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RESEARCH ARTICLE

Effects of anoxia on ATP, water, ion and pH balance in an insect (*Locusta migratoria*)

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ABSTRACT

When exposed to anoxia, insects rapidly go into a hypometabolic coma from which they can recover when exposed to normoxia again. However, prolonged anoxic bouts eventually lead to death in most insects, although some species are surprisingly tolerant. Anoxia challenges ATP, ion, pH and water homeostasis, but it is not clear how fast and to what degree each of these parameters is disrupted during anoxia, nor how quickly they recover. Further, it has not been investigated which disruptions are the primary source of the tissue damage that ultimately causes death. Here, we show, in the migratory locust (*Locusta migratoria*), that prolonged anoxic exposures are associated with increased recovery time, decreased survival, rapidly disrupted ATP and pH homeostasis and a slower disruption of ion ($[K^+]$ and $[Na^+]$) and water balance. Locusts could not fully recover after 4 h of anoxia at 30°C, and at this point hemolymph $[K^+]$ was elevated 5-fold and $[Na^+]$ was decreased 2-fold, muscle [ATP] was decreased to $\leq 3\%$ of normoxic values, hemolymph pH had dropped 0.8 units from 7.3 to 6.5, and hemolymph water content was halved. These physiological changes are associated with marked tissue damage *in vivo* and we show that the isolated and combined effects of hyperkalemia, acidosis and anoxia can all cause muscle tissue damage *in vitro* to equally large degrees. When locusts were returned to normoxia after a moderate (2 h) exposure of anoxia, ATP recovered rapidly (15 min) and this was quickly followed by recovery of ion balance (30 min), while pH recovery took 2–24 h. Recovery of $[K^+]$ and $[Na^+]$ coincided with the animals exiting the comatose state, but recovery to an upright position took ~90 min and was not related to any of the physiological parameters examined.

KEY WORDS: Anoxia tolerance, Anoxic stress, Ion homeostasis, Extracellular potassium, Migratory locust

INTRODUCTION

Most biological processes in animals are fueled by the continuous ATP delivery from aerobic respiration. Oxygen availability is therefore critical for the ability of animals to function and survive and most animals die soon after they are exposed to anoxia (von Brand, 1946; Lutz and Nilsson, 1997; Boutilier, 2001). The sensitivity to anoxia is particularly high for endothermic vertebrates because their high metabolic demands quickly deplete internal

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energy stores (Wegener and Moratzky, 1995; Lutz and Nilsson, 1997). Humans, for example, cannot recover fully from 5−8 min of anoxia because cardiac and brain function are compromised shortly after aerobic ATP production ceases (Wegener and Moratzky, 1995; Lutz and Nilsson, 1997). Nevertheless, anoxia tolerance varies considerably among animal species and some species have evolved the ability to tolerate anoxia for prolonged periods (von Brand, 1946; Lutz and Nilsson, 1997; Bickler and Buck, 2007). Anoxia-tolerant animals, such as painted turtles, crucian carp, brine shrimps (*Artemia*) and chironomid fly larvae, are all able to survive many months without oxygen, particularly at low temperatures (von Brand, 1946; Hoback and Stanley, 2001; Jackson, 2002; Bickler and Buck, 2007). These extremely tolerant species are often characterized by large glycogen stores (Bickler and Buck, 2007) as well as depressed and/or modified energy metabolism that allows increased reliance on anaerobic energy metabolism (Hoback and Stanley, 2001; Hochachka and Lutz, 2001; Bickler and Buck, 2007). Furthermore, extreme anoxia tolerance is universally associated with the ability to maintain cellular viability despite extreme metabolic depression (Hochachka, 1986; Hochachka and Lutz, 2001).

Terrestrial insects are obligate aerobes and are rarely considered among the most anoxia-tolerant species. Nevertheless, many insects are endowed with considerable anoxia tolerance (von Brand, 1946; Hoback, 2012). This is somewhat surprising considering that small insects have a relatively high mass-specific metabolic rate (Krogh and Weis-Fogh, 1951; Moratzky et al., 1993). The migratory locust (*Locusta migratoria*), for example, has an aerobic resting metabolic rate of ~4.70 ml O_2 kg^{-1} min^{-1} at 20°C (after Wegener and Moratzky, 1995). This is slightly higher than the resting human metabolic rate of ~3.50 ml O_2 kg^{-1} min^{-1} (Jetté et al., 1990). However, locusts can recover after >4 h of anoxia at 20°C, which is impressive considering that humans only tolerate a few minutes of anoxia (Wegener and Moratzky, 1995). Similarly, at 25°C, adult *Drosophila melanogaster* survive >6 h of anoxia and these insects have a resting normoxic metabolic rate of ~50 ml O_2 kg^{-1} min^{-1} (after Callier et al., 2015). Anoxia tolerance is often even more pronounced among the aquatic or semi-aquatic insects, which are also much more likely to experience hypoxia or anoxia in their natural habitats (von Brand, 1946; Hoback, 2012).

