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RESEARCH ARTICLE

Paralytic hypo-energetic state facilitates anoxia tolerance despite ionic imbalance in adult *Drosophila melanogaster*

Jacob B. Campbell[1,*], Mads Kuhlmann Andersen[2], Johannes Overgaard[2] and Jon F. Harrison[1]

ABSTRACT

Oxygen limitation plays a key role in many pathologies; yet, we still lack a fundamental understanding of the mechanisms responsible for variation in anoxia tolerance. Most vertebrate studies suggest that anoxia tolerance involves the ability to maintain cellular ATP despite the loss of aerobic metabolism. However, insects such as adult *Drosophila melanogaster* are able to survive long periods of anoxia (LT_{50}: ~8 h) in a hypo-energetic state characterized by low [ATP]. In this study, we tested for possible mechanisms that allow *D. melanogaster* adults to survive long periods of anoxia. Adults are paralyzed within 30 s, and after 2 h of anoxia, ATP was 3% of normal, extracellular potassium concentration ($[K^+]_o$) increased threefold, pH dropped 1 unit, yet survival was 100%. With 0.5–6 h of anoxia, adults maintained low but constant ATP levels while $[K^+]_o$ and pH_o continued to change. When returned to normoxia, adults restored $[K^+]_o$ and activity. With longer durations of anoxia, ATP levels decreased and $[K^+]_o$ rose further, and both correlated tightly with decreased survival. This response contrasts with the anoxia-sensitive larval stage (LT_{50}: ~1 h). During anoxia, larvae attempted escape for up to 30 min and after 2 h of anoxia, ATP was <1% of resting, $[K^+]_o$ increased by 50%, hemolymph pH fell by 1 unit, and survival was zero. The superior anoxia tolerance of adult *D. melanogaster* appears to be due to the capacity to maintain a paralytic hypometabolic state with low but non-zero ATP levels, and to be able to tolerate extreme extracellular ionic variability.

KEY WORDS: ATP, Ion homeostasis, Extracellular potassium, *D. melanogaster*

INTRODUCTION

Animals differ tremendously in their ability to tolerate oxygen deprivation. With a few noteworthy exceptions (e.g. killifish embryos, turtles and carp), vertebrates can only tolerate minutes of anoxia at body temperatures of 35°C or higher (Nilsson and Lutz, 2004; Podrabsky et al., 2007). Many of the champions of anoxia tolerance are found among the invertebrates that can tolerate hours or days of anoxia even at high temperature (Hoback and Stanley, 2001). This tolerance is even more impressive given that invertebrates are quite small and are characterized by high resting metabolic rates in normoxia. Additionally, ontogenetic variation in anoxia tolerance occurs in most animals, with tolerance often decreasing as development progresses (Singer, 1999). Despite the importance of anoxia tolerance to stress resistance, the physiological mechanisms responsible for the great variation in anoxia tolerance across animals are poorly known.

Cell damage in anoxia is thought to be caused by a decrease in pH, disrupted calcium homeostasis, increased intracellular osmotic pressure and/or mitochondrial damage. All these pathologies are related directly or indirectly to decreased ATP levels (Hochachka and Somero, 2002). Depletion of high-energy phosphates leads to reduced activity of Na^+/K^+- and Ca^{2+}-ATPases, leading to dissipation of ionic gradients (K^+ in particular) responsible for membrane polarization (Galli and Richards, 2014; Storey and Storey, 2007). Cessation of membrane ion pumps also causes intracellular ionic and osmotic imbalances that lead to unfolding and aggregation of proteins (Giffard et al., 2004). Eventually, a series of detrimental apoptotic and necrotic cascades are initiated that cause cell death (Murphy and Steenbergen, 2008). In theory, tolerance to anoxia might involve the capacity to conserve ATP; alternatively, anoxia tolerance could be related to mechanisms downstream of ATP depletion – i.e. the prevention or tolerance to ionic disturbances.

Variation in anoxia tolerance among vertebrates is tied to their ability to match ATP supply and demand to preserve cellular ATP (Boutilier and St-Pierre, 2000). For animals to preserve ATP during anoxia, they must suppress metabolic rate and/or upregulate anaerobic metabolism (Staples and Buck, 2009). Anoxia almost always causes metabolic depression, often to levels less than 10% of resting metabolic rate (Bickler and Buck, 2007; Hoback and Stanley, 2001). To suppress energy consumption, animals often reduce protein synthesis (Boutilier, 2001; Buc-Calderon et al., 1993; Land and Hochachka, 1994, 1995) and activity of ion-motive ATPases. Additionally, many anoxia-tolerant animals have large glycogen stores and considerable capacity for sustained anaerobic ATP production (Gorr et al., 2010; Müller et al., 2012). Accordingly, it is the combination of anaerobic ATP production and cessation of non-essential ATP use that allows preservation of cellular ATP in anoxia-tolerant vertebrates such as the crucian carp and painted turtle (Jackson, 2000; Lutz et al., 1984). Furthermore, these species have the ability to reduce membrane ion permeability during anoxia (Buck and Hochachka, 1993; Hochachka et al., 1996), which helps to defend extracellular potassium concentration ($[K^+]_o$) and membrane potential during prolonged periods of anoxia (Chih et al., 1989; Nilsson et al., 1993).

Many invertebrates (e.g. adult insects) and one notable vertebrate (killifish embryos) can survive long periods of anoxia with low levels of ATP, demonstrating that some animals can avoid or recover from the damage that normally ensues from low cellular ATP levels (Hoback and Stanley, 2001; Podrabsky et al., 2007; Wegener, 1993). Embryos of annual killifish can survive up to 100 days of anoxia during development (Podrabsky et al., 2007), yet ATP levels decline by 80% in the first 12 h of exposure (Podrabsky et al., 2012). Similarly, the majority of adult insects show a rapid depletion of

[1]School of Life Sciences, Arizona State University, Tempe, AZ, USA 85287. [2]Zoophysiology, Department of Bioscience, Aarhus University, 8000 Aarhus C, Denmark.

*Author for correspondence (Jacob.Campbell.1@asu.edu)

J.B.C., 0000-0003-3048-5419

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ATP, yet they show a great tolerance to anoxia (Hoback and Stanley, 2001; Wegener, 1993). Therefore, protective mechanisms downstream of ATP depletion must aid in surviving anoxic exposure. For example, protein aggregations increase during anoxia, and two mechanisms linked to survival involve the accumulation of heat shock proteins and trehalose to prevent protein unfolding and the accumulation of protein aggregates (Azad et al., 2009; Chen et al., 2002). These protective mechanisms that prevent damage associated with ATP depletion indicate that surviving despite low ATP is a key factor in enduring anoxic exposure in invertebrates, and perhaps in vertebrates that also experience rapidly declining ATP during oxygen deprivation.

