

## RESEARCH ARTICLE

# Paradoxical anaerobism in desert pupfish

Matt Heuton, Luis Ayala, Chris Burg, Kyle Dayton, Ken McKenna, Aldo Morante, Georgina Puentedura, Natasha Urbina, Stanley Hillyard, Spencer Steinberg and Frank van Breukelen\*

## ABSTRACT

In order to estimate metabolic demands of desert pupfish for conservation purposes, we measured oxygen consumption in fish acclimated to the ecologically relevant temperatures of 28 or 33°C. For these experiments, we used fish derived from a refuge population of Devils Hole pupfish (*Cyprinodon diabolis*). Measurement of routine oxygen consumption ( $\dot{V}_{O_{2,routine}}$ ) revealed some 33°C-acclimated fish (10% of 295 assayed fish) periodically exhibited periods of no measurable oxygen consumption despite available ambient oxygen tensions that were above the critical  $P_{O_2}$ . We call this phenomenon paradoxical anaerobism. The longest observed continuous bout with no oxygen consumption was 149 min, although typical bouts were much shorter. Fish maintained normal posture and ventilation rate (>230 ventilations per minute) during paradoxical anaerobism. Fish rarely demonstrated a compensatory increase in oxygen use following a period of paradoxical anaerobism. In contrast, only one out of 262 sampled fish acclimated at 28°C spontaneously demonstrated paradoxical anaerobism. Muscle lactate concentration was not elevated during periods of paradoxical anaerobism. However, the amount of ethanol released by the 33°C-acclimated fish was 7.3 times greater than that released by the 28°C acclimation group, suggesting ethanol may be used as an alternative end product of anaerobic metabolism. Exposure to exogenous ethanol, in concentrations as low as 0.1%, produced periods of paradoxical anaerobism even in 28°C-acclimated fish.

**KEY WORDS:** Oxygen consumption, Thermal acclimation, *Cyprinodon*

## INTRODUCTION

Several species of pupfish in the genus *Cyprinodon* inhabit warm springs of the Mojave Desert in southwestern North America where water temperatures may be as high as 33–35°C (for review, see Hillyard, 2011; Hillyard et al., 2015). Importantly, these fishes may not have spent much of their evolutionary history at these warm temperatures. The desertification that led to the Mojave Desert is relatively recent. Historically, southwestern North America was dotted by large lakes and interconnecting streams. Even Death Valley, currently one of the hottest and driest areas on Earth, was inundated with the 100 m-deep Lake Manley as recently as 10,000 years ago (Smith et al., 2002). Salt inclusions suggest Lake Manley was 4–15°C cooler than current Death Valley waters, such that it may have been <20°C (Lowenstein, 2002). As the pluvial waters receded following the last ice age, pupfish became isolated in warm springs (Echelle, 2008). Today, Ash Meadows National Wildlife Refuge in Nevada is home to ~20 such springs

with pupfish of either the *mionectes* or *pectoralis* subspecies of *Cyprinodon nevadensis* (Hillyard, 2011). Water temperatures of these springs vary from 22 to 35°C. One unique habitat in Ash Meadows is Devils Hole (Riggs and Deacon, 2002), a 3.5×22 m opening to a large aquifer, which experiences stable main pool temperatures of 33.2–33.9°C year round. The endemic pupfish species, *Cyprinodon diabolis*, is critically endangered and has the most restricted range of any described vertebrate species.

One possible reason for the small population size of *C. diabolis* is limited food availability. A remarkable feature of Devils Hole is its poor annual primary production of only ~5000 kJ for the entire system (Wilson and Blinn, 2007). For perspective, a similar aquatic habitat, Tecopa Bore, has an annual primary production of ~46,000 kJ m<sup>-2</sup> (Naiman, 1976). Contributing to the poor primary production of Devils Hole is its location; the opening to the water level in Devils Hole lies ~15 m below the surrounding desert (Riggs and Deacon, 2002). During the winter months, sunlight does not directly reach Devils Hole and primary production is limited. During the summer, there is a large amount of food available but shallow water temperatures may reach as high as 39°C. We measured oxygen consumption in order to estimate the energetic needs of these fish. Oxygen consumption was determined in *C. n. mionectes* and a population of fish derived from *C. diabolis* (refuge population; see Materials and methods) acclimated to ecologically relevant temperatures of 28 and 33°C. We found that despite the availability of ambient oxygen, fish may experience prolonged periods of anaerobic metabolism without any subsequent compensatory oxygen consumption. We call this process paradoxical anaerobism. Paradoxical anaerobism was evident in both species; however, all results shown here are from refuge fish because of their greater availability. Our rationale for calling this process paradoxical anaerobism is that most species exploit anaerobic metabolism only under conditions where oxygen availability is limited. Here, these fish may come from very energy-limited environments yet utilize the seemingly inefficient processes of anaerobic metabolism when they could be performing aerobic metabolism.

## MATERIALS AND METHODS

### Fish and husbandry

Because of the small population size and the restricted habitat of the critically endangered *C. diabolis* Wales 1930, a refuge population was established at Point of Rocks (POR) in Ash Meadows National Wildlife Refuge, Nevada (Riggs and Deacon, 2002). The refuge consisted of a concrete basin with a cooler water temperature (~28°C) than Devils Hole itself. Concerns that the POR refuge population of *C. diabolis* may have hybridized with the closely related species *C. n. mionectes* (Martin, 2005) prompted the US Fish and Wildlife Service (USFWS) to make available these fish for research purposes. It should be noted that the data supporting or refuting a hybridization event are not conclusive. Devils Hole fish typically lack a pelvic fin and this characteristic was long-considered a diagnostic characteristic even though the original description of *C. diabolis* as a species noted the presence of a pelvic fin on one specimen (Wales, 1930). Wales went on to describe fin ray loss at higher temperatures in other springs inhabited by *C. n. mionectes*. There is

School of Life Sciences, University of Nevada Las Vegas, 4505 S. Maryland Parkway, Las Vegas, NV 89154, USA.

\*Author for correspondence (frank.vanbreukelen@unlv.edu)

surprising genetic diversity in *C. diabolis*; there are 17 alleles in 29 sampled fish (locus A1) and 9 alleles in 22 sampled fish (locus A16) for two recognized microsatellite loci (Burg et al., 2002; Martin and Wilcox, 2004; Wilcox and Martin, 2006). This diversity is in spite of an effective population size that has been estimated to never be above 250 reproducing adults. When 23/110 of the POR refuge population displayed a pelvic fin in 2005, an assumption was made that the fish had hybridized with *C. n. mionectes*. Martin (2005) found 4 additional alleles for the A1 locus in the POR refuge population that were not previously found in the limited sampling of *C. diabolis*. Later, it was found that fish reared at 33°C frequently do not develop a pelvic fin (*C. n. amargosae*, Lema and Nevitt, 2006; our unpublished data for both refuge fish and *C. n. mionectes*). As there was no comprehensive DNA sampling of fish used to originally populate the refuge, there is no way to effectively ascertain whether refuge fish are pure *C. diabolis*. To examine whether paradoxical anaerobism is unique to the refuge fish, we also obtained *C. n. mionectes* Miller 1948 that inhabit flowing springs with a wider range of available temperatures. Fish were collected from Kings Pool at POR under permits from the USFWS and Nevada Department of Wildlife (NDOW). Both species thrive at 28°C in the laboratory and reproduce well. However, breeding at 33°C is very difficult. We observed use of paradoxical anaerobism in fish bred and reared for their entire lives at 33°C. However, the limited availability of these fish required we use fish reared at 28°C and subsequently acclimated to 33°C for a minimum of 3 weeks.

