Acidosis Maintains the Function of Brain Mitochondria in Hypoxia-Tolerant Triplefin Fish: A Strategy to Survive Acute Hypoxic Exposure?

Jules B. L. Devaux1*, Christopher P. Hedges1, Nigel Birch1, Neill Herbert2, Gillian M. C. Renshaw3 and Anthony J. R. Hickey1

1 School of Biological Sciences, The University of Auckland, Auckland, New Zealand, 2 Institute of Marine Science, The University Auckland, Auckland, New Zealand, 3 School of Allied Health Sciences, Griffith University, Gold Coast, QLD, Australia

The vertebrate brain is generally very sensitive to acidosis, so a hypoxia-induced decrease in pH is likely to have an effect on brain mitochondria (mt). Mitochondrial respiration (JO2) is required to generate an electrical gradient (∆Ψm) and a pH gradient to power ATP synthesis, yet the impact of pH modulation on brain mt function remains largely unexplored. As intertidal fishes within rock pools routinely experience hypoxia and reoxygenation, they would most likely experience changes in cellular pH. We hence compared four New Zealand triplefin fish species ranging from intertidal hypoxia-tolerant species (HTS) to subtidal hypoxia-sensitive species (HSS). We predicted that HTS would tolerate acidosis better than HSS in terms of sustaining mt structure and function. Using respirometers coupled to fluorimeters and pH electrodes, we titrated lactic-acid to decrease the pH of the media, and simultaneously recorded JO2, ∆Ψm, and H+ buffering capacities within permeabilized brain and swelling of mt isolated from non-permeabilized brains. We then measured ATP synthesis rates in the most HTS (Bellapiscus medius) and the HSS (Forsterygion varium) at pH 7.25 and 6.65. Mitochondria from HTS brain did have greater H+ buffering capacities than HSS mt (∼10 mU pH.mgprotein−1). HTS mt swelled by 40% when exposed to a decrease of 1.5 pH units, and JO2 was depressed by up to 15% in HTS. However, HTS were able to maintain ∆Ψm near −120 mV. Estimates of work, in terms of charges moved across the mt inner-membrane, suggested that with acidosis, HTS mt may in part harness extra-mt H+ to maintain ∆Ψm, and could therefore support ATP production. This was confirmed with elevated ATP synthesis rates and enhanced P:O ratios at pH 6.65 relative to pH 7.25. In contrast, mt volumes and ∆Ψm decreased downward pH 6.9 in HSS mt and paradoxically, JO2 increased (∼25%) but ATP synthesis and P:O ratios were depressed at pH 6.65. This indicates a loss of coupling in the HSS with acidosis. Overall, the mt of these intertidal fish have adaptations that enhance ATP synthesis efficiency under acidic conditions such as those that occur in hypoxic or reoxygenated brain.

Keywords: pH, hypoxia tolerance, mitochondria, lactate, acidosis, brain
INTRODUCTION

In hypoxic or anoxic conditions, $O_2$ becomes limiting and ATP production via mitochondrial (mt) oxidative phosphorylation (OXPHOS) is compromised. To support ATP requirements, vertebrate cells increase anaerobic metabolism activities, which is ∼15-fold less efficient than the OXPHOS. If hypoxia is sustained, glycolysis may become substrate limited, and diminishing ATP production mediates rapid depletion of ATP stores (Pamenter, 2014). ATP hydrolysis mediates proton (H$^+$) release (Wilson, 1988) alongside the accumulation of metabolic end-products (Azarias et al., 2011), which contributes to metabolic acidosis (Robergs et al., 2004). Although lactate is possibly oxidized by neurons (Quistorff et al., 2008; Gallagher et al., 2009; Barros, 2013; Riske et al., 2017) this requires oxygen, and lactate accumulation contributes to intracellular acidosis (reviewed in Kraut and Madias, 2014). In the ischemic brain, up to 60% of glucose can be metabolized to lactate (Teixeira et al., 2008; Dielen, 2012), which the accumulation of has been shown to associate with hypercapria and acidosis (Rehncrona, 1985a; Katsura et al., 1992b).

Acidosis alters mt respiration in ischemic mammalian brain (Hillered et al., 1984), enhances brain lipid peroxidation in vitro (Siesjo et al., 1985) and denatures proteins (Kraig and Wagner, 1987). Low pH (<6.8) also inhibits the hydrolytic role of FOF1-ATP synthase in isolated myelin vesicles (Ravera et al., 2009), and acidosis generally promotes irreversible cellular damage (Rehncrona, 1985a;b; Rehncrona and Kagstrom, 1983). In most vertebrates, acidosis occurs rapidly and compromises brain function within minutes of anoxia (Katsura et al., 1991). Hypoxia tolerant species (HTS) however, routinely survive hypoxic or anoxic environments for several hours to months, which make these animals useful model systems to explore adaptations against hypoxic damage.

Adult vertebrates such as the carp (Carassius carassius), its cousin goldfish (C. auratus) and the freshwater turtle (Chrysemys picta) have strategies that decrease lactate-mediated acidosis (Jackson, 2004; Vornanen et al., 2009). Among mammalian hibernators, the artic ground squirrel (Spermophilus parryii) suffers little damage from ischemia while torpid at body temperatures as low as −3°C (Barnes, 1989; Ma et al., 2005). Independent of hibernation cycle, normothermic brain slices of the ground squirrel tolerate $O_2$, ATP and glucose deprivation (Bhwomick et al., 2017). However, determining how such adaptations commensurate with physiological stressors associated with hypoxia. As mt respiration $(O_2)$ regulates the mt membrane potential ($ΔΨ_m$) and maintains a pH gradient (Mitchell, 2011), we tested the influence of lactate mediated acidosis on brain mt of triplefin fish, and predicted that mt of HTS would maintain function at lower pH compared to HSS.

MATERIALS AND METHODS

Animal Sampling and Housing

Adult specimens of four triplefin species (5–10 cm) were collected from different sites around the greater Auckland region using hand nets and/or minnow traps. Adult B. medius were caught from high rock-pools at low tide, F. lapillum and F. capito from rock-pools and off piers, and F. varium at 5–10 m depth. Individuals were maintained in 30 L tanks (20 fish per tank) in recirculating aerated seawater and were fed with a standard mixture of shrimps and green-lipped mussels every 2 days for a 2 weeks acclimation period prior to experiments procedure at 20 ± 1°C. All capture, housing and experimental procedures were performed with under the approval from the University of Auckland Ethic Committee (Approval R001551).