As in many vertebrates, there are strong developmental differences in anoxia tolerance in *Drosophila melanogaster*. Adults can survive anoxic exposure times approximately eight times longer than larvae (Callier et al., 2015), and adults can recover from hours of oxygen deprivation without evidence of cell damage (Ma et al., 2001). In response to anoxia, larvae and adults similarly reduce metabolic rates to ∼3% of normoxia during 30–120 min of exposure, so differences in anoxia tolerance cannot be explained by capacities to suppress metabolism (Callier et al., 2015). Nevertheless, adults are paralyzed within 30 s and larvae exhibit escape-like behavior for almost 30 min (Callier et al., 2015), so it is possible that survival differences are related to the different behavioral responses and ATP use during the first 30 min of anoxia.

Here, we present whole-body ATP concentrations, along with extracellular [K^+] and pH in adult and larval *D. melanogaster* exposed to varying durations of anoxia. We test whether the anoxia tolerance of *D. melanogaster* can be explained by the ability to maintain ATP levels, maintain extracellular ionic levels or tolerate low energetic state and ionic disruptions, and how this varies across developmental stages (adult versus late-stage third instar larvae).

MATERIALS AND METHODS

Insects, rearing conditions and experimental design

All flies used here were from the same population of Samarkand strain *D. melanogaster* Meigen 1830, maintained as described in Callier et al. (2015). All flies were maintained on the standard malt-based cornmeal diet (Fly Food B, LabExpress, Ann Arbor, MI, USA) at 25°C in 300 ml plastic bottles. Late third instar larvae were collected by choosing individuals that lacked colored food in the gut and were found wandering on the walls of the vials. For adults, vials were cleared and individuals were collected 24 h later; adults were then sexed under CO_2 anesthesia, separated into groups of 20 and then allowed to age until 3–4 days old. All flies were given a minimum of 24 h of recovery after exposure to CO_2 anesthesia before experimental treatments. All experiments were conducted at 25±1°C.

We designed these experiments based on previous studies in the same population of flies that showed large differences in anoxia tolerance between late third instar larvae and adults (e.g. Callier et al., 2015) (Fig. 1). For both larvae and adults, we measured whole-body ATP levels, $[K^+]_o$ and pH across anoxia exposure times associated with 0% to 100% mortality. Thus, we measured these parameters across times ranging up to 2 h in larvae and up to 12 h on adults. To statistically compare life stages, we tested for life-stage effects with a factorial ANOVA. Additionally, we tested for significant effects of time within each life stage using ANOVA across all times tested for that life stage. For $[K^+]_o$, we included samples taken after 15 min of recovery from anoxia. To compare the ability to significantly recover $[K^+]_o$, we used a factorial ANOVA for each life stage comparing recovery state (anoxia or 15 min of recovery) and time in anoxia. All data met assumptions of normality and homogeneity of variance for parametric tests. Main effects and *post hoc* tests were conducted at a family-wise alpha and Type I error rate of 0.05. All statistics were conducted using R software (https://www.r-project.org/) and various R packages [doBy (https://cran.r-project.org/web/packages/doBy/index.html), ggplot2 (Wickham, 2009), lsmeans (Lenth, 2016) and multcomp (Hothorn et al., 2008)].

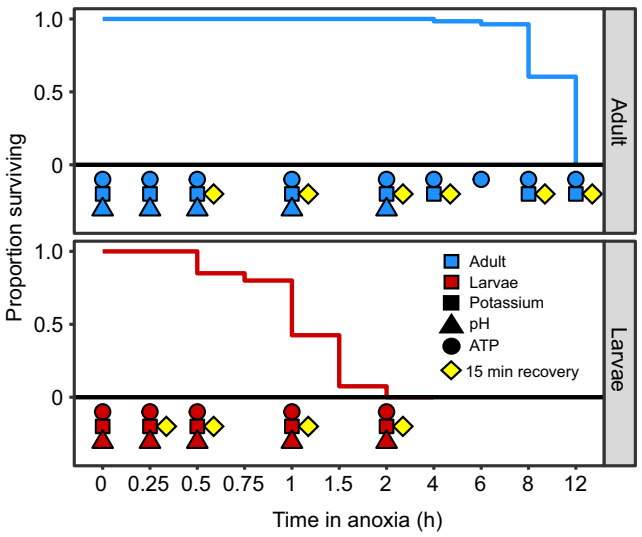

Fig. 1. Anoxia survival and sample collection times. Survival from anoxic exposure of various durations for adult (top panel) and larvae (bottom panel) *Drosophila melanogaster* from Callier et al. (2015). Points below the zero line represent the time points at which samples were collected for whole-body ATP, hemolymph [K^+], hemolymph [K^+] after 15 min of recovery from anoxia, and hemolymph pH. Note that the *x*-axis is categorical on this and many graphs to allow for ease of visualization of occurrences in the first hour.

Measurement of the effect of anoxic duration on whole-body ATP concentrations

Individual adults or larvae were put in groups of ∼20 into 25×95 mm plastic vials containing food. Vials were sealed with gas-permeable cotton plugs and placed into an airtight 1 liter chamber with two ports. To create humid, anoxic conditions, N_2 gas flowed (4 l min^{-1} regulated by a Mass Flow Bar, Sable Systems, Las Vegas, NV, USA) first through a glass flask of distilled water, then through the chamber and into an oxygen sensor (FoxBox, Sable Systems). After a measured duration of anoxic exposure, animals were flash-frozen in liquid nitrogen immediately after anoxic exposure and stored at −80°C until assays were performed.

The ATP assay was carried out using methods derived from Tennessen et al. (2014). Groups of five individuals were homogenized with a pellet pestle (Kimble Kontes, Rockwood, TN, USA) in 200 µl of homogenization buffer [6 mol l^{-1} guanidine HCl, 100 mmol l^{-1} Tris (pH 7.8), 4 mmol l^{-1} EDTA] on ice and a 50 µl aliquot was taken for protein assays. The remaining sample was boiled for 5 min and then centrifuged for 3 min at 12,000 ***g*** in a refrigerated centrifuge at 4°C. Next, 10 µl of the remaining supernatant was transferred to a new centrifuge tube and diluted 1:10 with dilution buffer [25 mmol l^{-1} Tris (pH 7.8), 100 mmol l^{-1} EDTA]. Then, 10 µl of the diluted samples and standards were added to each well of a white opaque 96-well plate (Corning Inc., Corning, NY, USA). To run the assay, 100 µl of the ATP reaction mix (Molecular Probes, Eugene, OR, USA) was added to each well and luminescence was measured using a Wallac Victor 2

luminometer (Perkin Elmer, Waltham, MA, USA); the reaction mix contained 1.25 µg ml^{-1} of luciferase, 50 µmol l^{-1} D-luciferin and 1 mmol l^{-1} DTT in the 1× reaction buffer per well. The masses of each sample were used to express ATP in µmol g^{-1} wet mass.