Many insects, but not all (e.g. fruitfly larvae; see Callier et al., 2015), enter a neuromuscular coma when exposed to anoxia (Wegener, 1993; Rodgers et al., 2010) and longer anoxic exposures reduce survival and increase the time it takes for insects to recover from anoxia (Wu et al., 2002; Lighton and Schilman, 2007). The lack of aerobic ATP production disrupts ATP homeostasis, causing ATP concentrations to drop rapidly (Wegener, 1993; Hoback, 2012; Campbell et al., 2018). ATP concentrations tend to decrease even further with prolonged anoxia as has been shown in *L. migratoria*

(Weyel and Wegener, 1996) and *D. melanogaster* (Campbell et al., 2018). It has been suggested that anoxic mortality only occurs when ATP levels are close to zero and anoxic survival may therefore involve some ability to regulate/maintain ATP at a low but non-zero level (Campbell et al., 2018). The low ATP levels are likely to reduce the activity of ion-motive ATPases, resulting in a gradual loss of ion homeostasis during anoxia. This is particularly problematic if extracellular K^+ concentration rises (Rodgers et al., 2010; Campbell et al., 2018), as a disrupted transmembrane $[K^+]$ gradient leads to cell membrane depolarization, which can initiate apoptotic or necrotic processes (Hochachka, 1986; Bortner et al., 2001; Armstrong et al., 2011; Bayley et al., 2018). Elevated extracellular $[K^+]$ has been observed in both brain (Armstrong et al., 2011; Rodriguez and Robertson, 2012) and hemolymph (Campbell et al., 2018) of anoxic *D. melanogaster*. Further, it is likely that the continued reliance on anaerobic metabolism causes severe acidification as has been demonstrated in the hemolymph of *D. melanogaster* (Campbell et al., 2018). This anaerobic acidosis is another possible source of anoxic injury (Storey and Storey, 1990) and it is also possible that injury is linked to oxidative damage, particularly during re-oxygenation upon recovery from anoxia (Hermes-Lima et al., 2015; Moreira et al., 2016).

Despite differences in tolerance, almost all animals will inevitably die if exposed to continuous anoxia. Yet, it is still not understood how depletion of energy stores, loss of homeostasis or accumulation of toxic waste products contributes to cell damage and death in insects (Moratzky et al., 1993; Harrison et al., 2018). Anoxic cell injury and death can in theory be a consequence of an extracellular K^+ surge and the associated membrane depolarization (Bayley et al., 2018). It may also be linked to acidification of tissues from extended anaerobiosis. Other downstream effects of ATP depletion may also contribute to anoxic injury [such as damage to proteins, disruption of osmotic homeostasis or excessive formation of reactive oxygen species (ROS) during re-oxygenation] (Harrison et al., 2018; Bergamini et al., 2004; Hermes-Lima et al., 2015; Lighton and Schilman, 2007). Several of these detrimental processes occur simultaneously and it is therefore possible that these factors act additively to cause anoxic injury. To understand how disruption of ATP, ion, water and pH homeostasis are linked to anoxic injury and loss of function in insects, the present study examined the effects of anoxic exposure and recovery on animal performance and *in vivo* ATP, ion, water and pH homeostasis. In addition, we investigated, for the first time, whether anoxia causes cellular damage *in vivo* in muscle tissue, and we examined *in vitro* whether injury is a result of the isolated or combined effects of hyperkalemia, acidosis and anoxia.

MATERIALS AND METHODS
Animals
All experimental animals used in this study were adult *Locusta migratoria* (Linnaeus 1758) aged 1–4 weeks after the final ecdysis. The animals were purchased from a commercial supplier (Peter Andersen Aps, Fredericia, Denmark) as 5th instar nymphs and housed in a ventilated 0.45 m³ container with metal mesh and egg trays for hiding and molting. Room temperature was set to 25°C and locusts were kept under a daily 16 h:8 h light:dark cycle. During light hours, animals had access to a heating lamp creating a temperature gradient (from 25 to ~45°C) and allowing behavioral thermoregulation. Animals were fed fresh wheat sprouts daily and had *ad libitum* access to wheat bran and water. Females and males were used in a 1:1 ratio in all experiments and data from the two sexes were pooled as no sex differences were observed.

Experimental protocol
Experiments were designed to investigate the effects of anoxia on animal performance (recovery time and recovery score), ion balance (intracellular and extracellular $[Na^+]$ and $[K^+]$), water balance (hemolymph volume), energy balance ([ATP]) and pH balance (hemolymph pH). The physiological measurements were made in separate experimental series in animals exposed to anoxia at 30°C for 0, 0.25, 0.5, 1, 2, 3 or 4 h and in animals that had recovered in normoxia for 0, 0.25, 0.50, 1, 2 or 24 h following a 2 h anoxic exposure.

On the day of the experiments, locusts were placed in glass jars (760 ml) with metal lids. The jars were placed in an incubator set at 30°C and each jar was connected to a RM-8 Flow Multiplexer (Sable Systems International, Las Vegas, NV, USA) that allowed for sequential flow of pure nitrogen (N_2) to eight individual jars. To minimize desiccation of experimental animals, the gas was hydrated using a gas wash bottle filled with water and humidified tissue paper was placed in each glass jar. Anoxic conditions in a jar were always initiated by 10 min of constant high flow rate of N_2 to ensure a quick purge of the original gas (room air) from the jars. The flow was not measured but anoxic coma was always induced in ≤3 min. After the initial purging phase, each jar was flushed at regular intervals (typically for 1 min every 5 min), thereby ensuring that all jars remained anoxic. Control animals were kept in an incubator at 30°C in a small cage with no food for 1 h before sampling. For experiments involving recovery (except for the experiment assessing recovery time and performance score; see below), animals were moved from the glass jars and allowed to recover at 20–22°C in normoxia for up to 2 h with no access to food and water. However, locusts that recovered for 24 h were kept at 30°C for the remaining 22 h and during this time the animals had access to both water and wheat bran *ad libitum*.