Brain Preparation and Tissue Permeabilization

Fish were euthanized by section of the spinal cord at the skull. The brain was immediately removed and placed in a modified ice-cold biopsy buffer containing (in mM from hereon unless stated) 2.77 $Ca_2$EGTA, 7.23 $K_2$EGTA, 5.77 $Na_2$ATP, 6.56 $MgCl_2$, $6H_2$O, 20 taurine, 15 $Na_2$-phosphocreatine, 20 imidazole, 0.5 DTT, 50 KMES, 50 sucrose, pH 7.1 at 30°C (Gnaiger et al., 2000). Cellular

**Abbreviations:** $ΔΨ_m$, mitochondrial membrane potential; BLac, buffered lactate; $C_{add}$, additional positive charges; ETS, electron transport system; HSS, hypoxia-sensitive species; HTS, hypoxia-tolerant species; $O_2$, mitochondrial respiration rate; LEAK, leak state respiration; Mt, mitochondria; OXPHOS, oxidative phosphorylation state respiration; ULac, unbuffered lactate; Vol$_{int}$, mitochondrial volume.
permeabilization was undertaken by the addition of 50 µg ml⁻¹ freshly prepared saponin and 30 min of gentle agitation within cell culture plastic plates held on ice. The permeabilized tissue was then removed and washed three times for 10 min ice-cold modified MiR05 respiration medium (Kuznetsov et al., 2000) containing 0.5 EGTA, 3.0 MgCl₂, 6.0 H₂O, 60 K-lactobionate, 20 taurine, 10 KH₂PO₄, 2.5 HEPES, 30 MES, 160 sucrose, 1 g l⁻¹ BSA, pH 7.1 at 30°C. Brain tissues were then split longitudinally into two halves, blotted dry on filter paper, and weighed before loading into respirometers.

Mitochondrial Isolation From Minimal Fish Brain Tissues

A miniaturized mt isolation was required to assess the mt swelling. Tripletin brain masses varied from only ~8–30 mg of tissue, hence the whole triplefin brain was required for mt isolation. The brain was first removed from the skull and gently homogenized in 1 ml cold MiR05 by expulsion and suction through a modified 1 ml syringe with decreasing gauge needles (16–25 gauge). Mitochondrial integrities were better preserved with this method compared to other standard homogenization methods and the small sample was also better retained (personal observation). The homogenate (600 µl) was centrifuged at 300 × g for 5 min at 4°C, the supernatant, which contained suspended mt was collected and spun at 11,000 × g for 10 min. The supernatant and the white lipid ring surrounding the brown mt rich pellet was discarded prior to the addition of 500 µl cold MiR05. The last step was repeated twice and isolated mt were then re-suspended in 50 µl ice-cold MiR05 and were held on ice for 1 h to permit recovery before respirometry assays. Post respirometry assays, the medium containing the mt was removed from the chambers and stored at −80°C for the determination of protein concentration. Prior to the protein assay, samples were slowly defrosted at 4°C and the protein concentration was determined with the Pierce™ BCA Protein Assay Kit as specified by the manufacturer, against a BSA standard and modified MiR05 control.

Acidification Protocol Optimization

To explore the influence of pH on mt function we chose to titrate lactic acid into a modified buffer to induce acidosis. Although acidosis results from ATP hydrolysis, other glycolytic and TCA intermediates and CO₂ (Roos and Boron, 1981), and whether lactate ionizes to an acid in vivo is contentious (Robergs et al., 2004), ULac provides an organic acid without inorganic ions [such as Cl⁻ if HCl were to be used (Selivanov et al., 2008)]. Given the high buffering capacity of typical respiratory media (MiR05) (Gnaiger et al., 2000), we decreased the pH buffering to mimic the pH changes expected in hypoxic brain, which may decrease to an extracellular pH of 6.3 in ischemic brain of non-hyperglycemic vertebrate brain (Katsura et al., 1991, 1992a; Kraut and Madias, 2014) and of ~6 in vivo, at least in hyperglycemic mammals (Katsura et al., 1991, 1992a). Assuming parallel changes in pH, these changes should equate to intracellular pH ~5.6–5.5. Therefore, we decreased the HEPES concentration to 2.5 mM and used 30 mM MES to buffer at low pH. With this buffer system, ULac/or BLac (pH ~7 adjusted with KOH) was titrated to cover physiological concentrations (Jokivarsi et al., 2007; Witt et al., 2017), and the pH changes mediated by ULac covered those that occur within vertebrate ischemic brain with a decrease 0.048 pH units per mM ULac. The media (or extra-mt pH) was recorded simultaneously with respiration and mt membrane potential (ΔΨm) using a solid state ISFET electrode (IQ Scientific Instruments) connected to the pX port of the O2k and calibrated using a three-point calibration (pH 4, 7, and 10) prior to experiments and allowed a ±0.001 pH U sensitivity. Measurements were performed on permeabilized brain and isolated mt, and pH buffering capacities were calculated relative to no-sample controls. As lactate is oxidized by LDH to produce NADH⁺ and pyruvate, which is further oxidized by mt for oxidative phosphorylation (OXPHOS) (Halestrap, 1975), it likely alters JO₂, ΔΨm, and pH in the presence of other respiratory substrates. Therefore, the influence of pH changes mediated by ULac was made relative to BLac controls. A representative trace of the protocol is displayed in Figure 1.

Respirometry

Between 5–12 mg of tissue was placed in respirometer chambers (Oroboros Instrument, Innsbruck, Austria) containing 2 ml (or 3.4 ml when extra-mt pH measured to accommodate the electrode) oxygen saturated modified-MiR05 (O₂ concentration = 290 µM at 20°C and 101.5 kPa barometric pressure). Two substrate uncoupler inhibitor titration protocols were performed. The first assay informed on the lactate-mediated respiration and consisted in the sequential addition of NAD⁺ (75 µM) and BLac (30 mM, pH 7.25) to initiate a leak state measurement (LEAK), followed by the addition of ADP (700 µM) to initiate OXPHOS. The second assay was designed to assess the effect of pH on mt at OXPHOS state and consisted of the subsequent addition of the NADH₂-generating substrates pyruvate (10 mM) malate (5 mM) and glutamate (10 mM) to initiate LEAK. OXPHOS supported by CI was then commenced by the addition of ADP (700 µM). The subsequent addition of 10 mM succinate activated parallel inputs from CI and CI1 to OXPHOS. NAD⁺ (75 µM) was added to the media to avoid cytosolic limitations (i.e., LDH and malate aspartate shuttle, discussed in Kane, 2014). BLac or ULac was then titrated to a 30 mM final concentration. Then, the FO/F₁-ATP synthase inhibitor oligomycin (2.5 µl) was added to place mt into artificial LEAK state. Subsequently, respiration was uncoupled from OXPHOS using three injections of the protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP, 0.5 µM each) to determine the maximal ETS capacity. O₂ concentration was maintained above 100 µM to avoid diffusion limitation. The protocol was applied on both isolated mt and permeabilized brain, however, only respiration and ΔΨm data from permeabilized brains are presented in the main manuscript (please refer to Supplementary Figures for isolated mt respiration data). The differences due to changes in pH within the same permeabilized brain in term of JO₂, ΔΨm and mt matrix volume (Volmt) were
Mitochondrial Volume (Vol\textsubscript{mt}) Dynamics