### Oxygen consumption

A flow-through system was constructed in which fish were placed in chambers (glass tubes, 25 mm diameter×120 mm length) connected to a peristaltic pump. The chambers were immersed in a 38 l aquarium with heating and aeration to maintain a stable temperature of water that was drawn into the chambers. Trials were performed at 25, 28, 31, 33, 34, 36 and 38°C for both 28 and 33°C-acclimated fish of varying mass. The oxygen partial pressure ( $P_{O_2}$ ) of the excurrent flow from the chambers was recorded continuously using a Strathkelvin 929 system. Oxygen consumption was calculated using the appropriate flow rates and oxygen solubility values. Excurrent flow was returned to the aquarium and flow rates were adjusted to keep excurrent oxygen tension above 90 Torr (where 1 Torr≈133 Pa), which is well above the critical  $P_{O_2}$  values but with a slow enough flow so fish could maintain position in the chamber with minimal observable swimming activity. After the ~2 h trials, background rates of oxygen consumption without the fish were determined. Defecation by the fish during the trials can lead to electrode drift.  $P_{O_2}$  values were corrected for small drifts in electrodes relative to the empty chamber by subtracting the final empty chamber  $P_{O_2}$  from the initial calibrated  $P_{O_2}$  and assuming a linear change in  $P_{O_2}$  across the time period of the trial.

The same flow-through system was used for the 24 h trials. During these longer periods, faeces and associated microbiological accumulation in the system required more extensive correction for background levels of oxygen consumption. We noted that drift in the electrodes was not constant but rather depended on how much fish had defecated. Based on our experiences with the shorter trials, variable oxygen consumption was not characterized by extended bouts of stable low oxygen use. However, paradoxical anaerobism resulted in near-zero oxygen consumption by the fish for more extended periods. Therefore, these periods provided an opportunity to adjust for background oxygen consumption during the 24 h trials. We exploited a conservative approach to the subsequent data analyses as values were compared with expected values for stable routine oxygen consumption (based on mass-specific values from stable fish; data not shown) and binned into four classes (see Results).

Critical  $P_{O_2}$  values were obtained by connecting the excurrent flow to the incurrent fitting of the chambers to create a closed system. Initial declines in  $P_{O_2}$  were relatively rapid, with a gradual transition to a lower value that, in some cases, levelled off. Data points for the initial decline and the subsequent levelling off were fitted by linear regression. Critical  $P_{O_2}$  was calculated as the intercept between the two lines.

### Ventilation

Fish ( $N=4$ ) were video recorded in the flow-through respirometer chamber before and after exposure to 2% exogenous ethanol. Slow speed analysis

**Table 1. Exogenous ethanol addition to aquarium water induces paradoxical anaerobism**

[Ethanol] (%)	Acclimation temperature	
	28°C	33°C
0.005	0/12	0/12
0.01	0/12	2/12
0.1	2/12	3/12
0.2	2/12	9/12
1	7/12	9/12
2	8/12	12/12

Values represent the number of fish out of 12 that demonstrated paradoxical anaerobism in the 1 h following ethanol addition. [Ethanol] represents the final concentration of ethanol in the water after addition of exogenous ethanol.

with manual counting of opercular movements for three 15 s periods was performed for periods of stable oxygen consumption and induced paradoxical anaerobism (both states were confirmed by examining oxygen consumption).

### Ethanol determination

Early experiments performed by spiking samples with exogenous ethanol demonstrated the lability of ethanol when fish defecated. We assumed the presence of ethanol-consuming microbes would interfere with recovery of ethanol from the water. Water was removed from fish tanks and boiled for 20 min prior to use. It was allowed to cool to 28°C and used to fill smaller sealed containers to which a single fish acclimated to either 28 or 33°C ( $N=9$  of each group) was added, and the apparatus was incubated at 28°C. A Strathkelvin oxygen electrode monitored  $P_{O_2}$  until it had declined to 90 Torr (up to 1 h). The fish was removed and 30 ml of water was immediately put into a headspace vial and 1.4 g of  $Na_2SO_4$  was added. Ethanol was adsorbed onto a polydimethylsiloxane (PDMS) fibre filter. Chromatographic analysis was performed using a Varian 3800 Gas Chromatograph interfaced with a Varian 2200 ion trap mass spectrometer using selective ion storage and compared with a standard. Only samples from fish that were observed not to defecate or swim during the sampling process were used.

### Lactate determination

Skeletal muscle was collected from  $N=5$  fish acclimated to 28 or 33°C and (a) demonstrating stable oxygen consumption patterns, (b) determined to be in a natural state of paradoxical anaerobism for 5 min, or (c) induced through addition of 2% exogenous ethanol to be in paradoxical anaerobism for 10 min (see below for details). Sampling states were confirmed using flow-through oxygen consumption as described above. Skeletal muscle lactate concentration was measured as per McGaw et al. (2009).

### Anoxia survival times

Fish ( $N=10$  of both 28 and 33°C-acclimated fish for each treatment) were placed into 38 l aquaria containing either 1 mmol l<sup>-1</sup> KCN in aerated water (chemical anoxia) or water bubbled with nitrogen (nominal anoxia was ensured using an oxygen electrode). Water temperature was 28°C. Fish were observed until they were unable to ventilate and maintain an upright position in the water. The endpoint of the experiment was death. A similar experiment was performed using 33°C-acclimated fish ( $N=10$  for each treatment) exposed to either  $N_2$  bubbled water confirmed to be nominally anoxic using an oxygen electrode or  $N_2$  bubbled water to which 2% ethanol had been added prior to bubbling.

### Ethanol induction

The same flow-through system was used to measure oxygen consumption as described above. After obtaining baseline data that demonstrated no paradoxical anaerobism, ethanol was added to the aquarium to give a final concentration as indicated in Table 1 ( $N=12$  of each group). To confirm Clark type electrode function in the presence of ethanol, similar results were obtained using a NeoFox Phase measurement system (which measures fluorescence lifetime of ruthenium to determine oxygen concentration; Ocean Optics Inc.).

### Animal welfare concerns

All experiments were approved by the University of Nevada Las Vegas Institutional Animal Care and Use Committee and are compliant with federal animal welfare regulations. Death as an endpoint was allowed for the anoxia tolerance experiments. Fish sampled for skeletal muscle lactate concentration were anaesthetized using MS-222 before decapitation and tissue collection.