Anoxic coma recovery time and performance score
Recovery time and performance score were used to assess the effects of anoxia on animal performance. After anoxic exposure (*N*=8 per treatment), locusts were observed for up to 3 h and the time to first movement and time to standing were noted for each individual. Locusts were stimulated during this recovery period by gently blowing air onto them every minute. After standing or after the 3 h had passed, locusts were placed in small cages at 30°C with *ad libitum* access to water and wheat bran. Twenty-four hours after the anoxic exposure, a performance score was obtained for each animal by scoring the animals from 0 to 5 [0=no observable movement (dead), 1=any observable movement, 2=able to stand, 3=able to walk, 4=able to jump and 5=able to fly].

ATP balance
Concentrations of ATP were measured in femur muscle tissue sampled during anoxic exposure and following recovery. All anoxic samples were prepared inside an airtight glove bag filled with nitrogen (Aldrich AtmosBag Z564451, Merck KGaA, Darmstadt, Germany). Samples were taken in a 30°C room and the glove bag was flushed with nitrogen several times to ensure that the samples did not come into contact with oxygen during the sampling procedure. The glove bag was equipped with inlet ports for gas inflow and outflow as well as electrical wires for instruments (i.e. the scale and Tissuelyser were placed in the glove bag during sampling). Samples taken during the recovery period were prepared at 20–22°C in normoxia.

Muscle samples were collected (inside the glove bag) by cutting off the hindlegs below the coxae and squeezing the femur muscle

tissue out with forceps. Muscle tissue was gently blotted on Kimwipes to remove residual hemolymph before it was transferred to a pre-weighed 2 ml microcentrifuge tube and weighed. A 40 μl homogenization buffer [6 mol l^{-1} guanidine HCl, 100 mmol l^{-1} Tris (pH 7.8) and 4 mmol l^{-1} EDTA] was added for each milligram of muscle together with a stainless-steel bead. This chaotropic buffer ensured stabilization of all adenylates and the tube containing muscle, buffer and stainless-steel bead was frozen in liquid nitrogen. After sampling, the frozen tubes were placed in a Tissuelyser LT (Qiagen, Hilden, Germany) and homogenized at 50 Hz while they were thawing. Samples remained cold until complete homogenization (typically 5–10 min) and were then returned to liquid nitrogen before being removed from the anoxic glove for storage at −80°C.

Later, all samples were warmed to 100°C (with closed lids to avoid evaporation) for 5 min, after which they were centrifuged at 14,000 *g* for 3 min in a 4°C centrifuge (3-18K, Sigma, Osterode am Harz, Germany). Samples of supernatant were then returned to −80°C until measurement of ATP. ATP was quantified using a bioluminescence assay (Molecular Probes #A22066, Eugene, OR, USA) as described by Campbell et al. (2018).

Ion balance

Extracellular concentration of K$^+$ and Na$^+$ ([K$^+$]$_o$ and [Na$^+$]$_o$) were estimated from hemolymph sampled during anoxia or following recovery (N=7–8 per treatment). Hemolymph samples (>5 μl) were obtained with a 25 μl capillary from the cervical membrane. If insufficient, additional hemolymph volume was collected from the hindlegs. A 5 μl volume of hemolymph was then pipetted from the capillary tube into a 2 ml microcentrifuge tube containing 2 ml 100 ppm Li$^+$ buffer (Sherwood Scientific Ltd, Cambridge, UK) for later determination of ion composition by flame photometry (Model 420, Sherwood Scientific Ltd). Measurements were referenced to standards of known concentration.

Intracellular concentration of K$^+$ and Na$^+$ ([K$^+$]$_i$ and [Na$^+$]$_i$) were estimated from measurements of muscle samples during anoxia or following recovery (N=7–8 per treatment). Collection of femur muscle tissue was performed using the same procedure as for adenine nucleotide measurements. After muscle collection, the muscles were dried for 24 h at 58°C. [K$^+$]$_i$ and [Na$^+$]$_i$ were determined by lysing dried muscle tissues in 200 μl Milli-Q water using a stainless-steel bead and a Tissuelyser at 50 Hz for 10 min. The samples were then centrifuged at 10,000 *g* for 10 min (3-18K, Sigma) and 20 or 5 μl of supernatant was placed in 2 ml 100 ppm Li$^+$ buffer for later determination of [Na$^+$]$_i$ and [K$^+$]$_i$, respectively. Concentrations were corrected according to the assumption that muscle tissues contain a 4% residual hemolymph volume (after MacMillan et al., 2012), using the following equation:

$$\text{Corrected}[X]_i = \frac{[X]_i - (0.04[X]_o)}{0.96}, \qquad (1)$$

where [X]$_i$ is the concentration of a specific ion measured in the muscle tissue, and [X]$_o$ is the concentration of a specific ion measured in the hemolymph of that animal.

Water balance

Organismal water balance was estimated gravimetrically by weighing the locust before and after withdrawal of its hemolymph (N=7–8). Hemolymph was withdrawn from the cervical membrane and hindlegs as described above. When no further hemolymph could be withdrawn, the animals were opened with a ventral incision and the body cavity was gently and quickly blotted with filter paper

(Filter Paper Type 138, Hounisen, Stilling, Denmark) to remove any remaining hemolymph. The total duration of this extraction was ≤1 min, after which the 'hemolymph-free' animal was reweighed. This method gave water content values comparable with those of a previously used inulin method in *L. migratoria* (O'Sullivan et al., 2017). Muscle water content was determined by comparing wet and dry mass of muscles from intracellular ion concentration measurements (N=7–8 per treatment). To determine gut water content, the entire gut (from the esophagus to the rectum) of the hemolymph-free animal was dissected out. Fatty tissues, connective tissues, Malpighian tubules, tracheal tubes, air sacs but not gastric ceca around the gut were cautiously removed with forceps beforehand. Guts were transferred to pre-weighed 2 ml microcentrifuge tubes while avoiding spillage of gut contents from either end. Total sampling time per locust was ~15 min. The wet mass was then determined, and the guts were dried at 58°C for 4–5 days before measurement of dry mass and calculation of fractional gut water content.