Mitochondria are dynamic organelles and shrink or swell when exposed to variable conditions (Cereghetti and Scorrano, 2006; Castella et al., 2011; Friedman and Nunnari, 2014). Changes in \textit{mt} volume (shrinkage or swelling) of isolated \textit{mt} were measured by following changes in absorption at 525 nm, which changes proportionally to the volume of the \textit{mt} matrix (Beavis et al., 1985; Garlid and Beavis, 1985; Das et al., 2003). Fluorescence sensors (green LED, 525 nm) were used without an emission filter, to follow the light absorption within the O2k. The voltages were recorded simultaneously with JO\textsubscript{19} and pH measurements on permeabilized brain and isolated \textit{mt}.

\begin{equation}
A_\lambda = -\log_{10}\left(\frac{I - D}{R - D}\right)/W
\end{equation}

Where \(A_\lambda\) corresponds to the total absorbance, \(I\) to the sample intensity, \(D\) to the dark intensity, \(R\) to the reference intensity and \(W\) to the amount of protein in mg. Changes in absorbance \(\Delta A_\lambda\) were normalized by \(A_\lambda\) in the OXPHOS state to account for the effect of pH on phosphorylating \textit{mt} only and where \textit{mt} shrinkage and swelling occurs when \(\Delta A_\lambda < 1\) and \(\Delta A_\lambda > 1\), respectively.

**\(\Delta \Psi m\) Measurement and Calculation**

Safranin-O was used to estimate \(\Delta \Psi m\), simultaneously with JO\textsubscript{2} and pH measurements on permeabilized brain and isolated \textit{mt}.

\begin{equation}
\Delta \psi m = 2.3026 \times \frac{RT}{zF} \times \log_{10}\left(\frac{[\text{Safr}]_{\text{out}}}{[\text{Safr}]_{\text{in}}}\right)
\end{equation}

Where \(R\) is the gas constant, \(T\) is the temperature in Kelvin, \(z\) the valence state of the ion (+1) and \(F\) the Faraday constant. While the safranin concentration outside the \textit{mt} \([\text{Safr}]_{\text{out}}\) corresponds to the calibrated fluorescent signal directly obtained from DatLab7, the safranin concentration in the \textit{mt} matrix \([\text{Safr}]_{\text{in}}\) is dependent on the Vol\textsubscript{mt}, back-calculated from OXPHOS state, which was assumed to be at \(-120\) mV (Huttemann et al., 2008; Perry et al., 2011). As \textit{mt} shape is dynamic (Fujii et al., 2004; Castella et al., 2011), the Vol\textsubscript{mt} was readdressed at each state following the estimation and the integration of the \(\Delta \Psi m\) swelling.
or shrinkage ($\Delta A_k$):

$$\text{Vol}_{\text{mt}} \left( \mu l \ mg^{-1} \right) = \frac{Safr \times 10^{-120}}{[\text{Safr}]_{\text{out}} \times W} \times 10^6 \times \Delta A_k$$

(3)

Where $Cst$ corresponds to $2.303 \times \frac{RT}{F}$ (58.17 mV) under our conditions, $Safr$ the amount of safranin in $mt$ in $\mu$mol, $[\text{Safr}]_{\text{out}}$ the recorded safranin concentration in $\mu$M, $W$ the amount of tissue in mg and $\Delta A_k$, the change in Vol$_{\text{mt}}$ (described above).

**Estimation of Mitochondrial Work to Maintain $\Delta \Psi_m$ With Additional External Charges**

We further estimated the energy required by $mt$ to maintain $\Delta \Psi_m$ as this summarizes the combined effects of $\text{JO}_2$, $\Delta \Psi_m$, and acidosis. The total energy of a closed system can be determined by:

$$J = C \cdot V$$

(4)

Where $J$ is the derived unit of energy transferred to an amount of work in Joules, $C$ is electric charge in Coulombs and $V$ is the electric potential in Volts. A Joule is the work required to move an electric charge of $1\text{C}$ against an electric potential of $1\text{V}$.

To better represent the effect $C_{\text{add}}$ at a given pH onto charges flux within $mt$, $J_{\text{mt}}$ was normalized by $C_{\text{add}}$ at corresponding pH.

**ATP Production**

ATP production was assessed based on previous work (Chinopoulos et al., 2009; Pham et al., 2014; Masson et al., 2017) in permeabilized brain of the most HTS $B. \text{medius}$ and the HSS $F. \text{varium}$. Due to the variable properties of the fluorescent dye Magnesium Green™ (Thermo Fisher Scientific, United States) to acidosis, we performed an experiment to assess ATP production at pH 7.25 and pH 6.65, which appears to be the pH at which $mt$ function is preserved in HTS but altered in the HSS (Figure 4). In separated experiments, O2k chambers were filled with modified MiR05 and mitochondrial substrates (pyruvate, malate, glutamate, and succinate) at concentrations described above. This was supplemented with 10 mM BLac or ULac to set the medium pH at 7.25 or 6.65, respectively. Magnesium Green™ (5 $\mu$M) was then added and MgCl$_2$ was titrated to calibrate the Mg$^{2+}$ free in the chamber. The equivalent of one permeabilized brain hemisphere was then added to the chamber and let to recover for around 15 min. Then, ADP was titrated (3 $\times$ 0.5 $\mu$M) to calibrate the Mg$^{2+}$ free signal to [ADP]. After 15 min, antimycin A (2.5 $\mu$M) was added to block proton pumping by the ETS. Once the $\text{JO}_2$ signal was stable (>20 min), oligomycin (10 nM) was added to block mitochondrial ATP synthesis and measure ATP hydrolysis. Data was exported into Excel and the rate of ATP was calculated as:

$$J_{\text{ATP}} = \frac{[\text{ADP}]_{t} - [\text{ADP}]_{t-1}}{t-t-1} \times Cst$$