Ion-selective microelectrodes for measurement of effects of anoxic duration on hemolymph [K$^+$] and pH

Hemolymph [K$^+$] and pH were measured on larvae or adults (n=12–14 for $[K^+]_o$, n=4–9 for pH) at each time point. Larvae and adults were placed individually into air-tight glass chambers (20 mm diameter×70 mm length) and connected to a multiplexer (Sable Systems) regulating the flow of humidified nitrogen to each chamber, allowing the duration of anoxia for each chamber to be individually controlled. After anoxic exposure, individuals were quickly removed from the anoxic chamber and hemolymph was collected as quickly as possible, usually within 60 s. We also measured $[K^+]_o$ for adults and larvae allowed to recover for 15 min in air after their assigned duration of anoxia.

Adult hemolymph was extracted as previously described (MacMillan and Hughson, 2014). Briefly, adult flies were placed head-first into 10-µl pipette tips fit to a system of tubing connected to the laboratory air supply; positive pressure was applied to the pipette tip, forcing a portion of the head to be exposed. We removed the first segment of the antennae with forceps to allow a droplet of hemolymph to flow out. The droplet was placed into hydrated paraffin oil for immediate measurement of $[K^+]_o$. For larval hemolymph collection, we submerged animals under hydrated paraffin oil and used a fine glass pipette to puncture the epidermis near the head; hemolymph then pooled around the larvae under the oil, and we collected the hemolymph with the pipette by capillary action. We took care to not rupture the gut, and any gut perforation was visible by the presence of cloudy fluid; therefore, only samples with clear hemolymph were used. The hemolymph was then transferred from the glass pipette into a secondary dish of hydrated paraffin oil set up for measurement of $[K^+]_o$. Although hemolymph volumes were not measured during this experiment, volumes were large enough for easy insertion of a reference and ion-selective electrode; similar methods were able to extract ∼50 nl of hemolymph for adults (MacMillan and Hughson, 2014) and ∼170 nl for larvae (Piyankarage et al., 2008).

$[K^+]_o$ and pH were measured using ion-selective microelectrodes prepared as described by MacMillan et al. (2015). Borosilicate glass capillaries (TW150-4, World Precision Instruments, Sarasota, FL, USA) were pulled to a ∼3–5 µm tip and silanized at 300°C with *N,N*-dimethyltrimethylsilylamine (Sigma-Aldrich, St Louis, MO, USA) vapors for 1 h. Immediately before use, [K$^+$] microelectrodes were filled with 100 mmol l^{-1} KCl solution, whereas pH microelectrodes were filled with 40 mmol l^{-1} KH_2PO_4, 15 mmol l^{-1} NaCl and 23 mmol l^{-1} NaOH, pH 7.0 (Lee et al., 2013). Next, the ion-selective ionophore was added to the tip of the ion-selective electrode (K$^+$ ionophore I, cocktail b; H$^+$ ionophore I cocktail b; Sigma-Aldrich). To prevent the displacement of the ionophore by paraffin oil, the tip of the electrodes were quickly dipped in a solution consisting of 10 mg of polyvinylchloride (Sigma-Aldrich) dissolved in 3 ml of tetrahydrofuran (Sigma-Aldrich). The ion-selective electrode was inserted into the hemolymph drop using micromanipulators, with the electrode tip observed with a dissecting microscope. For both $[K^+]_o$ and pH, a 2 mm OD glass reference electrode with filament (1B200F-4, World Precision Instruments) pulled to a long thin tip (∼1 µm) was filled with 0.5 mol l^{-1} KCl and inserted into the hemolymph to complete the circuit. The voltage difference between the two electrodes was measured with an FD223a differential electrometer (World Precision Instruments), an MP100A data acquisition system and AcqKnowledge software (Biopac Systems, Goleta, CA, USA). We calibrated the ion-selective electrode before and after each measurement using solutions differing 10-fold. Standards used for $[K^+]_o$ measurements were 10 and 100 mmol l^{-1} [KCl] solutions; the 10 mmol l^{-1} KCl solution also contained 90 mmol l^{-1} LiCl to balance osmolarity. A three-point calibration using standard pH buffer solutions ranging from pH 6 to 8 were used to determine hemolymph pH. Voltage from the ion selective electrode was converted to [K$^+$] or pH using:

$$[h] = [c] \times 10^{\frac{\Delta V}{S}}, \quad (1)$$

where $[h]$ is the ion concentration in the hemolymph, $[c]$ is the concentraion of one calibration solution, ΔV is the difference in voltage between the calibration solution and hemolymph, and S is the slope of the difference in voltage between the two calibration solutions. For pH calculations, $[H^+]$ was converted to pH by multiplying $[h]$ by –log10. The slopes of the ion-selective electrode averaged 53.85±2.50 mV per 10-fold difference in ion concentration, close to the expected Nernst relationship of 58 mV per 10-fold difference.

RESULTS

Whole-body ATP levels during anoxia

Time in anoxia and the time×life stage interaction significantly affected whole-body ATP levels (time: $F_{9,34}$=141.87, P<0.001; interaction: $F_{4,26}$=16.41, P<0.001), whereas the main effect of life stage was not different (life stage: $F_{1,34}$=3.25, P=0.080). Levels of whole-body ATP dropped near 100-fold for larvae after 2 h of anoxia (0.51±0.059 to 0.005±0.0001 µmol g^{-1} wet mass) and adults after 12 h of anoxia (1.00±0.044 to 0.009±0.0006 µmol g^{-1} wet mass); normoxic ATP levels were higher in adults (Fig. 2A). Adult ATP decreased significantly to 35% of resting values after 0.25 h of anoxia and to ∼3% of anoxic values after 0.5 h of anoxia; however, ATP then remained stable until another significant drop at 8 h (Fig. 2A). Larval ATP levels fell to 49% and 21% of resting levels after 0.25 and 0.5 h of anoxia, respectively, reaching less than 10% of normoxic values after 1 h and <1% of normoxic values after 1.5–2 h of anoxia (Fig. 2A). Adults survived at much lower levels of ATP than larvae during anoxia (larvae: 0.058±0.010, adults: 0.011±0.006; likelihood ratio test, χ^2=10.21, P=0.006; Fig. 2B). Thus, unlike larvae, adults maintain low but survivable ATP levels for long durations in anoxia.