pH balance

Hemolymph pH was measured using ion-selective micro-electrodes (ISMEs) during anoxic exposure or following recovery from anoxia (N=8–10). Hemolymph was sampled as described above and a 5 μl droplet of hemolymph was placed underneath a layer of hydrated paraffin oil in a Petri dish. Electrodes were constructed using borosilicate glass capillaries [TW150-4, World Precision Instruments (WPI), Sarasota, FL, USA]. Capillaries were pulled with an electrode puller (PC-84 Sachs-Flaming Micropipette Puller, Sutter Instruments, Novato, CA, USA) to form a ~3 μm tip. Afterwards, ISMEs were silanized in *N,N*-dimethyltrimethylsilylamine vapor for 1 h at 300°C. Electrodes were then backfilled with 40 mmol l^{-1} KH$_2$PO$_4$, 15 mmol l^{-1} NaCl, 23 mmol l^{-1} NaOH, pH 7.0, and front-filled with an H$^+$-selective ionophore (H$^+$ Ionophore I/Cocktail B, Sigma Aldrich) (Lee et al., 2013). The tips of the electrodes were then dipped in a PVC solution with 10 mg PVC dissolved in 3 ml tetrahydrofuran to prevent displacement of the ionophore when inserted in paraffin oil. A glass reference electrode (1B100F-4, WPI) filled with 500 mmol l^{-1} KCl was prepared to complete the electrical circuit. The voltage between the electrodes was recorded with a differential electrometer (FD223a, WPI) and a MP100A data acquisition system coupled to a computer running AcqKnowledge software (Biopac Systems, Goleta, CA, USA). Calibration of the ISME was done with calibration solutions at pH 6, 7 and 8. The 10-fold concentration differences in [H$^+$] of these calibration solutions allows conversion of voltage recordings to [H$^+$] using the formula:

$$[\text{H}^+]_o = [\text{H}^+]_c \cdot 10^{\Delta V/S}, \qquad (2)$$

where [H$^+$]$_o$ is the [H$^+$] in the hemolymph sample, [H$^+$]$_c$ is the [H$^+$] in the appropriate calibration solution, ΔV is the recorded voltage between the calibration solution and the hemolymph, and S is the slope of the recorded voltage between two calibration solutions with 10-fold differences in [H$^+$]. Measurements were only utilized if the electrodes generated slopes between 50 and 62 mV per log[H$^+$] change, as predicted by the Nernst relationship.

Tissue damage

Tissue damage was estimated in muscle tissue as described by MacMillan et al. (2015a). Here, *in vivo* and *in vitro* experiments were performed. *In vivo* experiments were performed to estimate tissue damage in locusts exposed to anoxia for 4 h while *in vitro* experiments were conducted on semi-intact locusts incubated in

modified buffers (see Table 1). These *in vitro* experiments were performed to estimate tissue damage by the combined and isolated effects of anoxia, acidosis and hyperkalemia.

In the *in vivo* experiments, locusts were either exposed to anoxia or normoxia for 4 h at 30°C before dissection (N=5–6). After treatment, the head, legs, wings, guts and ovaries/testes of the locusts were removed and the insects were placed in a Petri dish filled with 50 ml of buffer at 20–22°C. Control animals were placed in a buffer mimicking normoxic hemolymph composition (in mmol l^{-1}: 110 NaCl, 8 KCl, 3 CaCl$_2$, 2 MgCl$_2$, 1 NaH$_2$PO$_4$, 90 sucrose, 5 glucose, 5 trehalose, 1 proline, 10 Mops, pH 7.3; see Table 1) (modified from MacMillan et al., 2015a), whereas anoxic animals were placed in a modified buffer mimicking hemolymph composition after 4 h of anoxia (in mmol l^{-1}: 78 NaCl, 40 KCl, 3 CaCl$_2$, 2 MgCl$_2$, 1 NaH$_2$PO$_4$, 90 sucrose, 5 glucose, 5 trehalose, 1 proline, 10 Mops, pH 6.5; see Results). For this buffer, we only changed pH to mimic lactic acidosis, although lactate could possibly also influence the process involved in anoxic tissue damage. The mesothoracic posterior tergocoxal muscle (M90; Snodgrass, 1929) was used for these studies as it is easy to isolate without substantial mechanical damage. The muscle was exposed by carefully removing fatty tissues and trachea, and the muscle was then dissected out with micro-scissors and placed onto a glass slide to stain live and dead cells using the LIVE/DEAD Sperm Viability Kit (Thermo Fisher Scientific, Waltham, MA, USA) previously described by Yi and Lee (2003). Briefly, the muscles were incubated for 13 min in 35 µl of the appropriate buffer (i.e. that used for dissection) containing 38 µmol l^{-1} SYBR 14 (staining live cells green), after which 35 µl buffer containing 60 µmol l^{-1} propridium iodide (staining dead cells red) was added followed by incubation for 10 min. After the total 23 min incubation time, a glass coverslip was gently placed onto the muscle, and imaging was carried out as described by MacMillan et al. (2015a). Imaging of the muscle was performed through a Zeiss Axio Imager A2 fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany). To visualize live cells with DNA-bound SYBR 14 (excitation λ_{max}=475 nm, emission λ_{max}=516 nm), a filter for FITC was used (excitation λ=450–490 nm, emission λ=515–565 nm), and to visualize dead cells with DNA- and RNA-bound propridium iodide (excitation λ_{max}=535 nm, emission λ_{max}=617 nm) a filter for rhodamine was used (excitation λ=534–568 nm, emission λ=575–640 nm).