## RESULTS

### Oxygen consumption

Routine oxygen consumption was determined using flow-through respirometry. Fish acclimated to 28°C almost always demonstrated stable oxygen consumption for the entire sampling period (~2 h) at various ambient temperatures (25–38°C,  $N=262$ , ‘control’; Fig. 1A). In marked contrast, when fish were acclimated to 33°C ( $N=295$ ), oxygen consumption was often more irregular in pattern. Most fish (71% of those sampled for ~2 h) exhibited stable oxygen consumption patterns (‘stable’; Fig. 1B). The remaining fish demonstrated patterns of waxing and waning (19% of fish, ‘variable’; Fig. 1C) or even periods of essentially no oxygen consumption (10% of fish, ‘paradoxical anaerobism’; Fig. 1D). In contrast, only 1 of the 262 sampled fish acclimated to 28°C spontaneously demonstrated paradoxical anaerobism and another 10 fish demonstrated the variable pattern (at various assay temperatures; data not shown). An additional 9 examples of 33°C-acclimated fish using paradoxical anaerobism at various ambient temperatures are provided in Fig. S1. When assayed for 24 h, most fish acclimated to 33°C used paradoxical anaerobism of varying extent and duration (Fig. 2; 50% of 62 fish had at least one bout of 15 min or longer and 67% had a bout of 6 min or longer). Fish often entered into periods wherein paradoxical anaerobism may be more extensive (e.g. nearly 5 h interrupted by brief periods of  $O_2$  consumption in Fig. 2E). In one exceptional case, a fish experienced 149 consecutive minutes of virtually no oxygen use (data not shown).

### Ventilation

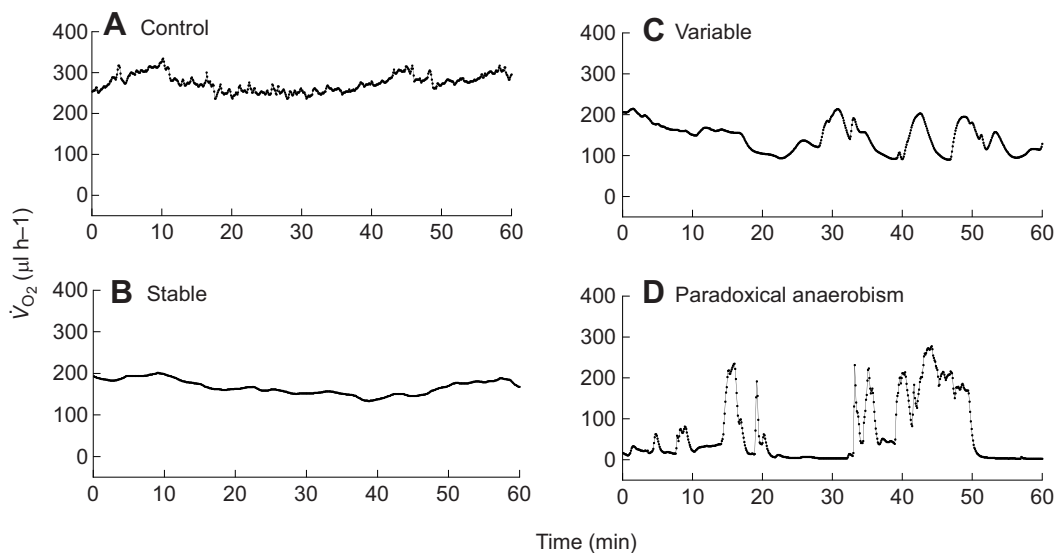
An important question is whether these anaerobic periods are simply a metabolic depression. Fish were observed to maintain posture and position in the metabolic chamber and ventilate during periods of paradoxical anaerobism. We measured opercular ventilations when fish were experiencing stable oxygen consumption and after induction of paradoxical anaerobism with exogenous ethanol (see below). Ventilation rate remained rapid ( $232.7 \pm 27.9$  ventilations  $\text{min}^{-1}$  during stable oxygen consumption versus  $247.7 \pm 23.1$  ventilations  $\text{min}^{-1}$  during periods of paradoxical anaerobism, means  $\pm$  s.e.m.;  $P > 0.05$ , paired  $t$ -test; Fig. S2). During this experiment, one fish spontaneously entered paradoxical anaerobism and there was no change in ventilation rate compared with its stable rate.

### Oxygen availability

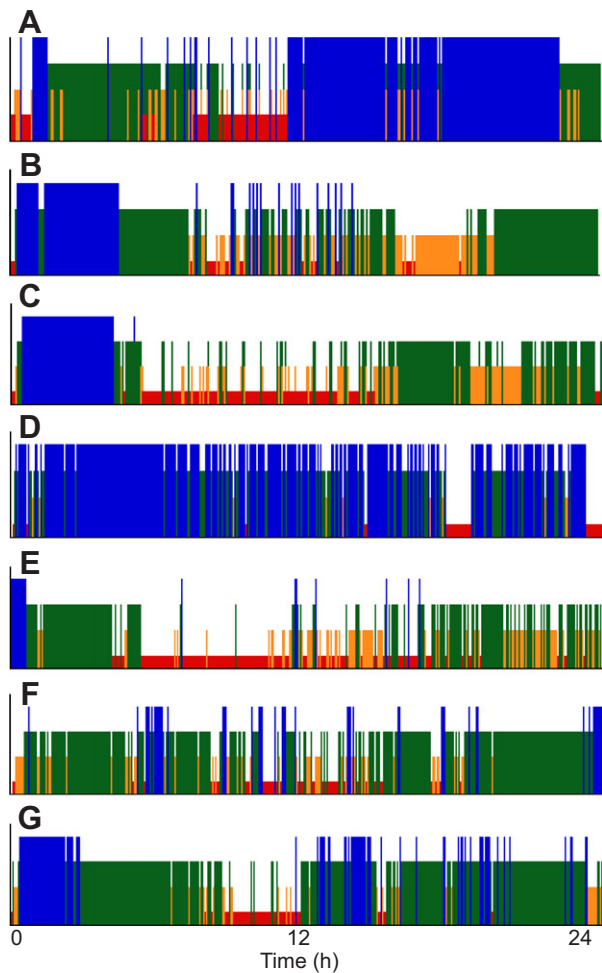
To test whether reduced oxygen consumption was due to restricted oxygen availability, we determined critical  $P_{O_2}$ . Critical  $P_{O_2}$  is defined as the partial pressure of oxygen where oxygen availability limits oxygen consumption. Oxygen consumption by fish in a closed system depleted oxygen in a linear fashion until the critical  $P_{O_2}$  was reached (Fig. 3A). When oxygen tension was depleted to below the critical  $P_{O_2}$ , oxygen consumption was reduced and often plateaued. Surprisingly, fish acclimated to 33°C and exhibiting stable oxygen consumption had a  $29 \pm 0.03\%$  higher critical  $P_{O_2}$  than 28°C-acclimated fish at ambient temperatures of 28 or 33°C (Fig. 3B; unpaired  $t$ -test;  $P < 0.05$ ). Ambient  $P_{O_2}$  was always maintained above critical  $P_{O_2}$  in the routine oxygen consumption experiments, indicating that animals could have used oxygen based on oxygen tensions.