Hemolymph [K$^+$] and pH during anoxia and recovery

Contrary to ATP, larvae tended to maintain $[K^+]_o$ better than adults during anoxic exposure. The main effects for life stage and time in anoxia, and their interaction, all significantly affected $[K^+]_o$ (time: $F_{7,148}$=64.12, P<0.001; life stage: $F_{1,148}$=32.44, P<0.001; interaction: $F_{4,148}$=3.73, P=0.006). Larvae had higher resting $[K^+]_o$ levels than adults in normoxia; however, adults reached levels of $[K^+]_o$ similar or higher to those of larvae by 30 min or more of anoxic exposure (Fig. 3). Adults increased $[K^+]_o$ by almost 2-fold after 2 h of anoxia and by 4-fold after 12 h of anoxia (Fig. 3A); in contrast, larvae only increased $[K^+]_o$ by ∼50% after 2 h of anoxia, with no significant changes from normoxic values until after 1 h of exposure (Fig. 3B). Adults survived much higher proportional increases in $[K^+]_o$ than larvae (LT_{50} for larvae: 45.89±1.19, and for adults: 78.71±0.30; likelihood ratio test, χ^2=10.87, P=0.004; Fig. 3C). Thus, the greater anoxia tolerance of adults relative to

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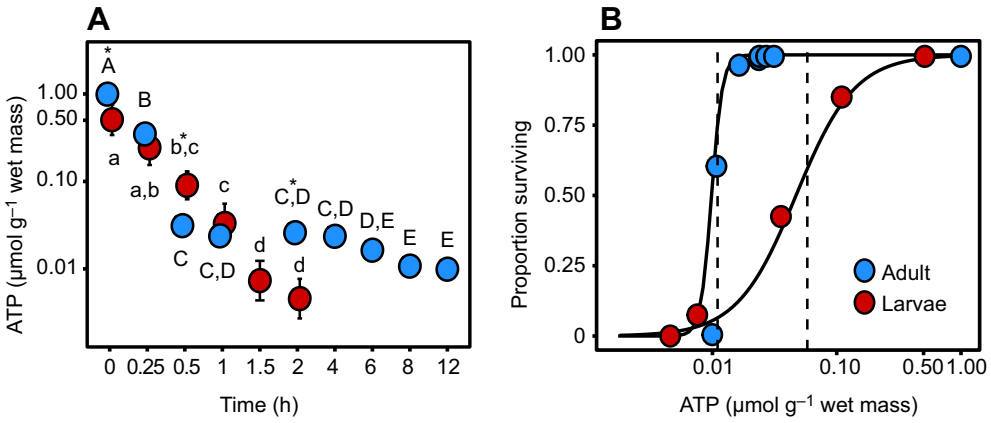

Fig. 2. Whole-body ATP levels throughout anoxia for *D. melanogaster* larvae and adults. ATP was measured for up to 2 h for larvae (red circles) and 12 h for adults (blue circles). (A) ATP in absolute concentration (µmol g^{-1} wet mass) for the duration of anoxic exposure. Note that data on the *x*-axis are presented categorically and the *y*-axis is on a log scale. Data shown are means ±95% CI; asterisks indicate a significant difference between life stages at that time point (Tukey *post hoc* test, $P<0.05$), and data with different letters (capital letters for adults and lowercase for larvae) represent time periods that have statistically different ATP levels within a life stage (Tukey, $P<0.05$). (B) The relationship between normoxic ATP and survival shows that adults survive with lower levels of ATP than larvae. Vertical dashed lines represent the LT_{50} for each life stage (larvae: 0.058±0.010, adults: 0.011±0.006). Note that the *x*-axis is on a log scale to help visualize the lower range of ATP.

larvae is due to greater tolerance of elevated $[K^+]_o$ rather than a better ability to maintain $[K^+]_o$ homeostasis.

Larvae and adults showed similar strong (approximately 1 pH unit) decreases in hemolymph pH during anoxia (Fig. 4A). There was no significant interaction between time and life stage ($F_{4,44}$=1.69, P=0.17), and no effect of life stage on pH_o during anoxia ($F_{1,44}$=0.79, P=0.38). However, the main effect of time in anoxia was significant ($F_{4,44}$=80.21, $P<0.001$); pH_o dropped from 7.4 to ∼6.7 within the first 15 min of anoxia and remained stable until the 2 h mark, at which it dropped to 6.4 (Fig. 4A). Although the pattern of decreasing pH_o was similar between life stages, adults were more capable of surviving the radical drop in pH_o than larvae (LT_{50} for larvae =6.52±0.18; likelihood ratio test, χ^2=14.70, $P<0.001$; Fig. 4B).

For adults, the capacity to restore $[K^+]_o$ to resting levels in normoxia was a good indicator of survival across various durations of anoxia. Adults quickly restored $[K^+]_o$ after 0.5–4 h of anoxic exposure, but not with longer anoxic exposures (Fig. 3A; interaction: $F_{5,138}$=15.49, $P<0.001$). In contrast, the larvae showed no significant capacity to lower $[K^+]_o$ levels during 15 min of normoxia after 0.25–2 h of anoxia (Fig. 3B; interaction: $F_{3,101}$=2.95, $P<0.05$).

DISCUSSION

Drosophila melanogaster adults maintain and tolerate a hypo-energetic state that seems key to their remarkable ability to survive long periods of anoxia. *Drosophila melanogaster* adults exposed to anoxia for 0.5–4 h exhibited a low, non-zero set-point for ATP at approximately 3% of those measured for resting flies, and over this duration, fly survival was near 100%. With longer periods of anoxia, ATP levels in adults fell to 1% of resting levels, correlating strongly with lethality. In contrast, *D. melanogaster* larvae exposed to anoxia exhibited steadily falling ATP levels that reached 1% of resting levels within 1–2 h, when survival fell to 0%.

We set out to investigate two non-alternative mechanisms that can explain the extraordinary (at least relative to most vertebrates) anoxia tolerance in *D. melanogaster* adults. Based on the leading hypothesis in anoxia tolerance for vertebrates, we first tested whether adults are able to attain a regulated state where ATP supply is matched to demand. Despite the observation that ATP levels fell dramatically in both adults and larvae during anoxic exposure, this hypothesis was partially supported, albeit with the observation that ATP levels in adults were regulated at 3% of resting levels for long periods of anoxia. A second hypothesis was that the anoxia-tolerant adults might be better endowed with capacities to preserve acid–base or ionic homeostasis. For extracellular ionic status this is clearly not true, as $[K^+]_o$ rose faster in adults than in larvae during anoxia (Fig. 3), and pH_o fell at a similar rate (Fig. 4A). Instead, a key aspect of the survival of adult *D. melanogaster* in anoxia is that they are able to tolerate and recover from dramatic departures from normal homeostatic conditions – tolerating ATP levels at 3% of resting, $[K^+]_o$ levels >4× resting and hemolymph $[H^+]$ >10× resting.

