conditions. However, it appears to fit well with the findings in cavefish, namely high blood glucose and low ketone levels even under KD feeding in this study, as well as higher GKI in cavefish than in surface fish in both the CD and KD groups (Figure 1D). Cavefish appear to have evolved to maintain a high GKI (high blood glucose and low ketone levels); therefore, the physiology of cavefish may allow them to survive in the low-oxygen condition by using anaerobic glycolysis. KD-fed cavefish behave similarly as wild cavefish because the balance between ketosis and glycolysis could reach a similar level as that in the wild after KD feeding. By contrast, if cavefish are fed a typical carbohydrate-rich lab fish diet, it may overactivate glycolysis and result in a higher GKI, which may lead to reduced social affinity and increased repetitive circling. The future use of a pharmacological glycolysis inhibitor (e.g., 2-deoxy-D-glucose; Yao et al., 2011) can reveal the relationship between GKI and cavefish behaviors.

Summary statement
Solitary animals surprisingly share a set of dysregulated genes and behavioral outputs. In this study, we demonstrated that a diet that induces ketosis shifts these behaviors toward the surface fish phenotype regardless of the presence of many dysregulated genes. In addition to the gene therapy approach, ketone body-based treatment may open a path for sustainable and less toxic therapy for multigenic psychiatric disorders, including autism, although the target pathways remain unclear. Concerning the genetics of behavior, differentially expressed metabolic genes have been largely overlooked because it was difficult to interpret. Because mitochondria-based disorders are highlighted in neuroscience (Chauhan et al., 2012; Rajasekaran et al., 2015), the balance between glycolysis and ketosis could be the starting point for identifying a therapeutic target. The known evolved behaviors should be also revisited by investigating whether the metabolic shift promotes the variations of behaviors.
Materials and Methods

Fish maintenance and rearing in the lab

*A. mexicanus* surface fish used in this study were the laboratory-raised descendants of original collections created in Balmorhea Springs State Park in Texas in 1999 by Dr. William R. Jeffery.

Cavefish were laboratory-raised descendants originally collected from Cueva de El Pachón (Pachón cavefish) in Tamaulipas, Mexico in 2013 by Dr. Richard Borowsky.

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

Fasting, KD and BHB treatment

The fish were fasted for 2 weeks (13 full days) before the behavior was recorded, while the control fish were fed live *Artemia* larvae. Following standard operating protocols (IACUC (17-2560)), water was changed, and home tanks were cleaned as usual.
To prepare the KD, we used a mixture of a human KD (KetoCal3:1) and zebrafish standard diet (adult zebrafish irradiated diet) in a 5:1 ratio. The gross caloric amounts were 6.99 kcal/g for KetoCal3:1 and 3.89 kcal/g for the zebrafish diet. Regarding the CD, we used the same KetoCal3:1 and zebrafish irradiated diet but mixed at a 1:5 ratio. The KetoCal3:1 powder and ground zebrafish irradiated diet were mixed in the aforementioned ratios and solidified with 1% agar at a final concentration of 20% w/v (2 g of mixture in 10 mL of 1% agar). After solidification, both KD and CD agar was cut into 3-mm³ cubes, and each four-fish group was given 1–2 pieces every morning (ZT 0:00–3:30) and afternoon (ZT 8:00–12:00). The fish were fed ad libitum in each feeding and the remaining amount was removed 1 h after feeding using a pipette.

To supplement BHB, we used a commercial fish diet (TetraColor Tropical Granules, Tetra, Blacksburg, VA, USA) mixed with BHB (DL-β-Hydroxybutyric acid sodium salt, MilliporeSigma, St. Louis, MO, USA) to be 10 mg/body g (78.7 µmol/body g). In detail, fish generally eat 3% of their body g per meal. Accordingly, the BHB supplemental diet contains 0.333 g/mL of BHB mixed with 0.2 g/mL of the ground Tetra fish diet (20% w/v) in 1% agar. Fish with 1 g body weight then eat 30 mg (3%, approx. 30 µL) of this diet, which contains 10 mg of BHB. The control diet was 20% w/v of the Tetra fish diet in 1% agar. After solidification, both BHB and control diet agar was cut into 3-mm³ cubes, and each four-fish group was given 1–2 pieces every morning (ZT 0:00–3:30) and afternoon (ZT 8:00–12:00). The fish were fed ad libitum in each feeding and the remaining amount was removed 1 h after feeding using a pipette. Surface fish and cavefish used in this BHB study were 10-11 months old (young adult: 2.0-2.5 cm in the standard length) when we started feeding.