### Alternative end product formation

In the absence of oxygen consumption, production of an alternative anaerobic end product like lactate or ethanol is required if glycolysis



**Fig. 1. Representative traces for routine  $O_2$  consumption at 28°C in pupfish from the refuge population.** Fish acclimated to 28°C and assayed at 28°C (control) almost always experience a stable pattern of resting  $O_2$  consumption with little variability at all temperatures (A; mass 430 mg). However, fish acclimated to 33°C but measured at 28°C (shown here) or other assay temperatures (see Fig. S1) may display different patterns: (1) oxygen consumption of stable fish is similar to that of fish acclimated to 28°C (B; mass 610 mg); (2) variable fish demonstrate a waxing and waning of  $O_2$  consumption below the typical mass-specific level (C; mass 620 mg); (3) fish may experience long periods of little or no  $O_2$  consumption wherein paradoxical anaerobism is evident (D; mass 571 mg). Note that in D there are a few periods where routine  $O_2$  consumption could be deemed compensatory.

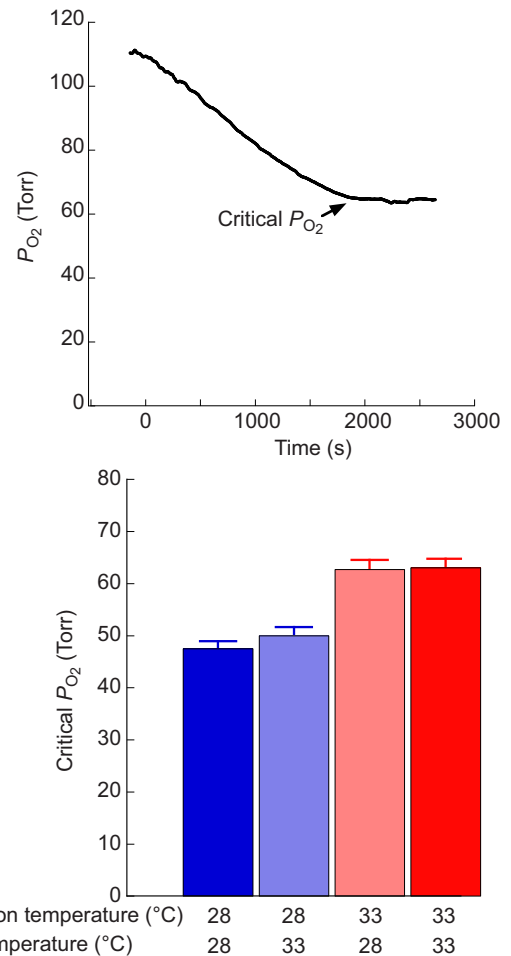


**Fig. 2. Oxygen consumption patterns of pupfish over a 24 h period demonstrating the variable nature of paradoxical anaerobism.** Electrode drift was corrected for as in Materials and methods. Data for seven pupfish (A–G) were compared with the expected stable routine  $O_2$  consumption based on fish mass, and binned: red, <20%; orange, 20–50%; green, 50–120%; and blue, >120% of expected  $O_2$  consumption.

is to continue. In some fish, ethanol and lactate are known to be produced during anoxia (Shoubridge and Hochachka, 1980; Mourik et al., 1982). We found fish acclimated to 33°C produced 7.3-fold more ethanol than fish acclimated to 28°C under aerobic conditions (Fig. S3;  $0.344 \pm 0.049$  versus  $0.047 \pm 0.019 \mu\text{mol g}^{-1} \text{h}^{-1}$  for 33 and 28°C-acclimated fish, respectively; unpaired  $t$ -test,  $P < 0.05$ ). There was no accumulation of skeletal muscle lactate as a function of acclimation temperature or with the use of paradoxical anaerobism (Fig. 4).

### Anoxia

Can fish simply avoid oxygen use completely in favour of anaerobic metabolism? Fish were placed into water either containing  $1 \text{ mmol l}^{-1}$  KCN to induce chemical anoxia or bubbled with nitrogen. Fish acclimated to 33°C and exposed to either form of anoxia experienced at most only a modest extension of survival time, suggesting that they were unable to exploit paradoxical anaerobism on demand (Fig. 5). One fish acclimated to 33°C survived for 47 min whereas most other fish died at times comparable to the 28°C-acclimated group. We suspect this fish may have been naturally exploiting paradoxical anaerobism (based on ~10% incidence as indicated in Fig. 1). Fish that were exposed to

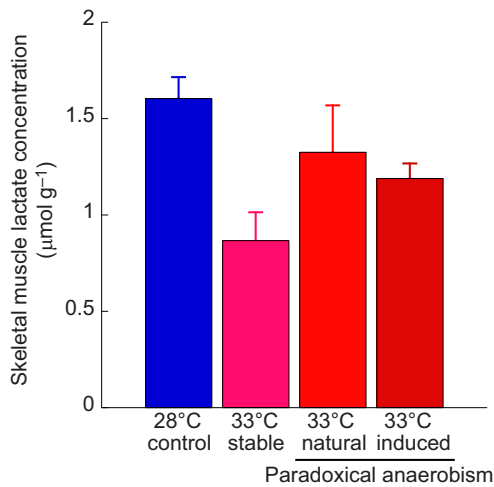


**Fig. 3. Determination of critical  $P_{O_2}$  in pupfish.** (A) The decrease in  $P_{O_2}$  as a fish consumed oxygen in a closed system. See Materials and methods for determination of critical  $P_{O_2}$ . (B) Critical  $P_{O_2}$  values for 28°C-acclimated fish were significantly lower than those of 33°C-acclimated fish regardless of ambient temperature (ANOVA,  $P < 0.05$ ,  $N = 21$ –24 fish per group).

2% ethanol, which should induce paradoxical anaerobism (see below), survived 35.6% longer in  $N_2$ -induced anoxia (Fig. S4; unpaired  $t$ -test,  $P < 0.05$ ). Taken together, fish appear unable to use paradoxical anaerobism on demand but if used, paradoxical anaerobism results in only a moderate extension of anoxia tolerance.