Analysis of images was performed with the Fiji distribution of ImageJ as described by MacMillan et al. (2015a) with 600×600 pixel subsamples. Individual green and red images were converted to grayscale, and live and dead cell nuclei were isolated from the background using the automatic threshold function. Cell viability

was found by counting the number of live and dead cells using the particle analysis function. The automated counting was validated by comparison with manual counting on a random subset of images. In a few cases, the automatic threshold did not work, and a manual threshold was used instead.

In vitro experiments were performed with semi-intact locusts as described by MacMillan et al. (2015a). All treatments were carried out at 30°C for 4 h with semi-intact locust preparations (e.g. without head, legs, wings, guts and ovaries/testes). Semi-intact locusts were then incubated in 50 ml of modified hemolymph buffer to mimic physiological anoxia, acidosis and/or hyperkalemia (see Table 1 for an overview of buffers). Buffers for hyperkalemia, anoxia and acidosis were made by increasing the buffer [K$^+$] (and decreasing [Na$^+$] to maintain osmolality), bubbling with a steady flow of N$_2$ and decreasing the buffer pH to 6.5, respectively. As mentioned above, we only changed pH to mimic lactic acidosis, although lactate could possibly also influence the process involved in anoxic tissue damage.

Treatments with the combined effects used buffers with high K$^+$ and low pH and had a steady nitrogen flow (same as the anoxic buffer used for the *in vivo* experiments). Control locusts were incubated in control buffer (same as control buffers used for the *in vivo* experiments). In all treatments, buffers were prewarmed to 30°C and bubbled with either N$_2$ or O$_2$ before use. All non-anoxic treatments had a steady oxygen gas flow. Subsequent dissection, staining, imaging and image analysis were executed following the same procedures as described above for the *in vivo* experiments.

Statistical analysis
Statistical analysis was performed in R (version 3.4.1). Effects of anoxic exposure and recovery on recovery time, ATP, ion concentrations, water content and pH were analyzed with one-way ANOVA, where significant differences between treatment groups were identified using a *post hoc* Tukey's HSD. Furthermore, a *post hoc* Dunnett's test was used in experiments evaluating the effects of recovery from anoxia to see when conditions had recovered to control levels. The effect of anoxic exposure on performance scores was analyzed with a Kruskal–Wallis test, where significant differences between treatment groups were identified using a *post hoc* multiple comparisons test using the kruskalmc() function in the pgirmess package. Effects of different treatments on tissue damage were analyzed with one-way ANOVA with *post hoc* Tukey's HSD to identify statistical significance. All results are presented as means ±s.e.m. and all statistics are related to a P<0.05 level of significance.

RESULTS
Anoxic coma recovery time and performance score
When locusts were exposed to anoxia, they quickly entered a comatose state; however, if they were returned to normoxia after short periods of anoxia, they recovered rapidly (Fig. 1A). Following 15 min of anoxia, it took the locusts 3.5±0.47 min to make their first movement and 17.7±1.49 min before they had returned to an upright position. As expected, recovery time increased with prolonged anoxic exposure ($F_{5,42}$=33.98, P<0.001 for time to first movement; $F_{3,28}$=47.53, P<0.001 for time to standing). Thus, after 4 h of anoxia, locusts made their first movement after 67.4 ±9.29 min and locusts that had been exposed to ≥3 h of anoxia were unable to recover to a standing position within the 3 h observation time.

Anoxic exposure time also had a clear and significant effect on survival and performance when the animals were examined 24 h after they had returned to normoxia (Kruskal–Wallis; χ^2=39.2,

Table 1. Buffer pH values, NaCl and KCl content and type of gas supply

Treatment	pH	NaCl (mmol l^{-1})	KCl (mmol l^{-1})	O$_2$/N$_2$
In vivo				
Control	7.3	110	8	O$_2$
Anoxia	6.5	78	40	N$_2$
In vitro				
Control	7.3	110	8	O$_2$
Combined effects	6.5	78	40	N$_2$
Anoxia	7.3	110	8	N$_2$
Hyperkalemia	7.3	78	40	O$_2$
Acidosis	6.5	110	8	O$_2$