Behavior assays

The protocol for social-like nearby interactions was described previously (Iwashita and Yoshizawa, 2021). Briefly, four fish raised in a home tray (15.6 × 15.6 × 5.7 cm³ Ziploc containers, S. C. Johnson & Sons, Inc, Racine, WI, USA) were released in a recording arena (49.5 × 24.2 × 6.5 cm³)
with a water depth of 3 cm on the stage of a custom-made infrared (IR) back-light system within a custom-built black box (75 × 50 × 155 cm, assembled with polyvinyl chloride pipe frame and covered by shading film). The IR back-light system was composed of bounce lighting of IR LED strips (SMD3528 850 nm strip: LightingWill, Guang Dong, China). The video was recorded at 20 frame/s using VirtualDub2 software (build 44282; http://virtualdub2.com/) with the x264vfw codec for 6 min, and the last 5 min were used for the analysis. After the recording, the fish were returned to the home tray. The X-Y coordinates of each fish were calculated using idTracker software (Pérez-Escudero et al., 2014) after the video image was processed for background subtraction using ImageJ (Iwashita and Yoshizawa, 2021). This X-Y coordinate was also used for the turning bias analysis. The duration and number of nearby interactions and swimming speed during and after nearby interaction events were calculated using custom-made MATLAB script (MathWorks Inc., Natick, MA, USA) (Iwashita and Yoshizawa, 2021).

The turning bias rate was calculated as $\frac{N_l}{N_s}$, where $N_s$ and $N_l$ represent a smaller ($N_s$) or larger ($N_l$) number of left or right turns. This turning bias rate indicates the extent to which fish turning is biased to the left or right, and ranging from “1” (L-R balanced) to infinity (L or R biased). The numbers of left or right turns were calculated as changes in the angles of swimming directions in every five frame window (0.25 s) as described previously (Iwashita and Yoshizawa, 2021). An automatic calculation of the total number of the left or right turns is implemented in the aforementioned homemade MATLAB script.

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

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

We assayed VAB as described previously (Yoshizawa et al., 2015, 2012, 2010). Briefly, fish were acclimatized for 4–5 days prior to the assay in a cylindrical assay chamber (325 mL glass dish, 10 cm × 5 cm, VWR, Radnor, PA, USA) filled with conditioned water (pH 6.8–7.0; conductivity 600–800 μS). During the assays, vibration stimuli were created using a glass rod that vibrated at 40 Hz. The number of approaches to the vibrating rod was video recorded during a 3-min period under infrared illumination. The number of fish approaches in a 1.3-mm radius from the vibrating glass rod were analyzed using the X-Y coordinate of each fish head detected by the trained DeepLabCut model (Mathis et al., 2018; Fernandez et al., *submitted*)

**Measurement of body**
Fish were anesthetized with 0.2 mg/mL ethyl 3-aminobenzoate methanesulfonate (MS-222: MilliporeSigma, Burlington, MA, USA) in ice-cold conditioned water (pH: 7.0; conductivity: 700 µS), and weight was measured after taking pictures with a standard camera (Pentax K-1 DSLR with 35-70 mm zoom lens, Ricoh, Tokyo, Japan). The standard body length and body depth were measured using ImageJ software (Schneider et al., 2012).