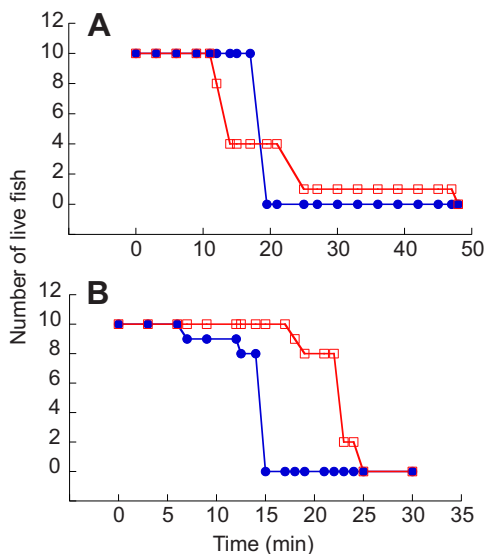
### Ethanol induction

We sought a putative mechanism for how fish might enter paradoxical anaerobism. Ethanol is able to freely diffuse across cell membranes. We exposed fish to ethanol in concentrations ranging from 0.005% to 2% in the aquarium water with the rationale that fish would experience increased tissue ethanol concentrations. We note that only one fish out of 262 acclimated to 28°C spontaneously used paradoxical anaerobism in the absence of exogenous ethanol (see Fig. 1). At concentrations as low as 0.01% environmental ethanol, fish were induced to enter paradoxical anaerobism (Table 1). At these lower concentrations, induction of paradoxical anaerobism was often delayed. For these assays, we restricted analyses for paradoxical anaerobism to a 1 h period immediately following ethanol addition. Trials with increased ethanol concentrations resulted in an increase in the number of fish that entered paradoxical anaerobism. For instance, upon exposure to 2% ethanol, 12/12 fish acclimated to 33°C and 8/12 fish acclimated

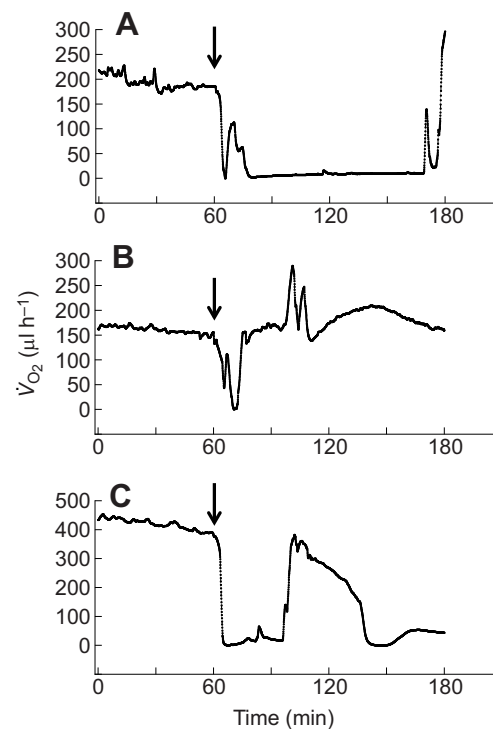


**Fig. 4. Skeletal muscle lactate concentration does not increase with the use of paradoxical anaerobism.** Concentrations are given as relative to skeletal muscle mass ( $N=5$  per group, ANOVA,  $P>0.05$  except between control fish acclimated to 28°C and 33°C-acclimated fish that were demonstrating stable oxygen consumption). Fish spontaneously used paradoxical anaerobism for 5 min (natural) or were induced to use paradoxical anaerobism for 10 min using 2% ethanol (induced). All states were verified using flow-through respirometry.

to 28°C immediately entered paradoxical anaerobism (Fig. 6, Table 1). As indicated in Fig. 6B, a few fish entered paradoxical anaerobism for short periods before spontaneously exiting. Interestingly, the represented animal does demonstrate apparent compensatory oxygen consumption. Other fish experienced stable and extended periods of no oxygen use. The average duration of the anaerobic periods after administration of 2% ethanol in 33°C-acclimated fish was  $32.04 \pm 7.21$  min. In the 2 h evaluation period following 2% ethanol addition, fish acclimated to 33°C spent  $49 \pm 7.22\%$  of their time in paradoxical anaerobism. Paradoxical anaerobism was observed more infrequently by fish acclimated to



**Fig. 5. Survival of anoxia.** Fish acclimated to either 28 or 33°C were placed into either (A)  $N_2$ -bubbled water (anoxia confirmed with oxygen electrode) or (B) water treated with  $1 \text{ mmol l}^{-1}$  KCN. Fish were observed until opercular movements ceased. Blue symbols represent 28°C-acclimated fish; red symbols represent 33°C-acclimated fish.



**Fig. 6. Representative oxygen consumption traces for pupfish showing induction of paradoxical anaerobism by exogenous ethanol.** Oxygen consumption in three fish (A–C) was measured by flow-through respirometry as described in Materials and methods. At the time indicated by the arrow (60 min), 2% ethanol was introduced into the system. Fish immediately entered into periods of paradoxical anaerobism of varying duration.

28°C at all concentrations of exogenous ethanol (Table 1; paired  $t$ -test,  $P<0.05$ ). We found 28°C-acclimated fish to be variable in their tolerance of exogenous ethanol. In one 28°C-acclimated fish, 2% ethanol induced a 75 min period of paradoxical anaerobism. However, in some 28°C-acclimated fish, it was necessary to stop the experiment 30 min after 2% ethanol administration because they had difficulty maintaining posture. No similar postural deficits were observed in 33°C-acclimated fish even with 2% exogenous ethanol.

## DISCUSSION

Utilization of aerobic pathways probably evolved as a result of their much higher energetic efficiency; aerobic metabolism yields ~15 times more ATP from glucose than does anaerobic metabolism (Hochachka and Somero, 1984). It follows then that Devils Hole fish would benefit energetically by avoiding anaerobic metabolism. A very unexpected finding of our study was the use of paradoxical anaerobism. Despite the availability of high ambient oxygen, many fish experienced long periods of dampened or even virtually no oxygen consumption (Fig. 1C,D; Fig. S1). We found periods as long as 149 continuous minutes with no oxygen consumption. The use of paradoxical anaerobism was widespread. When 62 fish acclimated to 33°C were assayed for 24 h, 2/3 experienced paradoxical anaerobism (Fig. 2). In contrast, fish acclimated to 28°C rarely used this novel metabolic strategy. Such a finding suggests a response to higher temperatures in these fish includes metabolic strategies that extend beyond simply trying to maximize energetic gain.

A simple explanation for paradoxical anaerobism would be that of metabolic depression. However, there was no change in the rapid ventilation rate with use of paradoxical anaerobism. Fish moved

about the small metabolic chamber in a deliberate manner in order to maintain position and posture irrespective of acclimation temperature or oxygen consumption patterns. These results do not support the use of metabolic depression. Another explanation for paradoxical anaerobism might be that low levels of oxygen availability are limiting oxygen consumption. We determined critical  $P_{O_2}$  in 28 and 33°C-acclimated fish (Fig. 3B). Sufficient ambient oxygen was available to support normal oxygen consumption in our assays. An inability to use oxygen might also be attributable to deficits in mitochondrial function. Importantly, animals are able to spontaneously exit from paradoxical anaerobism and experience normal oxygen consumption suggesting that any alterations to mitochondrial function are not permanent or the result of damage.