(9)

Where $[\text{ADP}]_{t}$ corresponds to the concentration of ADP at the end of a $mt$ state and $[\text{ADP}]_{t-1}$ the ADP concentration at the start of the $mt$ state and $t$ and $t-1$ the time at which corresponding [ADP] were taken. Cst corresponds to the relative difference between $K_{\text{DADP}}$ and $K_{\text{DATP}}$ extracted from Chinopoulos et al. (2009). Then ATP consumption rate determined after the addition of oligomycin ($J_{\text{ATP}} < 0$), was subtracted to the other state to determine the net ATP production rate ($J_{\text{ATP}} > 0$) in pmol s$^{-1}$ mg$^{-1}$. The PO ratio was calculated as:

$$\text{PO} = \frac{J_{\text{ATP}}}{\text{JO}_2}$$

(10)

**Data and Statistical Analysis**

Respirometry and simultaneous spectrometry data were extracted from DatLab 7.0 software. All data were copied and processed in Excel © 2016. GraphPad Prism v7 was used to perform two-way ANOVA to test for the effect of pH between species and the $mt$ parameters ($\text{JO}_2$, $\Delta \Psi_m$, Vol$_{\text{mt}}$, and ATP) within $mt$ states. Two-way ANOVA repeated-measures were performed to analyze the effect of BLac, ULac, and associated pH titrations on the $mt$ parameters between the species of fish. Post hoc tests using Turkey’s correction were used for pairwise comparisons and a $P$-value of 0.05 was chosen to represent statistical difference.
RESULTS

Mitochondrial pH Buffering Capacities
Permeabilized brain of *B. medius* had a 4.5–6% (∼0.05 pH-unit mg⁻¹) greater buffering capacities than the HSS *F. varium* (P = 0.006; Table 1). Brain tissue pH buffering capacities of *F. lapillum* were similar to those of the more HTS *B. medius*, while mt of *F. capito* had similar buffering to *F. varium*. In isolated mt, however, only *F. varium* differed, with a lower pH buffering capacity (P < 0.04), indicating most, but not all, of the buffering results from non-mt components. Estimated contributions of mt to brain buffering capacities approach ∼20% in HTS and 10% in HSS brain.

Overall Mitochondrial Oxygen Flux and pH Effects on the Mitochondrial Function
In phosphorylating mt (presence of sufficient mt substrates and ADP), Blac addition did not alter OXPHOS JO₂ in any species (F10,60 = 0.381, P = 0.95; Figure 2A). However, in the absence of other mt substrates, lactate mediated JO₂ in the HTS mt > 22% more than in *F. varium* mt (P < 0.03; Figure 2B) and *F. lapillum* JO₂ was also 30% greater than *B. medius* (P < 0.01).

The JO₂ differed among species for permeabilized brain and isolated mt held at a physiological pH (species effect F3,48 = 15.8 and F3,72 = 10.5, respectively, P < 0.01; Figure 2). While the LEAK JO₂ in permeabilized brain was similar among species (Figure 2C), OXPHOS and ETS JO₂ were greater in *B. medius* and *F. capito* relative to *F. varium* (P < 0.05). No difference was observed between *F. lapillum* and *F. capito*, which JO₂ at both OXPHOS and ETS states, sat between JO₂ of the two other species. All species had greater ETS fluxes than OXPHOS fluxes (P < 0.02) indicating some limitation of the OXPHOS system relative to the ETS, and this was further observed in isolated mt (Figure 2D).

In the presence of 30 mM ULac, which mediated a decrease of pH to 5.75, JO₂ was similar between species and across all states in permeabilized brain (P = 0.26; Figure 2C). However, while LEAK was increased by ∼50% with ULac relative to BLac (F1,6 = 47.4, P < 0.001), ETS was significantly decreased by ∼50% (P1,6 = 139, P < 0.001). In OXPHOS, there was interaction between species and pH (F3,18 = 4.35, P = 0.02), mediated by a significant decrease in JO₂ in *B. medius* (P = 0.02) only. In isolated mt (Figure 2D), acidosis did not affect *F. capito*, but decreased OXPHOS and ETS by > 50% in all other species (P < 0.05). OXPHOS was 50% lower and ETS was 65% lower in *F. capito* than in *F. varium* mt (P = 0.05 and 0.009, respectively).

We then assessed the effect of graded acidosis on OXPHOS, which overall, mediated a contrasting response between the HTS and the HSS (main effect of species F3,18 = 4.42, P = 0.02; Figure 3). First, JO₂ was gradually decreased in HTS until around pH 6.4 to ∼18% relative to OXPHOS_initial (at pH 7.25, Figure 3A). Below pH 6.4, the response was more variable in the *Forstergyrum* genus, whereas JO₂ was more stable in *B. medius*. In *F. varium*, however, JO₂ increased by 12% and was more variable below pH 6.9. JO₂ was significantly different between *B. medius* and *F. varium* below pH 7 (P < 0.01).

Membrane Potential and Changes in Mitochondrial Volume (Volmt)
The Volmt in HTS was 30% higher than *F. varium* in OXPHOS (P < 0.04, Table 1). Below pH 6.9, acidosis mediated swelling in HTS mt, and decreased Volmt in *F. varium* mt (interaction between species and pH of F30,120 = 3.59, P < 0.001; Figure 3B). While the Volmt increased to around 50% in *F. capito* and *F. lapillum*, it decreased by 12% in *F. varium*, relative to Volmt at pH 7.25 (P < 0.04). Differences between *B. medius* and *F. varium* were significant below pH 6.6 (P < 0.03).

Using estimates of Volmt dynamics with pH changes, we incorporated Volmt into the Nerst equation to better derive the ∆Ψm relative to pH (Supplementary Figure S2). Two-way ANOVA revealed an interaction between ∆Ψm, species and pH (F30,180 = 3.71, P < 0.001, Figure 3C). While the main effect of pH was significant for all species (F3,18 = 9.39, P = 0.001), only ∆Ψm at pH 5.75 (around −100 mV) differed from its original value at pH 7.25 (−120 mV) in the HTS. In contrast, ∆Ψm in *F. varium* mt gradually decreased down to around −50 mV at pH 5.75 and was significantly lower than ∆Ψm in *B. medius* mt (P < 0.05 from pH 7). For comparison, a ∆Ψm of −110 mV was reached at pH 7 in *F. varium* and 6.12–6 in the HTS.