Blood ketone and glucose measurements

All blood samples were collected 2–3 h after feeding. Fish were then deeply anesthetized in ice-cold water, and blood was collected from the tail artery. Blood ketone and glucose levels were measured using either the Abbott Precision Xtra (Abbott Laboratories, Abbott Park, Illinois, USA) or Keto-Mojo GK+ (Keto-Mojo, Napa, California, USA) blood glucose and ketone monitoring system according to the manufacturers’ instructions. The reads of Abbott Precision Xtra were standardized by the reads of the same blood sample with Keto-Mojo GK+. Both reads from the Abbott and Keto-Mojo meters were highly linearly correlated ($R^2 = 0.93$, $P = 0.000103$ and $R^2 = 0.74$, $P = 0.00565$ for glucose and ketone reads, respectively; $N = 8$).

Statistical analysis

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

For statistical comparisons of our data, we performed tests including Student’s $t$-test and two- or three-way generalized linear model analyses to compare surface and cavefish, treatment and non-
treatment, and pre-treatment and post-treatment. We applied the linear model to non-processed data, generalized liner model (Poisson family) to discrete data, and generalized linear model (gamma family) to processed data (i.e., turning index). Holm’s post hoc correction was used to understand which contrasts were significant (Holm, 1979).

Regarding replicates of experiments, we used different individuals for the replicates, namely two biological replicates, using different individuals in each trial (e.g., Figure 2 and Figure 4—figure supplement 1). There was no repeated usage of individual fish excluding the time-course experiment (Figure 2). For the experiments measuring sleep and VAB in addition to nearby interactions and turning bias (Figures 5, Figure 5-figure supplement 1–2), we used two biological replicates and confirmed that the averages of experimental data did not largely differ from each other. We then merged the data acquired in two biological replicates and presented the data as a single set of results.

The aforementioned calculations were performed using R version 4.0.4 software (packages of car, lme4, and lmerTest) (Bates et al., 2015; Fox and Weisberg, 2019; Kuznetsova et al., 2017), and all statistical scores are available in Supplementary file 1, the figure legends, or the text.
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**Figure 1. Blood glucose and ketone levels under the control diet (CD) or ketogenic diet (KD).**

(A) Experimental procedure. After fish were raised for 3–4 months on a brine shrimp larva diet, fish were fed the CD or KD for 5 weeks. After 5 weeks, blood glucose and ketone levels were measured.

(B) Blood ketone level (mmol/L). Ketone levels were significantly reduced by KD feeding in both surface fish (SF) and cavefish (CF). Data are presented as the mean ± standard error of the mean. Dots indicate individual data.

(C) Blood glucose level (mg/dL). Glucose levels were significantly reduced by KD feeding in both SF and CF, suggesting that this diet altered the balance between glucose and ketone.


*: P < 0.05, **: P < 0.01, ***: P < 0.001. All detailed statistical data are available in Supplementary file 1.
Figure 1—figure supplement 1. Two weeks fasting reduced glucose ketone index (GKI) in both surface fish and cavefish, and increased nearby interactions in cavefish.

(A) Experimental procedure. After fish were fasted (Fasting) or fed with brine shrimp (CD) for 2 weeks, blood glucose and ketone levels were measured. (B) Blood ketone level (mmol/L). Ketone level change was not detectable by the 2-week fasting in either surface fish (SF) or cavefish (CF). Data are presented as the mean ± standard error of the mean. Dots indicate individual data. (C) Blood glucose level (mg/dL). Glucose levels were significantly reduced by fasting in both SF and CF, suggesting that this diet altered the balance between glucose and ketone. (D) Nearby interaction duration. The time periods when a fish was nearby (≤5 cm) and spent more than 4 s in a 5 min assay were summed in each fish. Only cavefish result was shown due to a loss of surface fish individuals during fasting. Fasted cavefish showed longer nearby interaction than the fed control. (F) Nearby interaction event number. Nearby events (≤5 cm and ≥4 s) in a 5 min assay were counted. Fasted cavefish showed longer nearby interaction than the fed control.

SF: N = 8 for CD feeding, N = 5 for fasting. CF: N = 8 for CD feeding, N = 8 for fasting. *: P < 0.05, **: P < 0.01, ***: P < 0.001. All detailed statistical data are available in Supplementary file 1.
Figure 2. Time-course of nearby interaction changes during 9 weeks of control diet (CD) or ketogenic diet (KD) feeding.