In order to continue glycolysis,  $NAD^+$  must be regenerated (Hochachka and Somero, 1984). The sustained use of anaerobic metabolism necessitates the use of an alternative end product such as ethanol or lactate. Indeed, fish like goldfish produce ethanol and lactate as alternative end products of anaerobic metabolism and are able to withstand extended periods of anoxia (Shoubridge and Hochachka, 1980). For instance, 12 h of anoxic exposure at 4°C in goldfish results in tissue accumulation of ethanol to  $4.58 \mu\text{mol g}^{-1}$  fish, and excretion of an additional  $6.63 \mu\text{mol g}^{-1} \text{ fish}^{-1}$  into the water. Pupfish are phylogenetically distant from goldfish. Nevertheless, we found fish acclimated to 33°C voided 7.3-fold more ethanol than fish acclimated to 28°C under aerobic conditions (Fig. S2). We do not believe that this value represents a complete accounting of ethanol production. Our preliminary experiments suggest a robust microbial community consumes ethanol in aquarium water. Time-dependent increases in ethanol concentration were only obtained after we carefully boiled water prior to addition of the fish, rinsed fish with fresh water, and ensured that the fish did not defecate during the trial. An obvious advantage of ethanol production over lactate accumulation is its ability to pass through membranes and diffuse from the fish. The practicable voiding of ethanol would allow for sustained anaerobic use whereas production of the less voidable lactic acid could result in significant metabolic issues like acidosis and muscle fatigue (Hochachka and Somero, 1984). One major disadvantage to the voiding of ethanol to the environment is loss of potential energy associated with the metabolite, i.e. ethanol lost to the environment cannot be catabolized by the fish. When one considers the low availability of energy in the systems inhabited by fish such as *C. diabolis*, this loss of potential energy is even more surprising. We suspect additional costs to performing aerobic metabolism such as the production of reactive oxygen species may outweigh the energetic costs of ethanol voiding. In contrast to what is observed in anoxic goldfish, there were no increases in tissue lactate concentration with use of paradoxical anaerobism (Fig. 4). These lactate data as well as the data that fish are unable to survive extended periods of anoxia (Fig. 5) indicate paradoxical anaerobism is a distinct physiological event from the anaerobiosis that goldfish experience when placed into anoxia.

We sought a putative mechanism for paradoxical anaerobism. Substrates entering the outer mitochondrial membrane pass through voltage-dependent anion channels (VDACs; Lemasters and Holmuhamedov, 2006; Lemasters et al., 2012; Holmuhamedov et al., 2012). During normal metabolism, it is assumed that VDACs are open, supporting the commonly accepted paradigm that small molecular weight molecules pass through the outer mitochondrial membrane freely. In partially permeabilized rat hepatocytes, ethanol metabolism is able to suppress oxygen

consumption (Holmuhamedov and Lemasters, 2009). Additional experiments demonstrated acetaldehyde produced by ethanol metabolism resulted in the closure of VDACs. A closed VDAC would limit substrate entry into the mitochondrion; reduced substrate entry would limit oxygen consumption. We exposed fish to varying concentrations of exogenous ethanol. Even very small concentrations were able to induce paradoxical anaerobism in both 28 and 33°C-acclimated fish (Fig. 6). The incidence of paradoxical anaerobism increased in a dose-dependent fashion (Table 1). We propose that ethanol metabolism and the likely accumulation of acetaldehyde results in closure of the VDACs in living pupfish. This model is consistent with the observation of no changes in ventilation rate with paradoxical anaerobism. Unlike that in terrestrial vertebrates, ventilation in fish is regulated by reduced tissue  $P_{O_2}$  (Randall, 1982). It follows that during paradoxical anaerobism, tissue levels of oxygen may be high and ventilation rate would remain unchanged.

The use of paradoxical anaerobism is more complex than simply being a result of the presence or absence of high tissue-ethanol concentrations. Fish are able to spontaneously exit from paradoxical anaerobism despite the continued presence of exogenous ethanol as in Fig. 6. This observation suggests that the effects of ethanol are not simply due to toxicity. Furthermore, the ability to spontaneously exit from paradoxical anaerobism despite the continued presence of as much as 2% exogenous ethanol suggests a more complex regulation than simple absence or presence of ethanol. Fish acclimated to 33°C were more tolerant of 2% ethanol than were their 28°C counterparts. Fish acclimated to 33°C were also more likely to enter paradoxical anaerobism at lower concentrations of ethanol. We suspect that 33°C-acclimated fish utilize alternative approaches towards dealing with chronic ethanol exposure. In other models, chronic ethanol use like alcoholism results in tremendous changes to how ethanol is metabolized within the cell (e.g. Laposata and Lange, 1986; Lieber, 2005).

Water at higher temperatures and/or elevations has a decreased capacity for carrying oxygen. Fully oxygen-saturated water in the main pool of Devils Hole (elevation 719 m; Hoffman, 1988) would be expected to have an oxygen solubility of  $\sim 5.22 \text{ ml l}^{-1}$  and a  $P_{O_2}$  of  $\sim 146.6$  Torr depending on daily barometric pressure. For reference, the oxygen carrying capacity of 28 versus 33°C water in our laboratory is  $\sim 5.64$  versus  $5.27 \text{ ml l}^{-1}$ , respectively (elevation=640 m). Importantly, water in the main pool of Devils Hole is not oxygen saturated;  $P_{O_2}$  is  $\sim 43$ –63 Torr during most of the day (Bernot and Wilson, 2012). This  $P_{O_2}$  is lower than the critical  $P_{O_2}$  of the fish acclimated to a temperature (33°C) that approximates that of Devils Hole (Fig. 3B). It is questionable whether a native Devils Hole fish would experience reduced oxygen consumption under natural conditions. We note that Devils Hole is not the only warm spring inhabited by fish in the Ash Meadows complex where water may be  $>33^\circ\text{C}$ . Although data are not presented here, we have observed paradoxical anaerobism in *C. n. mionectes* (Ash Meadows pupfish) acclimated to 33°C.

We suggest metabolism in pupfish is frequently a mosaic of both aerobic and anaerobic processes. A plausible explanation for the oscillations characteristic of variable oxygen consumption might be that they are a result of the degree and extent to which some tissues experience aerobic metabolism. It follows that the absence of oxygen consumption during paradoxical anaerobism is simply a more global extension of variable oxygen consumption. We note that variable oxygen consumption was evident in many fish that did not enter paradoxical anaerobism in the ethanol induction experiments.

Despite the obvious energetic advantages of using aerobic metabolism in an energy-starved ecosystem, fish readily exploit variable oxygen consumption and paradoxical anaerobism. It might be tempting to speculate that exploitation of these novel and alternative metabolic strategies could conceivably avoid some deleterious aspect of oxygen use. However, the use of paradoxical anaerobism may also simply be a consequence of a regulatory process with no real benefit. Nevertheless, that fish use paradoxical anaerobism begs questions as to how this impacts on longevity, energy budgets and the overall ecology of the fish. Perhaps these factors explain why these fish are critically endangered. If our current model is correct, our data shed new light on the importance of VDACs in regulating metabolism at the organismal level. The evidence presented here reveals some of the mechanism at the whole-organism level. We hope future studies at other levels such as the tissue or cellular level will complement these findings and provide elucidation of how and why these animals use paradoxical anaerobism.

#### Acknowledgements

We thank the various regulatory and permitting agencies for facilitating this work. In particular, Sean Harris from Nevada Division of Wildlife (NDOW) was instrumental in initiating this project.