### TABLE 1 | pH buffering capacities, mitochondrial volume and efficiency of permeabilized tissue and isolated mt from triplefin brain.

|                  | pH buffering | Volmt (nl mg⁻¹) | RCR         |
|------------------|--------------|----------------|-------------|
|                  | Permeabilized (pH-unit mgbrain⁻¹) | Isolated mt (pH-unit mgprotein⁻¹) | OXPHOS (at −120 mV) | Permeabilized | Isolated mt |
| *B. medius*      | 0.940 ± 0.007 cv | 0.193 ± 0.059 v | 2.06 ± 0.24 v | 2.66 ± 0.25 v | 6.64 ± 1.64 v |
| *F. lapillum*    | 0.929 ± 0.011 r | 0.169 ± 0.072 t | 2.09 ± 0.25 v | 3.38 ± 0.51 v | 3.17 ± 1.29 v |
| *F. capito*     | 0.892 ± 0.005 m | 0.177 ± 0.028 v | 2.14 ± 0.18 v | 2.42 ± 0.10 v | 1.79 ± 0.10 v |
| *F. varium*     | 0.889 ± 0.008 m | 0.089 ± 0.062 mc | 1.53 ± 0.21 mc | 1.94 ± 0.15 v | 6.74 ± 0.73 v |

Buffering capacities were calculated relative to controls (no sample) in permeabilized brain and isolated mt at OXPHOS state exposed tograded acidosis, mediated by acid lactic titration (0–30 mM, 7.24–5.73 pH equivalent). Results expressed as mean (n = 5) ± SEM. The mt volume (Volmt) was back-calculated from the membrane potential signal as per described in the “Materials and Methods” section, with the assumption that in phosphorylating mt, the membrane potential equates −120 mV. Data expressed as mean of n = 7 ± SEM. Respiratory control ratio (RCR) in both permeabilized and isolated mt and relates to coupling efficiencies. Data expressed as mean of 7 ± SEM. Significant difference at P < 0.05 from designated species between *B. medius*, *F. lapillum*, *F. capito* or *F. varium* indicated as m, l, c and v, respectively. The difference between sample preparation states was chosen at P < 0.05 and indicated as # (all tested with two-way ANOVA with Turkey correction).
Energy Attributed to Sustaining ΔΨm

Combined JO2, ΔΨm, and extra-<i>mt</i> pH data allowed an estimate of the work by <i>mt</i> to develop ΔΨm in OXPHOS state in regard to additional positive charges mediated by addition of acid ("C<sub>add</sub>\text{add}," additional H<sup>+</sup>) (Figure 4). Brain <i>mt</i> from <i>F. varium</i> showed graded increase in work with C<sub>add</sub>, up to ~50 µJ s<sup>−1</sup> mg<sup>−1</sup> at 100 µC charges (pH 6.6 equivalent, Figure 4A). In contrast, the remaining species showed a graded decrease in work down to ~50 µJ s<sup>−1</sup> mg<sup>−1</sup> at ~25 µC (pH 7). We then normalized the <i>mt</i> work by C<sub>add</sub>, which represents the use of C<sub>add</sub> against or used to develop ΔΨm (Figure 4B). While internal work was maximized in <i>F. varium</i> with the first decrease in pH (from 7.25 to 7.12), C<sub>add</sub> were fully used by <i>B. medius</i> to develop ΔΨm (P = 0.02). Between pH 7.12–6.4, internal work was further decreased in <i>B. medius</i> (P < 0.04), although increased for <i>F. varium</i> (P < 0.04). C<sub>add</sub> was appeared to be utilized by <i>F. lapillum</i> and <i>F. capito</i> <i>mt</i> between pH
Devaux et al. Acidosis Maintains Hypoxia-Tolerant Mitochondria

7.05–6.6 and pH 6.9–6.6, respectively (P < 0.05). Progressively, mt work (positive in F. varium and negative in HTS) returned to near zero values at approximately pH 6.

ATP Production
Two-way ANOVA revealed an interaction between species and acidosis (F<sub>1,10</sub> = 9.01; P = 0.01; FIGURE 5B) as well as a difference between the two species (F<sub>1,10</sub> = 7.8; P = 0.02). While ATP production is suppressed with acidosis in *F. varium*, it is increased by ~3.6-fold in *B. medius*. With a simultaneous decrease in JO<sub>2</sub> (FIGURE 5A), this significantly increases the P:O ratio in *B. medius* from 1.14 ± 0.36 at pH 7.25 to 4.95 ± 1.67 at pH 6.65 (P = 0.032; FIGURE 5C). For *F. varium*, at pH 6.65 the JO<sub>2</sub> trended higher (P = 0.06), while the P:O ratio decreased from 1.56 ± 0.65 to −0.59 ± 0.43 (P < 0.05).

DISCUSSION
In the present study, we assessed the mt function across a range of pH down to those experienced by hypoxic brain (Katsura et al., 1991, 1992a; Kraut and Madias, 2014; Witt et al., 2017). It is the first study to explore these effects through pH titration and on a range of species with different tolerance to hypoxia, and it revealed significant differences among species that are consistent with species distribution. This differs from studies that test mt function with a large variation of pH, as here we titrated unbuffered lactic acid sequentially to modulate pH in order to mimic in vivo lactate accumulation and associated pH changes. This includes the progressive nature of pH changes and the duration of changes. Here, we were able to follow mt respiration, ΔΨmt and pH or JO<sub>2</sub>, Volmt and pH simultaneously. However, as lactate may modulate the mt function and is a possible substrate of neurons (Philp et al., 2005; Chen et al., 2016; Caruso et al., 2017), we divided tissues from single brains to provide a control and reference for comparisons between buffered and unbuffered samples. We show that while intertidal HTS suppress JO<sub>2</sub> they preserve ΔΨmt and ATP production as pH declines. However, the ΔΨmt decreased with the suppression of ATP synthesis despite JO<sub>2</sub> increases in the subtidal species *F. varium*. We contend that with decreasing pH the HTS mt may harness the H<sup>+</sup> accumulation to maintain ΔΨmt and ATP synthesis rates.