(A) Experimental procedure. After rearing fish for 3–4 months on a brine shrimp larva diet, the pre-treatment recording was performed, followed by CD or KD feeding for 5 weeks. Nearby interactions were recorded every week until week 5 of feeding. Subsequently, all groups including KD-fed fish were given the CD until week 9. (B) An example of nearby interaction events among surface fish (SF). The left panel presents an example frame of the video. The colored lines indicate the trajectories of individual fish. A red-labeled fish was followed by a blue-labeled fish. Each nearby event that met the detection criteria, namely a distance of ≤ 5 cm between two fish that was maintained for more than 4 s, was counted as a nearby interaction event. The right panel presents an example of the detected events...
presented in a raster plot (each yellow bar indicates a nearby interaction event). Each pair of fish (six pairs among four fish) is presented in the rows. (C) Duration of nearby interactions. Although SF did not exhibit any differences in the duration of nearby interactions (s) between CD (green) and KD (blue) feeding, differences were detected among cavefish (CF) in week 5. However, the nearby interaction duration was indistinguishable from that of the CD group starting in week 6 when the KD was withdrawn from the experimental group. (D) Number of nearby interactions. Whereas SF exhibited no differences between CD and KD feeding, differences were observed in CF in weeks 4–6. After the KD was withdrawn in week 6, the number of events decreased to the level observed with CD feeding. Data are presented as the mean ± standard error of the mean. Dots indicate individual data. N = 20 for each group. *: P < 0.05, **: P < 0.01, ***: P < 0.001. All detailed statistical data are available in Supplementary file 1.
Figure 2—figure supplement 1. Shift 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. By contrast, in cavefish, the swimming distance and activity were suppressed by KD feeding starting in week 1, and these values remained smaller than those in CD-fed fish until week 9. KD-fed cavefish subsequently exhibited an increased swimming distance (week 0).
Data are presented as the mean ± standard error of the mean. Dots indicate individual data. N = 20 in all groups. *: P < 0.05, **: P < 0.01, ***: P < 0.001. All detailed statistical data are available in Supplementary file 1.
Figure 3. Ketogenic diet (KD) feeding induced surface fish (SF)-like speed profiles during nearby interactions in cavefish (CF).

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

(A) Diagram and the calculation formula for the turning bias index. The changes in the left or right traveling directions were calculated every five frames (every 0.25 s) across all trajectories and expressed as radians. Positive radian values represent left (anticlockwise) turning, and negative values indicate right turning. The ratio between the numbers of clockwise and anticlockwise turns was used as the turning rate (1–infinity, positive value). (B) Turning biases of surface fish (left) and cavefish (right). There was no difference between CD and KD feeding in surface fish, whereas the turning index in CD-fed cavefish was larger than in KD-fed cavefish (see week 6).

Data are presented as the mean ± standard error of the mean. Dots indicate individual data. N = 20 for all groups. *: P < 0.05, **: P < 0.01, ***: P < 0.001. All detailed statistical data are available in Supplementary file 1.
Figure 4—figure supplement 1. 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) The duration of nearby interactions, (B) the number of nearby interactions, (C) swimming distance, and (D) turning bias are presented. The overall tendencies were the same as those observed in the original experiments except the 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 the turning bias (D). The duration (A) and number of nearby interactions (B) were maintained in KD-fed cavefish, whereas they were reduced in CD-fed cavefish, which also exhibited a higher level of turning bias (D). The swimming distance was not significantly reduced in KD-fed cavefish compared to that in CD-fed controls in this repeated experiment (C).

Data are presented as the mean ± standard error of the mean. Dots indicate individual data. Surface fish: N = 28 for CD, N = 32 for KD. Cavefish: N = 28 for CD, N = 32 for KD. *: P < 0.05, **: P < 0.01, ***: P < 0.001. All detailed statistical data are available in Supplementary file 1.
Figure 5. Day and night sleeping durations and swimming distances were not altered by ketogenic diet (KD) feeding.