In addition to NaCl and KCl at the indicated concentrations, buffers contained (in mmol l^{-1}): 3 CaCl$_2$, 2 MgCl$_2$, 1 NaH$_2$PO$_4$, 90 sucrose, 5 glucose, 5 trehalose, 1 proline and 10 Mops.
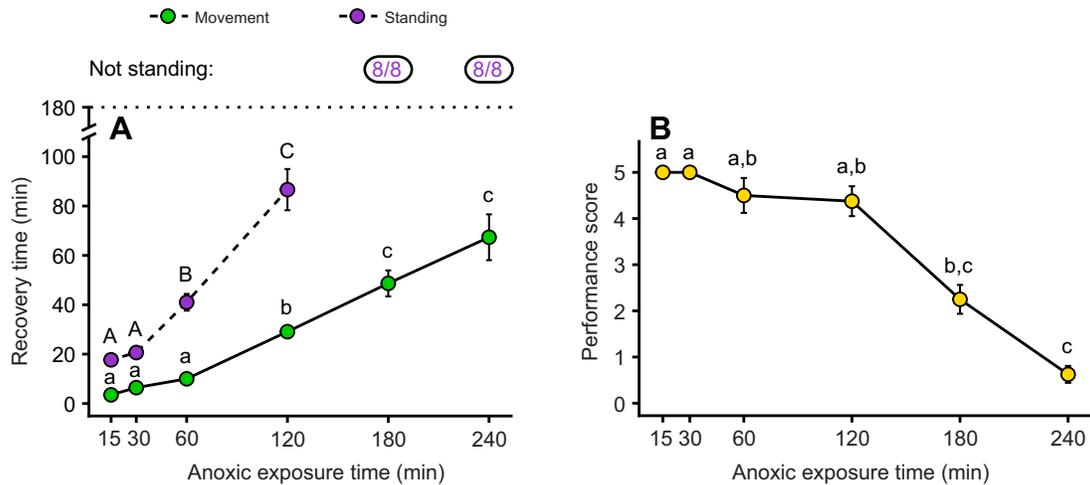
Fig. 1. Recovery after anoxic coma in *Locusta migratoria*. (A) Recovery time measured as the time to first movement (green circles, solid lines) and time to standing (purple circles, dashed lines) after different durations of anoxic exposure. Anoxia was carried out at 30°C and recovery at 20–22°C. Numbers above the dotted line indicate the number of locusts that did not stand within 180 min. $N=8$ locusts per sample point. Dissimilar lowercase letters indicate differences in means for time to move (green circles), and dissimilar capital letters indicate differences in means for time to stand (purple circles) (Tukey's HSD).
(B) Performance score in *L. migratoria* 24 h after different durations of anoxic exposure. The first 3 h of the recovery period were carried out at 20–22°C followed by 21 h at 30°C. Estimation of performance was achieved with a performance score ranging from 0 to 5 (0=no observable movement, 1=any observable movement, 2=able to stand, 3=able to walk, 4=able to jump and 5=able to fly). Dissimilar letters indicate differences in means (Kruskal–Wallis test). All values are means±s.e.m. (non-visible error bars are obstructed by symbols).

$P<0.001$; Fig. 1B). Thus, locusts exposed to ≤30 min of anoxia scored 5, the highest score on the performance scale (0–5, see Materials and Methods). One hour of anoxia resulted in a non-significant tendency for the score to decrease, and the score was lower than control after 3 h of exposure (score of 2.25±0.31). After 4 h of anoxia, all animals had a score of either 1 (movement but not standing) or 0 (dead) (average=0.63±0.18).

ATP balance
Muscle ATP concentrations decreased quickly and dramatically during anoxia ($F_{6,33}=45.48$, $P<0.001$; Fig. 2). Muscle ATP concentration was reduced by ~40% after 15 min exposure, and
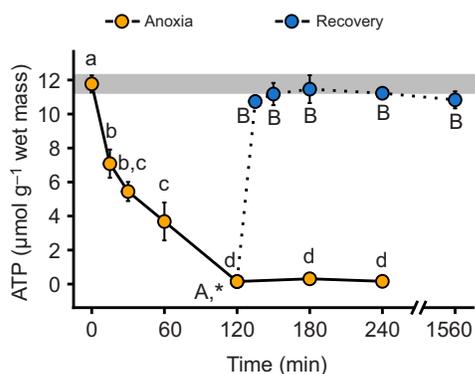

Fig. 2. Muscle ATP of *L. migratoria* exposed to anoxia for different durations or recovering from a 2 h anoxic exposure for different durations in normoxia. Note the break on the *x*-axis allowing inclusion of the 1560 min time point (24 h). The gray area indicates mean±s.e.m. values of control locusts. Asterisks indicate that a recovery value is significantly different from the non-anoxic control value (gray area) (Dunnett's test). Dissimilar lowercase letters indicate differences in means for anoxic exposure (orange circles), and dissimilar capital letters indicate differences in means for anoxic recovery (blue circles) (Tukey's HSD). $N=5$–6 locusts per sample point. All values are means±s.e.m. (non-visible error bars are obstructed by symbols).

this depletion continued steadily until 2 h of exposure, after which ATP was almost fully depleted with a concentration of less than 3% of control values.

Recovery in normoxia after 2 h of anoxia had a significant effect on muscle ATP levels ($F_{5,30}=80.83$, $P<0.001$) and was characterized by an extremely rapid ATP recovery (Fig. 2). Thus, locusts had completely recovered ATP concentration at the first measurement, 15 min after the return to normoxia.

Ion balance
The changes in ion concentration in muscle tissues were generally small during anoxic coma (Fig. 3A,B). There was a slight increase in $[Na^+]_I$, which was significantly higher than control values after 4 h of anoxia ($F_{6,49}=2.548$, $P=0.032$; Fig. 3A), while muscle $[K^+]_i$ remained at control levels during the entire anoxic exposure ($F_{6,49}=0.666$, $P=0.677$; Fig. 3B). The changes in hemolymph ion concentration were much more extensive and were characterized by a significant decrease in $[Na^+]_o$ ($F_{6,49}=18.19$, $P<0.001$; Fig. 3C) and a large increase in $[K^+]_o$ ($F_{6,49}=134.1$, $P<0.001$; Fig. 3D). $[Na^+]_o$ was nearly halved in locusts after 4 h of anoxia, going from 73.3 ±3.5 to 37.5±2.8 mmol l^{-1}, and $[K^+]_o$ increased more than 5-fold from 7.8±0.5 to 41.2±1.6 mmol l^{-1} after 4 h of anoxia.