Lactate Management of Triplefin Brain mt
In the brain, the lactate anion is a putative substrate for aerobic metabolism (reviewed in Barros, 2013; Kane, 2014). While lactate had little influence on JO<sub>2</sub> for any species in the presence of other mt substrates (FIGURE 2A), lactate and NAD<sup>+</sup> could sustain OXPHOS at higher rates in the HTS than in *F. varium* (FIGURE 2B). This indicates a greater capacity for lactate oxidation in HTS, which is likely advantageous post-hypoxia (Dienel, 2012).
pH Buffering Capacities in the Brain of Triplefin Fish

Under acidosis, cells rely on bicarbonate and non-bicarbonate buffering capacities (Roos and Boron, 1981), and despite the low apparent pH buffering capacity of mt (Poburko et al., 2011), mt may play a role in the pH regulation when required. We measured the overall buffering capacities of permeabilized brain and isolated mt. In both preparations, HSS brains displayed a lower buffering capacity than HTS (Table 1). Although permeabilized tissue buffering differed only marginally (~5%) across species, isolated mt buffering differed by ~2-fold between B. medius and F. varium. The mt pH buffering contributes to approximately 17% of the cell pH buffering capacity, and while this suggests that some cytosolic components may remain following the permeabilization process, which may significantly buffer pH changes within cells, mt also contribute to some pH buffering and protects the mt as well and this varies in accordance with hypoxia tolerance.

Comparison of Volmt also revealed that mt from HTS exposed to acidosis swelled by 45% of their initial volume at pH 7.25 (Figure 3). This dilutes matrix solutes, which includes enzymes, substrates and ions including H\(^+\). A 1.4-fold swelling of the matrix (observed in F. lapillum) should mediate an alkalization of approximately 0.15 pH units, preventing excess acidification of the cells. Although the mt integrity was similar (RCRs) across preparations (Table 1).

**Acidosis Mediates Contrasted Responses in the Brain mt of Hypoxia-Sensitive and Hypoxia-Tolerant Species**

Notably in mammalian models, intracellular acidosis has generally been shown to impact mt function with some loss of ΔΨm (Tiefenthaler et al., 2001; Bento et al., 2007) and a partial decrease in JO2 (Hillered et al., 1984), putatively through CII inhibition (Lemarie et al., 2011). We observed a significant elevation of JO2 coincided with a decrease in ΔΨm in F. varium (Figure 3). This indicates a loss of OXPHOS efficiency as pH decreases, thereby decreasing ATP synthesis alongside an elevated O2 turnover. Moreover, with a substantial drop in ΔΨm, i.e., below ~110 mV the ATPFp–F1 can reverse and act as a hydrolase, thereby elevating ATP consumption (Chinopoulos et al., 2010).

In contrast, mt of the remaining HTS decreased JO2 which would appear to be deleterious for OXPHOS (Hillered et al., 1984). However, with these species maintained ΔΨm to moderately low pH (Figure 3). The lesser O2 utilization for ΔΨm maintenance suggests that there is either a decrease in proton leak and/or the [H\(^+\)] increase in the media (or cytosol) diffuses into the IMS, may contribute to maintaining the ΔΨm. Notably the permeability of the mt outer membrane is high (Cooper, 2000).

**Mitochondria of Hypoxia Tolerant Species May Harness the Extra-mt Protons to Maintain Function**

The ΔΨm represents the repartition of charge, between the mt matrix and the IMS and drives, in part, ATP production (Mitchell, 1961). ΔΨm dissipates with H\(^+\) transfer into the matrix through three “negative fluxes”; (1) Constitutive leak (not

---

**TABLE 1**

| Species | Brain | mt |
|---------|-------|----|
| HSS     | 0.15  | 0.05 |
| HTS     | 0.18  | 0.12 |

Frontiers in Physiology | www.frontiersin.org 9 January 2019 | Volume 9 | Article 1941
Acidosis Maintains Hypoxia-Tolerant Mitochondria

Devaux et al. Acidosis Maintains Hypoxia-Tolerant Mitochondria

FIGURE 5 | Acidosis enhances ATP production in brain mitochondria of the intertidal species. Permeabilized brain hemispheres were placed in respiratory chamber with mitochondrial substrates (described in the "Materials and Methods" section) and 10 mM buffered lactate or unbuffered lactic acid to set the respiratory medium pH at 7.25 or 6.65, respectively. Mitochondrial respiration (\( \text{JO}_2 \)) was measured simultaneously with ATP production (B) using the fluorescent probe Mg-Green\textsuperscript{TM}. ATP consumption was measured with the addition of saturated antimycin A and oligomycin to block ATP production. P:O ratios were then calculated as ATP produced per oxygen element consumed (C). Data presented as scattered plot of six individuals and mean ± SEM. Statistical difference presented as * for \( P < 0.05 \), tested with two-way ANOVA followed by Turkey’s post hoc test.

regulated) results from the basal proton diffusion across the inner \( mt \) membrane; (Jastroch et al., 2010; Divakaruni and Brand, 2011); (2) Inducible leak (regulated), resulting from the proton exchange through proteins (UCPs, ANT, NXHs) (Bernardi, 1999; Halestrap, 2009) that can be modulated by ROS, fatty acids and GDP (Stuart et al., 1999; Jastroch et al., 2010; Masson et al., 2017); and (3) the proton flux used to produce ATP by the F0-F1ATP synthase (Mitchell, 2011). These all act in opposition to the proton flux transferred by the ETS (Mitchell, 2011), or if some of the F0-F1ATP synthases have reversed (Chinopoulos et al., 2010). Work by the ETS (i.e., the positive transfer of \( H^+ \)) can be estimated by \( O_2 \) consumption rates and balances these negative fluxes to sustain the \( \Delta \Psi_m \). With physiological intracellular pH, the proton gradient (and therefore \( \Delta \Psi_m \)) that drives ATP production is maintained by the work performed by the ETS.

In 1966, André Jagendorf manipulated the extra-thylakoid pH of chloroplast vesicles to drive ATP synthesis in the dark (Jagendorf and Uribe, 1966), confirming Mitchell’s chemiosmotic hypothesis (Mitchell, 1961) and indicating that ATP synthesis can be mediated with the manipulation of pH changes not generated by the ETS. Here, we sought to assess whereas pH modulation would partially assist \( \Delta \Psi_m \) and ATP synthesis and if this would influence the work performed by the ETS (illustrated in Figure 6A).