Examination of the literature indicates that ATP patterns in anoxia are highly variable among anoxia-tolerant animals. With the exception of annual killifish embryos, anoxia-tolerant vertebrates maintain ATP levels near normal during anoxia while most invertebrates survive long periods of anoxia with low levels of ATP (Bickler and Buck, 2007; Hoback and Stanley, 2001). Unlike anoxia-tolerant vertebrates, the majority of insect species studied show patterns similar to those of anoxia-sensitive mammals in that ATP is usually substantially depleted in anoxia (Hoback and Stanley, 2001; Wegener, 1993). However, the extent and timeline of ATP depletion varies tremendously between species and tissues. For example, migratory locusts show minimal survival after 4 h of anoxia whereas whole-body ATP levels are depleted to one-third of normal, and ATP levels in the brain and flight muscle are depleted to ∼6% and 40% of normal, respectively (Hochachka et al., 1993; Wegener, 1987, 1993). Whether the pattern of maintenance of ATP concentrations at a low, non-zero level occurs commonly in anoxic insects is unclear; although this has not been previously reported, we did not notice this pattern until after plotting log-transformed ATP levels, which most studies have not done.

Plausible mechanisms allowing adults but not larvae to maintain a prolonged, low ATP state that is linked to better survival of anoxia

Maintenance of ATP levels (even at 3% of normoxic levels) requires a matching of ATP supply to demand. Adults may have lower ATP use during the first 30 min of anoxia, when they are paralyzed and

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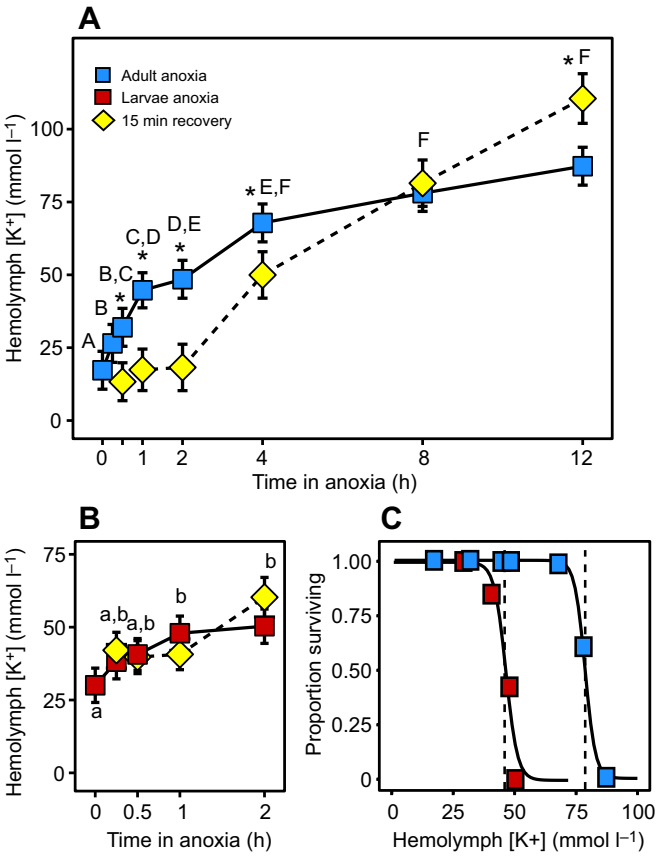

Fig. 3. Hemolymph [K^+] during anoxic exposure for *D. melanogaster* larvae and adults and after 15 min of recovery. Hemolymph [K^+] (mmol l^{-1}) during 12 h of anoxia and after 15 min of recovery (yellow) from anoxia for adults (A, blue) and larvae (B, red). Asterisks represent significant differences between anoxia and recovery [K^+] (ANOVA, Tukey, $P<0.01$), and data with different letters (capital letters for adults and lowercase for larvae) represent time periods that have statistically different hemolymph [K^+] levels within a life stage (Tukey, $P<0.05$). Data shown are means ±95% CI. (C) The relationship between hemolymph [K^+] and anoxia survival shows that adults are more capable of surviving higher proportional changes in hemolymph [K^+]. Vertical dashed lines represent the LT_{50} for each life stage (larvae: 45.89±1.19, adults: 78.71±0.30).

larvae are attempting escape. Anoxia causes adult flight muscles and neurons to lose excitability (Gu and Haddad, 1999; Krishnan et al., 1997), suggesting a response similar to the classical vertebrate channel and spike arrest wherein depolarization causes a slow inactivation of voltage-gated channels that limits ionic flux and neuronal activity, thereby preserving ATP (Lutz and Milton, 2004; Rodgers et al., 2010). Larvae likely do not exhibit this paralysis (at least initially) in anoxia because they have been selected to attempt escape from anoxic, semi-liquid media into air. The cellular mechanisms underlying the differential behavioral responses of adults and larvae to anoxia would be very interesting to explore. In adults, paralysis occurs in less than a minute (Callier et al., 2015), when, at the whole-body level, ATP levels have fallen by less than 50% (Fig. 2).

Calorimetric measurements demonstrate that larvae and adults have similar metabolic rates during 30–120 min of anoxia; thus, a better ability of adults to suppress metabolism is unlikely to completely explain their superior ability to regulate a hypo-energetic ATP level (Callier et al., 2015). The prolonged maintenance of ATP at 3% of normoxic levels shows that adults are more able to sustain a match of anaerobic ATP production to ATP use. One possible explanation for this is that adults may use a more diverse array of anaerobic pathways with reduced negative impact of intracellular acid–base status. Lactate accumulation (three times higher in larvae than in adults) accounts for nearly all heat production in anoxic *D. melanogaster* larvae, while only accounting for only approximately one-third in adults (Callier et al., 2015). Adults accumulate high levels of acetate and alanine, potentially improving ATP generation from glycogen with less H^+ production (Feala et al., 2007, 2009). If larvae experience a higher rate of intracellular H^+ accumulation, this could compromise cellular functions such as glycolysis sooner, inhibiting anaerobic ATP production and survival. Another possibility is that larvae might more quickly exhaust their fuel stores (such as glycogen, trehalose and glucose) owing to possession of lower stores or during their initial high-intensity locomotory response to anoxia. Finally, it is conceivable that adults are more capable of defending or repairing cellular damage such as aggregations of unfolded proteins during anoxia. Adult *D. melanogaster* upregulate heat shock proteins (Azad et al., 2011), and have high levels of trehalose that protect protein denaturation (Chen et al., 2004), and it is possible that such protective mechanisms are lower in larvae.