(A) Sleep duration (min/h) during the day (left) and night (right). During 5 weeks of growth, the sleep duration decreased in surface fish and cavefish regardless of the diet. (B) 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. (C) Swimming distance during the day (left) and night (right). Control diet (CD)-fed cavefish exhibited a longer swimming distance during the day and night. Conversely, surface fish fed either diet and cavefish fed the KD exhibited a significantly increased swimming distance only at night.

Data are presented as the mean ± standard error of the mean. Dots indicate individual data. Surface fish: N = 28 for CD, N = 32 for KD. Cavefish: N = 28 for CD, N = 32 for KD. *: P < 0.05, **: P < 0.01, ***: P < 0.001. All detailed statistical data are available in Supplementary file 1.
Figure 5—figure supplement 1. Daytime and nighttime number of sleeping events (/ hr) under control diet (CD) or ketogenic diet (KD) feeding.

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

Data are presented as the mean ± standard error of the mean. Dots indicate individual data. Surface fish: N = 28 for CD, N = 32 for KD. Cavefish: N = 28 for CD, N = 32 for KD. *: P < 0.05, **: P < 0.01, ***: P < 0.001. All detailed statistical data are available in Supplementary file 1.
Figure 5—figure supplement 2. Vibration attraction behavior (VAB) and swimming distance during VAB under control diet (CD) or ketogenic diet (KD) feeding.

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

Data are presented as the mean ± standard error of the mean. Dots indicate individual data. Surface fish: N = 28 for CD, N = 32 for KD. Cavefish: N = 28 for CD, N = 32 for KD. *: P < 0.05, **: P < 0.01, ***: P < 0.001. All detailed statistical data are available in Supplementary file 1.
Figure 6. Body length and weight under control diet (CD) or ketogenic diet (KD) feeding.
(A) Standard length (cm). KD-fed surface fish and cavefish were significantly smaller than their CD-fed counterparts. (B) Body weight (g). KD-fed surface fish and cavefish weighed less than their CD-fed counterparts.

Data are presented as the mean ± standard error of the mean. Dots indicate individual data. Surface fish: N = 28 for CD, N = 32 for KD. Cavefish: N = 28 for CD, N = 32 for KD. *: P < 0.05, **: P < 0.01, ***: P < 0.001. All detailed statistical data are available in Supplementary file 1.
Figure 7. Nearby interactions and other behaviors under control diet (CD) or beta-hydroxybutyrate–supplemented diet (BHB) feeding.

(A) Duration of nearby interactions (s). After 4 weeks, the duration of nearby interactions was decreased in BHB-treated surface fish and increased in BHB-treated cavefish. (B) Number of nearby interactions. The number of nearby interactions was increased in BHB-treated cavefish. (C) Swimming distance. No difference was detected between the CD and BHB groups. (S) Turning bias ratio. BHB-treated cavefish tend 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. N = 20 for all groups. *: P < 0.05, **: P < 0.01, ***: P < 0.001. All detailed statistical data are available in Supplementary file 1.
Figure 7—figure supplement 1. Body size and weight under control diet (CD) or beta-hydroxybutyrate-supplemented diet (BHB) feeding.

(A) 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 supplement. (B) Body weight (g). BHB-treated surface fish exhibited significantly reduced weight, whereas the weight of BHB-treated cavefish increased more than the control diet-treated cavefish. Data are presented as the mean ± standard error of the mean. Dots indicate individual data. N = 20 for all groups. *: P < 0.05, **: P < 0.01, ***: P < 0.001. All detailed statistical data are available in Supplementary file 1.
Figure 7—figure supplement 2. 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 in surface fish increased in BHB feeding but not in the 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 diets. Data are presented as the mean ± standard error of the mean. Dots indicate individual data. N = 20 for all groups. *: P < 0.05, **: P < 0.01, ***: P < 0.001. All detailed statistical data are available in Supplementary file 1.