Modulation of the cytosolic pH (i.e., medium pH in permeabilized tissue) mediated an increase of the work performed by the ETS in \( mt \) of HSS brains (Figure 4), associated with a decrease in \( \Delta \Psi_m \) (Figure 3C) and ATP synthesis (Figure 5B). With the dogma that acidosis as detrimental to cellular functions, a deleterious effect of acidosis was somewhat expected. However, in brain \( mt \) of HTS, \( \Delta \Psi_m \) was maintained despite a decrease in ETS work and ATP production was further increased. This was associated with an elevation of P:O ratios that exceeded 2.7. This indicates that extra-\( mt \) \( H^+ \) may participate in the proton motive force in phosphorylating \( mt \) (illustrated in Figure 6B). Such findings are in concordance with a recent study, which showed that in mammalian cortical neurons, mild acidosis (pH 6.5) mediates \( mt \) remodeling and helps to sustain ATP production regardless of \( O_2 \) levels (Khacho et al., 2014).

CONCLUSION

With the increase in glycolytic flux and overall increase in ATP hydrolysis, acidosis mediated by hypoxia generally impairs \( mt \) function of most vertebrates. In this study, we demonstrate that brain \( mt \) of hypoxia-tolerant intertidal fish species buffer \( H^+ \) better than the subtidal species \( F. varium \). In their natural habitat, as \( O_2 \) dwindles during nocturnal low-tides, intertidal triplefins may turn the problem of acidosis into a temporary solution, and means to sustain ATP synthesis. As opposed to the subtidal \( F. varium \), intertidal hypoxia tolerant triplefins appear to take advantage of extra-\( mt \) protons that helps for maintenance of \( \Delta \Psi_m \) and ATP production. Acidosis also partially depresses proton pumping by the ETS and resulted in a significant increase in P:O ratio. The increase in \( mt \) volume also appears to help with \( \Delta \Psi_m \) maintenance since this dilutes matrix compounds, including protons. We note that a similar process was recently proposed to occur...
Devaux et al. Acidosis Maintains Hypoxia-Tolerant Mitochondria

FIGURE 6 | Simplified representation of how acidosis may help to maintain the mitochondrial function. (A) ATP production relies on the proton motive force $\Delta p$, which in part consists of a proton gradient. In physiological conditions, $O_2$ consumption reflects "work" performed by the ETS, which is seen here as proton pumping. At a given $\Delta p$, proton pumping equally opposes the combined negative proton fluxes (inward), which includes proton leak (constitutive and active) and "efficient" flux through the F0-F1-ATP synthase. As $O_2$ becomes limiting, proton pumping decelerates, decreasing $\Delta p$ and ATP synthesis. However, as ATP hydrolysis rates surpass synthesis rates, protons accumulating in the cytosol may freely diffuse to the IMS (Cooper, 2000) and these extra-mt protons may contribute to $\Delta p$, independently ETS "work." (B) In this study, triplefin fish brain mitochondria fish were exposed to gradual acidosis, mediated by the titration of lactic acid. Relative to control (pH 7.25), mitochondria in the subtidal HSS shrank with decreased $\Delta p$ and ATP consumption rates, but $\Delta \psi_m$ and ATP production decreased. In contrast, intertidal HTS mitochondria swelled and maintained $\Delta \psi_m$ with increased ATP production rates while $O_2$ consumption rates were decreased. $\Delta pH$, differential pH; ETS, electron transport system; HSS, hypoxia sensitive species; HTS, hypoxia tolerant species; IMS, inter-membrane space; $\Delta \psi_m$, mitochondrial membrane potential; MCT, monocarboxylate transporter; OXPHOS, oxidative phosphorylation; $\Delta p$, proton motive force; TCA, tricarboxylic acid cycle; VDAC, voltage dependent anion channel.
in mammalian cortical neurons (Khacho et al., 2014). The partial suppression of JO2 may also slow O2 depletion in a hypoxic environment. While the mechanisms underlying the difference between the responses in HSS and HTS are yet to be resolved, these species provide natural strategies that have evolved to support mt function in the acidifying brain.

**AVAILABILITY OF SUPPORTING DATA**

Data supporting the results presented in this article are available at the University of Auckland repository.

**AUTHOR CONTRIBUTIONS**

JD, NB, and AH designed the research. JD performed the research. JD, CH, and AH analyzed the data. JD, CH, NB, NH, GR, and AH wrote the paper.

**REFERENCES**

Akerman, K. E., and Wikstrom, M. K. (1976). Safranine as a probe of the mitochondrial membrane potential. FEBS Lett. 68, 191–197. doi: 10.1016/0014-5793(76)80434-6

Azarias, G., Perreten, H., Lengacher, S., Poburko, D., Demaurex, N., Magistretti, P. J., et al. (2011). Glutamate transport decreases mitochondrial pH and modulates oxidative metabolism in astrocytes. J. Neurosci. Off. J. Soc. Neurosci. 31, 3550–3559. doi: 10.1523/JNEUROSCI.4578-10.2011

Barnes, B. M. (1989). Freeze avoidance in a mammal: body temperatures below 0 degree C in an Arctic hibernator. Science 244, 1593–1595. doi: 10.1126/science.2749095

Barros, L. F. (2013). Metabolic signaling by lactate in the brain. Trends Neurosci. 36, 396–404. doi: 10.1016/j.tins.2013.04.002

Beavis, A. D., Brannan, R. D., and Garlid, K. D. (1985). Swelling and contraction of the mitochondrial matrix I. A structural interpretation of the relationship between light scattering and matrix volume. J. Biol. Chem. 260, 13424–13433.

Bento, L. M., Fagian, M. M., Vercesi, A. E., and Gontijo, J. A. (2007). Effects of NH4Cl-induced systemic metabolic acidosis on kidney mitochondrial coupling and calcium transport in rats. Nephrol. Dial. Transplant. 22, 2817–2823. doi: 10.1093/ndt/gfm306

Bernardi, P. (1999). Mitochondrial transport of cations: channels, exchangers, and permeability transition. Physiol. Rev. 79, 1127–1155. doi: 10.1152/physrev.1999.79.4.1127

Bhowmick, S., Moore, J. T., Kirschner, D. L., and Drew, K. L. (2017). Arctic ground squirrel hippocampus tolerates oxygen glucose deprivation independent of hibernation season even when not hibernating and after ATP depletion, acidosis, and glutamate efflux. J. Neurochem. 142, 160–170. doi: 10.1111/jncc.13996

Caruso, J. P., Koch, B. J., Benson, P. D., Varughese, E., Monterey, M. D., Lee, A. E., et al. (2017). pH, Lactate, and Hypoxia: reciprocity in regulating high-affinity monocarboxylate transporter expression in glioblastoma. Neoplasia 19, 121–134. doi: 10.1016/j.neo.2016.12.011

Castella, L., Devin, A., Carriere, A., Salin, B., Schaeffer, J., Rigoulet, M., et al. (2011). Control of mitochondrial volume by mitochondrial metabolic water. Mitochondrion 11, 862–866. doi: 10.1016/j.mito.2011.06.008

Cereghetti, G. M., and Scorrano, L. (2006). The many shapes of mitochondrial death. Oncogene 25, 4717–4724. doi: 10.1038/sj.onc.1209605

Chen, Y. Jr., Mahieu, N. G., Huang, X., Singh, M., Crawford, P. A., Johnson, S. L., et al. (2016). Lactate metabolism is associated with mammalian mitochondria. Nat. Chem. Biol. Adv. 12, 937–943. doi: 10.1038/nchembio.2172

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.01941/full#supplementary-material

**FUNDING**

This work was fully supported by the Royal Society of New Zealand Marsden fund (14-UOA-210).