#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

M.H., S.H. and F.v.B. were responsible for data collection and analyses, experimental design and interpretation, and manuscript preparation. L.A., C.B., K.D., K.M., A.M., G.P. and N.U. were responsible for fish husbandry, data collection and data analyses. S.S. designed and performed the GC-MS experiments for ethanol concentration determination.

#### Funding

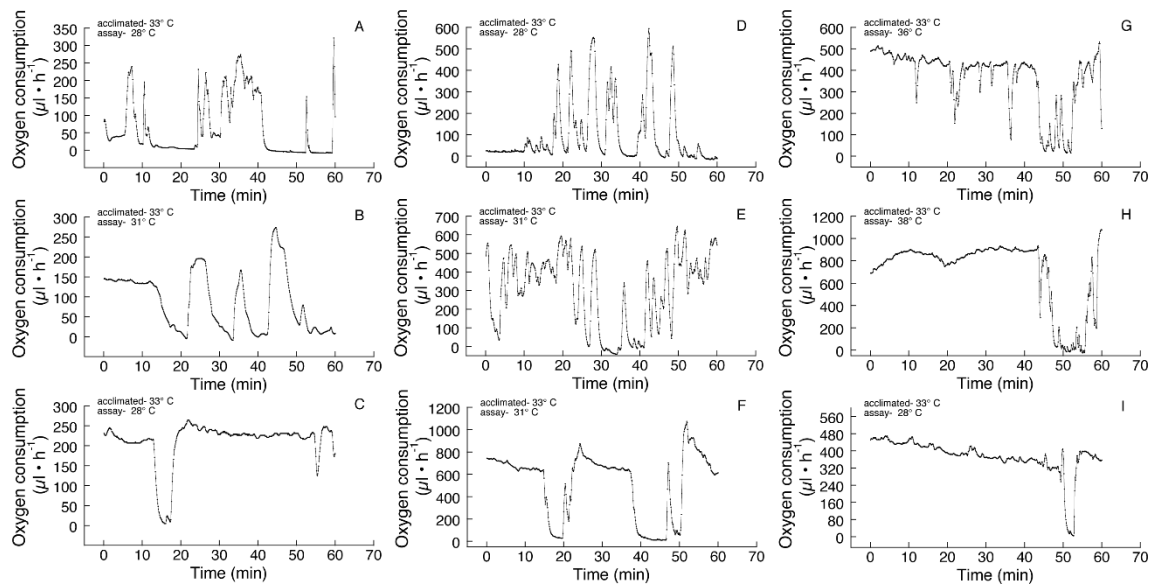
This work was funded by grants from University of Nevada Las Vegas, the US Fish and Wildlife Service (USFWS), the Nevada Division of Wildlife (NDOW), and the National Science Foundation Nevada Experimental Program to Stimulate Competitive Research (EPSCoR).

#### Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.130633/-/DC1>

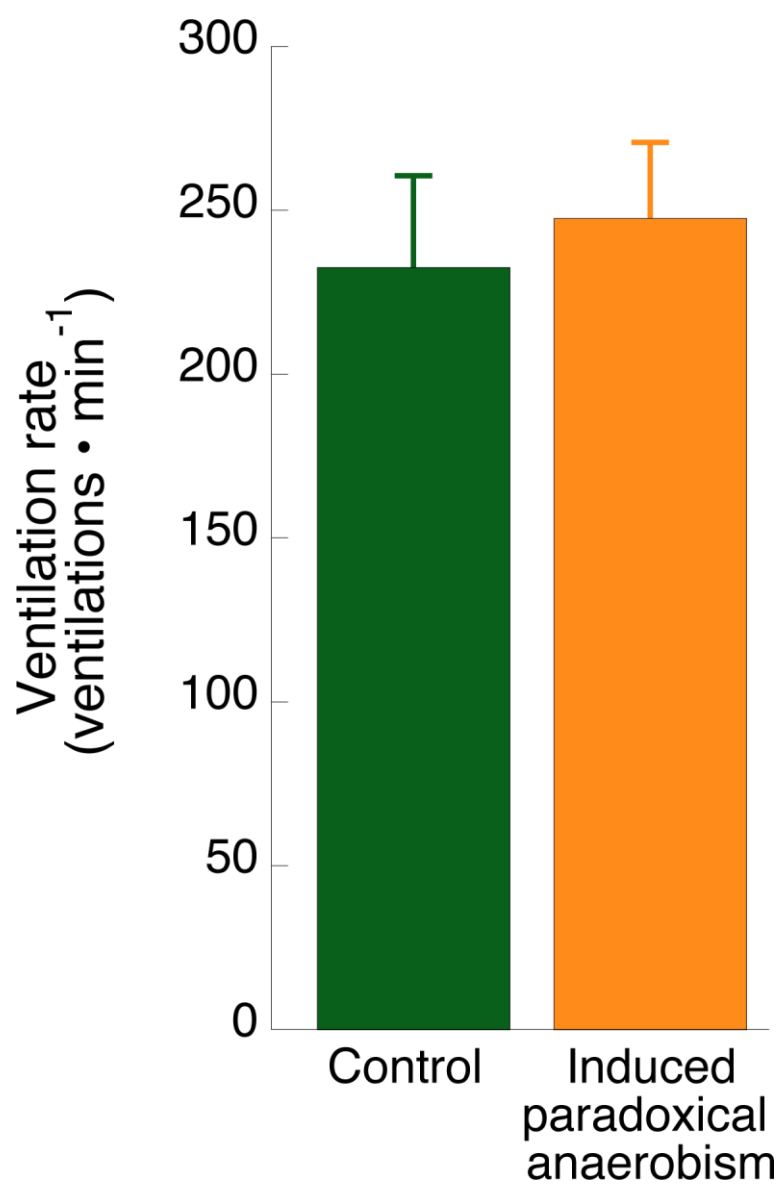
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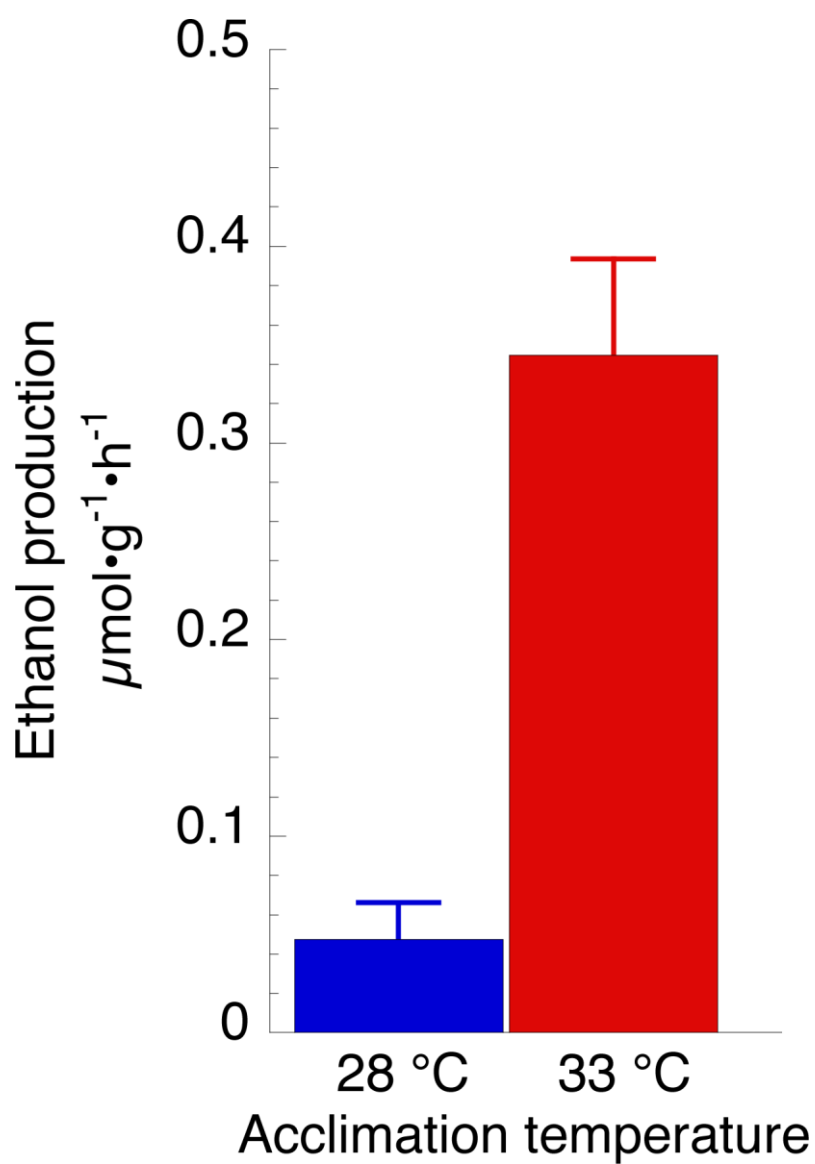