During recovery from 2 h of anoxia, $[Na^+]_i$ showed a tendency to return to control values (Fig. 3A; $F_{5,49}=2.58$, $P=0.039$), and after 30 min of recovery, $[Na^+]_i$ was no longer significantly different from controls. Curiously, we observed a significant increase in $[Na^+]_i$ after 24 h of recovery (corresponding to the 1560 min mark on the *x*-axis in Fig. 3A). There were no clear changes in $[K^+]_i$ during recovery (Fig. 3B; $F_{5,49}=0.764$, $P=0.58$). Recovery from anoxia was associated with a substantial and fast recovery of both $[Na^+]_o$ and $[K^+]_o$ (Fig. 3C,D; $F_{5,49}=14.91$, $P<0.001$ for $[Na^+]_o$; $F_{5,49}=140.7$, $P<0.001$ for $[K^+]_o$), which returned to control values within 30 min.

Water balance
Anoxia led to a significant decrease in hemolymph volume ($F_{6,48}=4.163$, $P=0.002$), which was more than halved after 4 h of
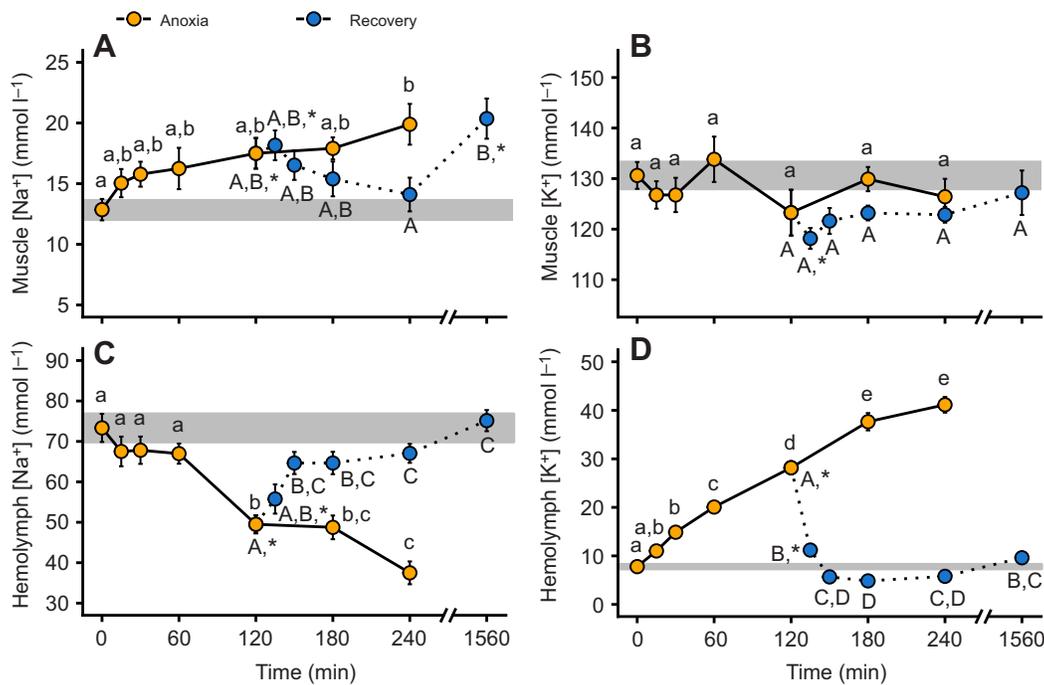
Fig. 3. Intracellular and extracellular concentrations of Na⁺ and K⁺ in *L. migratoria* exposed to anoxia for different durations or recovering from a 2 h anoxic exposure for different durations in normoxia. (A,B) Femur muscle tissue and (C,D) hemolymph levels of Na⁺ (A,C) and K⁺ (B,D). Note the break on the *x*-axis allowing inclusion of the 1560 min point (24 h). Gray areas indicate mean±s.e.m. values of control locusts. Asterisks indicate that a recovery value is significantly different from the non-anoxic control value (gray area) (Dunnett's test). Dissimilar lowercase letters indicate differences in means for anoxic exposure (orange circles), and dissimilar capital letters indicate differences in means for anoxic recovery (blue circles) (Tukey's HSD). *N*=7–8 locusts per sample point. All values are means±s.e.m. (non-visible error bars are obstructed by symbols).

anoxia (Fig. 4A). However, neither muscle ($F_{6,9}$=0.832, P=0.551; Fig. 4B) nor gut ($F_{6,48}$=1.431, P=0.222; Fig. 4C) showed significant changes in water content.

pH balance
Control locusts had a hemolymph pH of ∼7.30 and exposure to anoxia induced a significant decrease in hemolymph pH ($F_{6,54}$=17.97, P<0.001; Fig. 5). After 4 h of anoxia, pH had decreased by approximately 0.8 pH units, corresponding to a ∼6.3-fold increase in $[H^+]_o$.

Locusts recovering from 2 h of anoxia had a gradual and significant rise in hemolymph pH ($F_{5,43}$=35.31, P<0.001; Fig. 5). However, pH recovery was still in progress at 2 h of recovery and pH was not recorded as fully recovered until the animals had recovered for 24 h in normoxia (there were no measurements between 2 and 24 h of recovery).

Tissue damage
Exposure to 4 h of anoxia caused a significant rise in *in vivo* tissue damage, which increased from 6% to 39% ($F_{1,9}$=7.717, P=0.022; Fig. 6A). Muscle cell death was low in control experiments (6%), indicating that the muscle dissection had a relatively small effect on cell survival.