Higher survival in anoxia in *D. melanogaster* adults is associated with tolerance to extreme ionic imbalance

According to most classical models of anoxia tolerance, a key factor is prevention of substantial elevation of $[K^+]_o$ that depolarizes membranes (Erecińska and Silver, 1989; Katsura et al., 1994; Knickerbocker and Lutz, 2001). However, hemolymph $[K^+]_o$ increases more rapidly in adults than in larvae, and adults survive

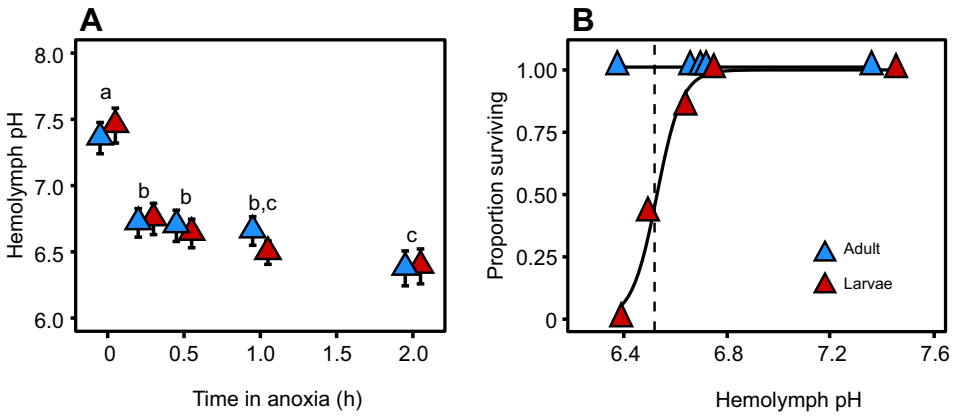

Fig. 4. Change in hemolymph pH of *D. melanogaster* during 2 h of anoxic exposure. (A) Hemolymph pH declined over time, but there were no statistically significant differences between larvae and adults. Data shown are means ±95% CI; data with different letters represent time periods that have statistically different hemolymph pH levels (Tukey, $P<0.05$). (B) The relationship between hemolymph pH and anoxia survival indicates that adults were more tolerant to the drop in pH. Vertical dashed line represents the LT_{50} for larvae (6.52±0.18).

much higher levels of $[K^+]_o$ than larvae (Fig. 3). For hemolymph pH, the changes are similar in larvae and adults (Fig. 4). Thus, the higher anoxia tolerance of adults is associated with a greater tolerance of extracellular ionic disruption rather than better extracellular ionic regulation (Fig. 3).

In both larval and adult *D. melanogaster*, $[K^+]_o$ increases in a nonlinear fashion during anoxia, wherein changes in $[K^+]_o$ are at their highest early on and begin to slow as exposure continues. For adults, during hour 1, when ATP levels fall dramatically, $[K^+]_o$ rises by 300%, whereas during the next 6 h, when ATP is statistically constant, $[K^+]_o$ rises only by 50%. This pattern is likely due to the dynamic changes in passive and active forces on K^+ balance. As ATP declines, Na^+/K^+-ATPase activity and passive reuptake decrease, leading to increased extracellular K^+; this change in $[K^+]_o$ disrupts the electrochemical gradient and leads to further loss of intracellular K^+. Furthermore, editing of ion channels by adenosine deaminase improves hypoxia survival in adult *D. melanogaster*, likely by reducing transmembrane ion flux (Ma et al., 2001).

Adult hemolymph [K^+] recovery facilitates rapid recovery of behavior after anoxia

Following a detrimental disruption of ion homeostasis and the loss of neuronal activity, successful recovery from anoxia requires the ability to clear excess $[K^+]_o$ and recover ion gradients (Rodgers et al., 2007). Fitting with their higher anoxia tolerance, adults demonstrate a higher capacity to restore ionic homeostasis after anoxia. Within 15 min of reoxygenation (after 2 h of anoxia), adults return $[K^+]_o$ to normal levels. Only at the anoxia durations of 8 h or more are adults unable to make a significant recovery of $[K^+]_o$, coinciding with the rapid decline in survival (Callier et al., 2015). The rate of recovery of $[K^+]_o$ correlates well with reported times for flies to behaviorally recover from anoxia. Adult flies exposed to less than 2 h of anoxia exhibit the first signs of movement within 15 min of recovery, and a 4 h exposure of anoxia extends mobility recovery time to 60 min (Krishnan et al., 1997). However, larvae exhibit very little recovery of $[K^+]_o$ when returned to normoxia after anoxic exposures. This may be partly due to the fact that the changes in $[K^+]_o$ were smaller than in adults, making it more challenging to observe recovery.

Speculations on the links between ATP, extracellular ionic changes and survival

It seems difficult to believe that preservation of ATP at 3% of normoxic levels could be essential to sustaining life during anoxia exposure in adults; however, the tight correlations between ATP, $[K^+]_o$ and survival (Figs 2 and 3) are certainly suggestive. It is possible that Na^+/K^+-ATPases are able to sustain some level of transport at very low ATP, slowing the rate of ionic fluxes that eventually lead to cell death. In support of this possibility, larvae have approximately three times the hemolymph volume as adults (larvae: 0.178 µl, adults: 0.064 µl; Folk and Bradley, 2003; Folk et al., 2001; Piyankarage et al., 2008); assuming that hemolymph volume remains stable throughout anoxia, adults have an approximately four times lower rate of K^+ efflux to the hemolymph than larvae during anoxia (Fig. S1), perhaps because of higher Na^+/K^+-ATPase activity or differences in membrane conductance. This low level of ATP might also be able to sustain some extrusion of H^+ or Ca^{++} to prolong survival. Direct measurement of intracellular conditions and intracellular to extracellular ionic fluxes in anoxic larvae and adults will be necessary to test this hypothesis. It is also plausible that these low but non-zero ATP levels are sufficient for other protective functions such as translation of heat shock proteins, or synthesis of protective organic osmolytes.

Conclusions

When metazoans are broadly considered, it is clear that anoxia tolerance can be achieved without preservation of near-normoxic ATP levels, contrary to the paradigm for anoxia-tolerant vertebrates such as turtles and carp. Here we have identified that survival from anoxic exposure in *D. melanogaster* is associated with maintenance of extremely low but non-zero ATP levels, likely made possible by behavioral paralysis that reduces metabolic rate and use of diverse metabolic pathways to generate ATP anaerobically. Additionally, we show that surviving anoxia in *D. melanogaster* adults is related to the ability to tolerate large disturbances in extracellular ionic homeostasis. Given the many similarities in response to anoxia with mammals, identification of the molecular and cellular mechanisms that allow *D. melanogaster* to survive low energetic state and large ionic disruptions may be useful for advancing the understanding of variation in anoxia tolerance in a biomedical context.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.B.C., J.O., J.F.H.; Methodology: J.B.C., M.K.A., J.O., J.F.H.; Software: J.B.C.; Formal analysis: J.B.C.; Investigation: J.B.C., M.K.A., J.O., J.F.H.; Resources: M.K.A., J.O., J.F.H.; Writing - original draft: J.B.C., J.F.H.; Writing - review & editing: J.B.C., M.K.A., J.O., J.F.H.; Visualization: J.B.C., J.F.H.; Supervision: J.O., J.F.H.; Project administration: J.B.C., J.O., J.F.H.; Funding acquisition: J.O., J.F.H.