**Fig. S1.** Representative traces from fish showing paradoxical anaerobism of varying durations and periods. Note the range of assay temperatures. Traces for D, G, and H were not corrected for the minimal electrode drift. All other traces were corrected for electrode drift ( $5.9 \pm 0.6\%$  drift for the 6 corrected traces).

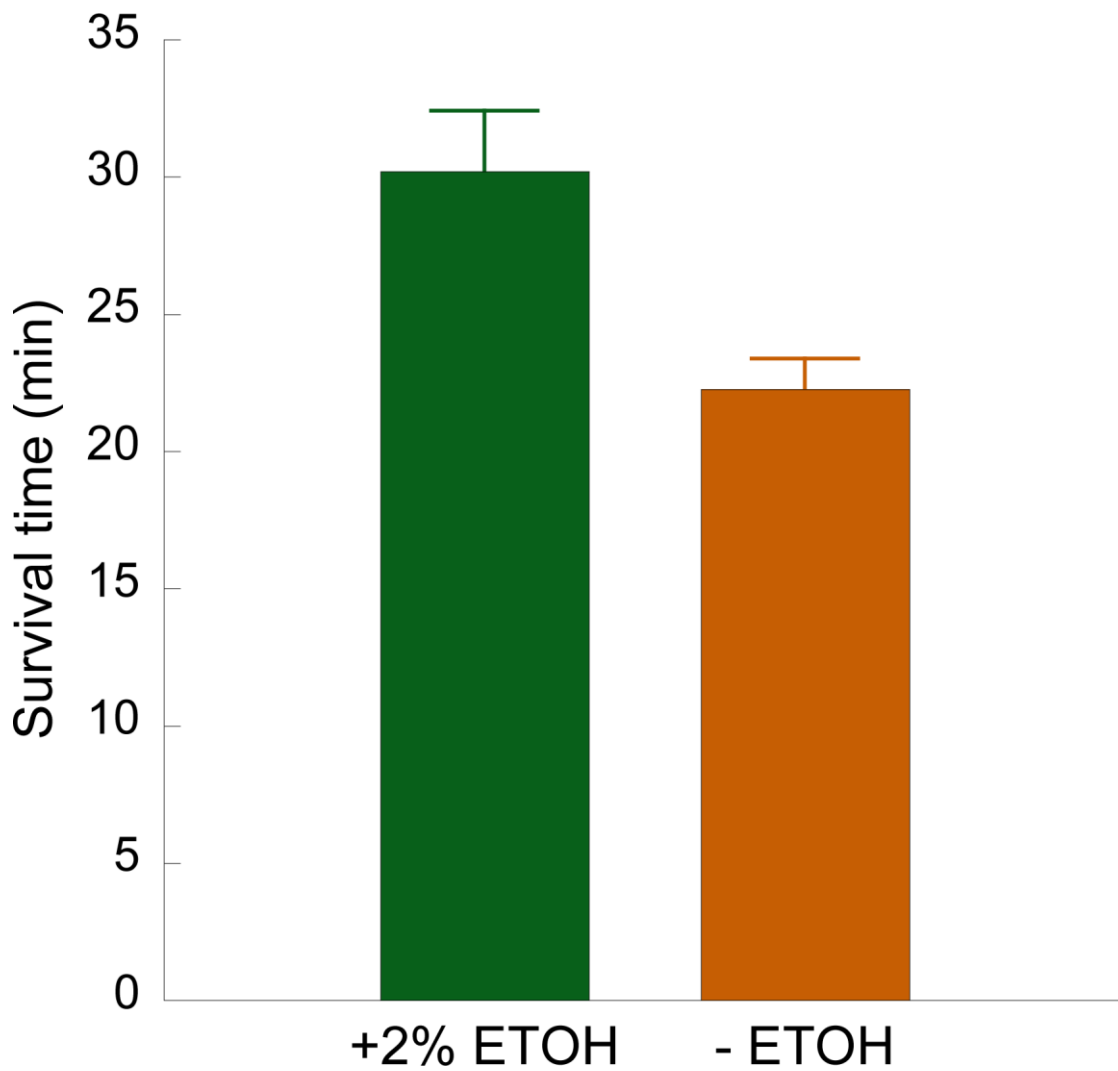




**Fig. S2.** Ventilation rates for 33°C acclimated fish ( $n = 4$ ) demonstrating stable  $O_2$  consumption rates (control) and after the induction of paradoxical anaerobism by 2% ethanol. There was no difference between ventilation rates during periods of stable and paradoxical anaerobism (paired t-test;  $p > 0.05$ ).



**Fig. S3.** Fish acclimated to 33°C produced 7.3-fold more ethanol than fish acclimated to 28°C (unpaired t-test;  $p < 0.05$ ;  $n = 9$  fish at each temperature).



**Fig. S4.** Survival of anoxia. Fish acclimated to 33°C were exposed to water  $\pm$  2% ethanol and bubbled with N<sub>2</sub>. Fish exposed to ethanol are presumed to have entered paradoxical anaerobism based on the results of Table 1. Nominal anoxia was confirmed using an oxygen electrode. Fish exposed to ethanol survive 35.6% longer in N<sub>2</sub> induced anoxia (unpaired t-test;  $p < 0.05$ ;  $n = 10$  per group).