**ACKNOWLEDGMENTS**

The authors would like to thank Tristan McArley, Lucy Van Oosterom, Peter Schlegel, and Craig Norrie for their precious help with the animal collection. The authors must acknowledge NB, who sadly passed away during this study. He was an excellent scientist, teacher, mentor, and friend.

**SUPPLEMENTARY MATERIAL**

Chinopoulos, C., Gerencser, A. A., Mandi, M., Mathe, K., Töröcsik, B., Doczi, J., et al. (2010). Forward operation of adenine nucleotide translocase during F0F1-ATPase reversal: critical role of matrix substrate-level phosphorylation. FASEB J. Off. Publ. Federat. Am. Soc. Exp. Biol. 24, 2405–2416. doi: 10.1096/fj.09-149898

Chinopoulos, C., Vajda, S., Csánydi, L., Mándi, M., Mathe, K., Adam-Vizi, V., et al. (2009). A novel kinetic assay of mitochondrial ATP-ADP exchange rate mediated by the ANT. Biochem. J. 396, 2493–2504. doi: 10.1042/bj200812.3915

Cooper, G. M. (2000). The Cell a Molecular Approach. Sunderland, MA: Sinauer Associates.

Das, M., Parker, J. E., and Halestrap, A. P. (2003). Matrix volume measurements challenge the existence of dioxazoxide/glibenclamide-sensitive KATP channels in rat mitochondria. J. Physiol. 547(Pt 3), 893–902. doi: 10.1113/physiol2002.03006

Dienel, G. A. (2012). Brain lactate metabolism: the discoveries and the controversies. J. Cereb. Blood Flow Metab. 32, 1107–1138. doi: 10.1038/jcbfm.2011.175

Divakaruni, A. S., and Brand, M. D. (2011). The regulation and physiology of mitochondrial proton leak. Physiology 26, 192–205. doi: 10.1152/physiol.00046.2010

Friedman, J. R., and Nunnari, J. (2014). Mitochondrial form and function. Nature 505, 335–343. doi: 10.1038/nature12985

Fujii, F., Nodasaka, Y., Nishimura, G., and Tamura, M. (2004). Anoxia induces matrix shrinkage accompanied by an increase in light scattering in isolated brain mitochondria. Brain Res. 999, 29–39. doi: 10.1016/j.brainres.2003.11.017

Gallagher, C. N., Carpenter, K. L., Grice, P., Howe, D. J., Mason, A., Timofeev, I., et al. (2009). The human brain utilizes lactate via the tricarboxylic acid cycle: a 13C-labelled microdialysis and high-resolution nuclear magnetic resonance study. Brain 132( Pt 10), 2839–2849. doi: 10.1093/brain/awp202

Garlid, K. D., and Beavis, A. D. (1985). Swelling and contraction of the mitochondrial matrix. II. Quantitative application of the light scattering technique to solute transport across the inner membrane. J. Biol. Chem. 260, 13434–13441.

Gnaiger, E., Kuznetsov, A. V., Schneeberger, S., Seiler, R., Brandacher, G., Steurer, W., et al. (2000). “Mitochondria in the cold,” in Life in the Cold: Eleventh International Hibernation Symposium, eds G. Heldmaier and M. Klingenspor (Berlin: Springer), 431–442. doi: 10.1007/978-3-662-04162-8_45

Halestrap, A. P. (1975). The mitochondrial pyruvate carrier. Kinetics and specificity for substrates and inhibitors. Biochem. J. 148, 85–96. doi: 10.1042/bj1480808

Halestrap, A. P. (2009). What is the mitochondrial permeability transition pore? J. Mol. Cell Cardiol. 46, 821–831. doi: 10.1016/j.yjmcc.2009.02.021
Stuart, J. A., Brindle, K. M., Harper, J. A., and Brand, M. D. (1999). Mitochondrial proton leak and the uncoupling proteins. *J. Bioenerg. Biomembr.* 31, 517–525. doi: 10.1023/A:1005456725549

Teixeira, A. P., Santos, S. S., Carinhas, N., Oliveira, R., and Alves, P. M. (2008). Combining metabolic flux analysis tools and 13C NMR to estimate intracellular fluxes of cultured astrocytes. *Neurochem. Int.* 52, 478–486. doi: 10.1016/j.neuint.2007.08.007

Tiefenthaler, M., Amberger, A., Bacher, N., Hartmann, B. L., Margreiter, R., Koller, R., et al. (2001). Increased lactate production follows loss of mitochondrial membrane potential during apoptosis of human leukaemia cells. *Br. J. Haematol.* 114, 574–580. doi: 10.1046/j.1365-2141.2001.02988.x

Vornanen, M., Stecyk, J. A. W., and Nilsson, G. E. (2009). “Chapter 9 the anoxia-tolerant Crucian carp (Carassius carassius L.),” in *Fish Physiology*, Vol. 27, eds G. Jeffrey, A. P. F. Richards, and J. B. Colin (Cambridge, MA: Academic Press), 397–441. doi: 10.1016/S1546-5098(08)0009-5

Wilson, J. L. (1988). Biochemistry; Third edition (Stryer, Lubert). *J. Chem. Educ.* 65:A337.

Witt, A. M., Larsen, F. S., and Bjerring, P. N. (2017). Accumulation of lactate in the rat brain during hyperammonaemia is not associated with impaired mitochondrial respiratory capacity. *Metab. Brain Dis.* 32, 461–470. doi: 10.1007/s11011-016-9934-7

Zanotti, A., and Azzone, G. F. (1980). Safranine as membrane potential probe in rat liver mitochondria. *Arch. Biochem. Biophys.* 201, 255–265. doi: 10.1016/0003-9861(80)90510-X

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Devaux, Hedges, Birch, Herbert, Renshaw and Hickey. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.