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Supplementary information

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References

Azad, P., Zhou, D., Russo, E. and Haddad, G. G. (2009). Distinct mechanisms underlying tolerance to intermittent and constant hypoxia in *Drosophila melanogaster*. *PLoS ONE* **4**, e5371.

Azad, P., Ryu, J. and Haddad, G. G. (2011). Distinct role of Hsp70 in *Drosophila* hemocytes during severe hypoxia. *Free Radic. Biol. Med.* **51**, 530-538.

Bickler, P. E. and Buck, L. T. (2007). Hypoxia tolerance in reptiles, amphibians, and fishes: life with variable oxygen availability. *Annu. Rev. Physiol.* **69**, 145-170.

Boutilier, R. G. (2001). Mechanisms of cell survival in hypoxia and hypothermia. *J. Exp. Biol.* **204**, 3171-3181.

Boutilier, R. G. and St-Pierre, J. (2000). Surviving hypoxia without really dying. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **126**, 481-490.

Buc-Calderon, P., Lefebvre, V. and Van Steenbrugge, M. (1993). Inhibition of protein synthesis in isolated hepatocytes as an immediate response to oxygen limitation. In *Surviving Hypoxia: Mechanisms of Control and Adaptation* (ed. P. W. Hochachka, P. L. Lutz, T. J. Sick and M. Rosenthal), pp. 271-280. Boca Raton, FL: CRC Press.

Buck, L. T. and Hochachka, P. W. (1993). Anoxic suppression of Na^+-K^+-ATPase and constant membrane potential in hepatocytes: support for channel arrest. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **265**, R1020-R1025.

Callier, V., Hand, S. C., Campbell, J. B., Biddulph, T. and Harrison, J. F. (2015). Developmental changes in hypoxic exposure and responses to anoxia in *Drosophila melanogaster*. *J. Exp. Biol.* **218**, 2927-2934.

Chen, Q., Ma, E., Behar, K. L., Xu, T. and Haddad, G. G. (2002). Role of trehalose phosphate synthase in anoxia tolerance and development in *Drosophila melanogaster*. *J. Biol. Chem.* **277**, 3274-3279.
Chen, L., Rio, D. C., Haddad, G. G. and Ma, E. (2004). Regulatory role of dADAR in ROS metabolism in *Drosophila* CNS. *Mol. Brain Res.* **131**, 93-100.
Chih, C. P., Feng, Z. C., Rosenthal, M., Lutz, P. L., Sick, T. J., Feng, Z. and Lutz, P. L. (1989). Energy metabolism, ion homeostasis, and evoked potentials in anoxic turtle brain. *Am. J. Physiol.* **257**, R854-R860.
Erecińska, M. and Silver, I. A. (1989). ATP and brain function. *J. Cereb. Blood Flow Metab.* **9**, 2-19.
Feala, J. D., Coquin, L., McCulloch, A. D. and Paternostro, G. (2007). Flexibility in energy metabolism supports hypoxia tolerance in *Drosophila* flight muscle: metabolomic and computational systems analysis. *Mol. Syst. Biol.* **3**, 99.
Feala, J. D., Coquin, L., Zhou, D., Haddad, G. G., Paternostro, G. and McCulloch, A. D. (2009). Metabolism as means for hypoxia adaptation: metabolic profiling and flux balance analysis. *BMC Syst. Biol.* **3**, 91.
Folk, D. G. and Bradley, T. J. (2003). Evolved patterns and rates of water loss and ion regulation in laboratory-selected populations of *Drosophila melanogaster*. *J. Exp. Biol.* **206**, 2779-2786.
Folk, D. G., Han, C. and Bradley, T. J. (2001). Water acquisition and partitioning in *Drosophila melanogaster*: effects of selection for desiccation-resistance. *J. Exp. Biol.* **204**, 3323-3331.
Galli, G. L. J. and Richards, J. G. (2014). Mitochondria from anoxia-tolerant animals reveal common strategies to survive without oxygen. *J. Comp. Physiol. B.* **184**, 285-302.
Giffard, R. G., Xu, L., Zhao, H., Carrico, W., Ouyang, Y., Qiao, Y., Sapolsky, R., Steinberg, G., Hu, B. and Yenari, M. A. (2004). Chaperones, protein aggregation, and brain protection from hypoxic/ischemic injury. *J. Exp. Biol.* **207**, 3213-3220.
Gorr, T. A., Wichmann, D., Hu, J., Hermes-Lima, M., Welker, A. F., Terwilliger, N., Wren, J. F., Viney, M., Morris, S., Nilsson, G. E. et al. (2010). Hypoxia tolerance in animals: biology and application. *Physiol. Biochem. Zool.* **83**, 733-752.
Gu, X. Q. and Haddad, G. G. (1999). *Drosophila* neurons respond differently to hypoxia and cyanide than rat neurons. *Brain Res.* **845**, 6-13.
Hoback, W. W. and Stanley, D. W. (2001). Insects in hypoxia. *J. Insect Physiol.* **47**, 533-542.
Hochachka, P. W. and Somero, G. N. (2002). *Biochemical Adaptation: Mechanism and Process in Physiological Evolution*. New York: Oxford University Press.
Hochachka, P. W., Nener, J. C., Hoar, J. and Saurez, R. K. (1993). Disconnecting metabolism from adenylate control during extreme oxygen limitation. *Can. J. Zool.* **71**, 1267-1270.
Hochachka, P. W., Buck, L. T., Doll, C. J. and Land, S. C. (1996). Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. *Proc. Natl. Acad. Sci. USA* **93**, 9493-9498.
Hothorn, T., Bretz, F. and Westfall, P. (2008). Simultaneous inference in general parametric models. *Biometrical J.* **50**, 346-363.
Jackson, D. (2000). Living without oxygen: lessons from the freshwater turtle. *Comp. Biochem. Physiol. Part A* **125**, 299-315.
Katsura, K., Kristián, T. and Siesjö, B. K. (1994). Energy metabolism, ion homeostasis, and cell damage in the brain. *Biochem. Soc. Trans.* **22**, 991-996.
Knickerbocker, D. L. and Lutz, P. L. (2001). Slow ATP loss and the defense of ion homeostasis in the anoxic frog brain. *J. Exp. Biol.* **204**, 3547-3551.
Krishnan, S. N., Sun, Y. A., Mohsenin, A., Wyman, R. R. J. and Haddad, G. G. (1997). Behavioral and electrophysiologic responses of *Drosophila melanogaster* to prolonged periods of anoxia. *J. Insect Physiol.* **43**, 203-210.
Land, S. C. and Hochachka, P. W. (1994). Protein turnover during metabolic arrest in turtle hepatocytes: role and energy dependence of proteolysis. *Am. J. Physiol. Cell Physiol.* **266**, C1028-C1036.
Land, S. C. and Hochachka, P. W. (1995). A heme-protein-based oxygen-sensing mechanism controls the expression and suppression of multiple proteins in anoxia-tolerant turtle hepatocytes. *Proc. Natl. Acad. Sci. USA* **92**, 7505-7509.
Lee, S.-K., Boron, W. F. and Parker, M. D. (2013). Monitoring ion activities in and around cells using ion-selective liquid-membrane microelectrodes. *Sensors* **13**, 984-1003.
Lenth, R. V. (2016). Least-squares means: the R package lsmeans. *J. Stat. Softw.* **69**, 1-33.
Lutz, P. L. and Milton, S. L. (2004). Negotiating brain anoxia survival in the turtle. *J. Exp. Biol.* **207**, 3141-3147.
Lutz, P. L., McMahon, P., Rosenthal, M. and Sick, T. J. (1984). Relationships between aerobic and anaerobic energy production in turtle brain *in situ*. *Am. J. Physiol.* **247**, R740-R744.
Ma, E., Gu, X.-Q., Wu, X., Xu, T. and Haddad, G. G. (2001). Mutation in pre-mRNA adenosine deaminase markedly attenuates neuronal tolerance to O_2 deprivation in *Drosophila melanogaster*. *J. Clin. Invest.* **107**, 685-693.
MacMillan, H. A. and Hughson, B. N. (2014). A high-throughput method of hemolymph extraction from adult *Drosophila* without anesthesia. *J. Insect Physiol.* **63**, 27-31.
MacMillan, H. A., Andersen, J. L., Loeschcke, V. and Overgaard, J. (2015). Sodium distribution predicts the chill tolerance of *Drosophila melanogaster* raised in different thermal conditions. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* **308**, R823-R831.
Müller, M., Mentel, M., van Hellemond, J. J., Henze, K., Woehle, C., Gould, S. B., Yu, R.-Y., van der Giezen, M., Tielens, A. G. M. and Martin, W. F. (2012). Biochemistry and evolution of anaerobic energy metabolism in eukaryotes. *Microbiol. Mol. Biol. Rev.* **76**, 444-495.
Murphy, E. and Steenbergen, C. (2008). Ion transport and energetics during cell death and protection. *Physiology* **23**, 115-123.
Nilsson, G. E. and Lutz, P. L. (2004). Anoxia tolerant brains. *J. Cereb. Blood Flow Metab.* **24**, 475-486.
Nilsson, G. E., Pérez-Pinzón, M., Dimberg, K. and Winberg, S. (1993). Brain sensitivity to anoxia in fish as reflected by changes in extracellular K+ activity. *Am. J. Physiol.* **264**, R250-R253.
Piyankarage, S. C., Augustin, H., Grosjean, Y., Featherstone, D. E. and Shippy, S. A. (2008). Hemolymph amino acid analysis of individual *Drosophila* larvae. *Anal. Chem.* **80**, 1201-1207.
Podrabsky, J. E., Lopez, J. P., Fan, T. W. M., Higashi, R. and Somero, G. N. (2007). Extreme anoxia tolerance in embryos of the annual killifish *Austrofundulus limnaeus*: insights from a metabolomics analysis. *J. Exp. Biol.* **210**, 2253-2266.
Podrabsky, J. E., Menze, M. A. and Hand, S. C. (2012). Long-term survival of anoxia despite rapid ATP decline in embryos of the annual killifish *Austrofundulus limnaeus*. *J. Exp. Zool. A Ecol. Genet. Physiol.* **317**, 524-532.
Rodgers, C. I., Armstrong, G. A. B., Shoemaker, K. L., LaBrie, J. D., Moyes, C. D. and Robertson, R. M. (2007). Stress preconditioning of spreading depression in the locust CNS. *PLoS ONE* **2**, e1366.
Rodgers, C. I., Armstrong, G. A. B. and Robertson, R. M. (2010). Coma in response to environmental stress in the locust: a model for cortical spreading depression. *J. Insect Physiol.* **56**, 980-990.
Singer, D. (1999). Neonatal tolerance to hypoxia: a comparative-physiological approach. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **123**, 221-234.
Staples, J. F. and Buck, L. T. (2009). Matching cellular metabolic supply and demand in energy-stressed animals. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **153**, 95-105.
Storey, K. B. and Storey, J. M. (2007). Tribute to P. L. Lutz: putting life on "pause"–molecular regulation of hypometabolism. *J. Exp. Biol.* **210**, 1700-1714.
Tennessen, J. M., Barry, W. E., Cox, J. and Thummel, C. S. (2014). Methods for studying metabolism in *Drosophila*. *Methods* **68**, 105-115.
Wegener, G. (1987). Insect brain metabolism under normoxic and hypoxic conditions. In *Arthropod Brain: Its Evolution, Development, Structure, and Functions* (ed. A. P. Gupta), pp. 369-397. New York, NY: Wiley-Interscience.
Wegener, G. (1993). Hypoxia and posthypoxic recovery in insects: physiological and metabolic aspects. In *Surviving Hypoxia: Mechanisms of Control and Adaptation* (ed. P. W. Hochachka, P. L. Lutz, T. J. Sick and M. Rosenthal), pp. 417-434. Boca Raton, FL: CRC Press.
Wickham, H. (2009). *Ggplot2: Elegant Graphics for Data Analysis*. New York: Springer-Verlag.

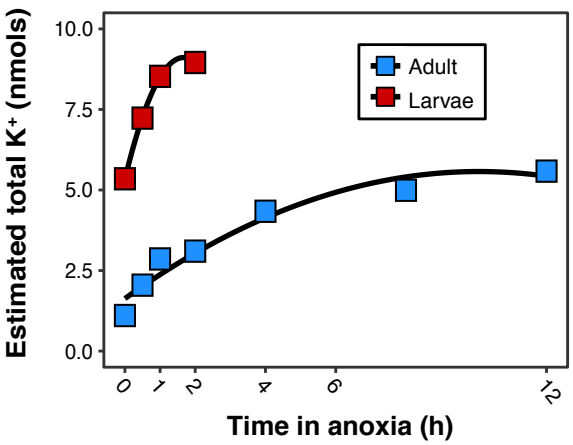

Figure S1. Estimated K+ efflux (nmols) after correcting for differences in hemolymph volumes (adults: 0.064 µl, larvae: 0.178 µl).

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