In vitro experiments were designed to examine the isolated and combined effects of acidification, anoxia and hyperkalemia by placing muscle tissue in different experimental conditions. The control group was more damaged under *in vitro* (23%) than *in vivo* conditions (6%) (compare Fig. 6A and B). However, the treatments with acidification, anoxia and hyperkalemia clearly increased tissue damage ($F_{4,30}$=6.912, P<0.001; Fig. 6B) beyond the control values. The isolated effects on tissue damage of hyperkalemia, acidosis and anoxia did not significantly differ from each other or from the

combined effects of all three perturbations. All treatment groups had injury in 44–54% of cells, corresponding to an approximate doubling of tissue damage compared with controls (Fig. 6B). Thus, no clear additive effects of the single variables were evident from these experiments.

DISCUSSION
Anoxia causes disruption of homeostasis
The ability to survive anoxic exposure in most animals is tightly linked to their ability to match energy demand and supply at a suppressed metabolic level (Boutilier, 2001; Hochachka and Lutz, 2001). Many insects show high anoxic tolerance despite very low ATP concentrations (Campbell et al., 2018), and survival in insects may therefore involve tolerating other detrimental effects that are downstream of ATP depletion. Our experiments demonstrated considerable anoxia tolerance of the migratory locust despite it experiencing large physiological changes. Anoxic exposure led to a decrease in muscle ATP concentration that reached a stable plateau of 1–3% of control levels after 2 h of anoxia (Fig. 2). This response is consistent with previous studies on insects (Wegener, 1993; Weyel and Wegener, 1996; Campbell et al., 2018) and the stability of a low but non-zero ATP tissue concentration could indicate a regulated ATP homeostasis (Boutilier, 2001; Campbell et al., 2018). Entering coma when exposed to environmental stressors such as anoxia can in some respects be considered an adaptive mechanism as coma decreases energy demand (Rodgers-Garlick et al., 2011). Energy production of the hypometabolic *L. migratoria* is seemingly supported by anaerobic metabolism, as indicated by the hemolymph pH decrease of ∼0.8 units within the first 2 h of anoxia (Fig. 5). This acidification is probably caused by acidic waste products from anaerobic metabolism and a similar decrease of 1 pH unit was recently observed by Campbell et al. (2018) in *D. melanogaster* after 2 h of anoxia at 25°C.
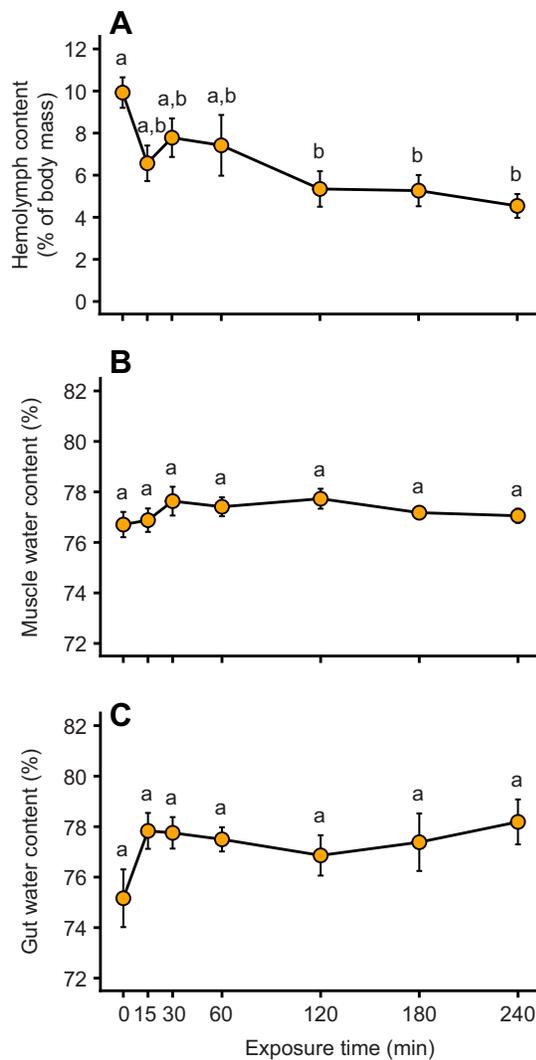
Fig. 4. Water balance in hemolymph, muscle and gut of *L. migratoria* after anoxic exposure for different durations. (A) Hemolymph, (B) femur muscle tissue (C) and gut. Dissimilar letters indicate differences in means (Tukey's HSD). N=7–8 locusts per sample point. All values are means±s.e.m. (non-visible error bars are obstructed by symbols).
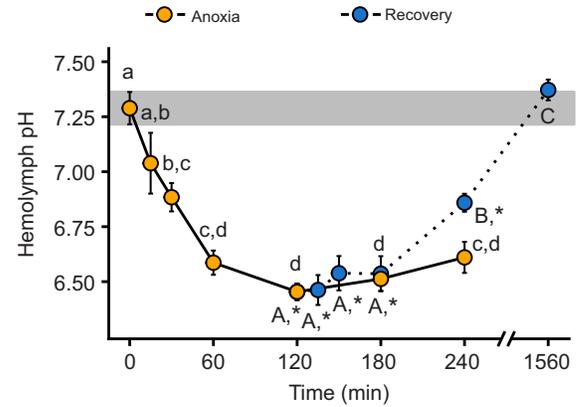

Fig. 5. Hemolymph pH of *L. migratoria* exposed to anoxia for different durations or recovering from a 2 h anoxic exposure for different durations in normoxia. Note the break on the *x*-axis allowing inclusion of the 1560 min point (24 h). The gray area indicates mean±s.e.m. values of control locusts. Asterisks indicate that a recovery value is significantly different from the non-anoxic control value (gray area) (Dunnett's test). Dissimilar lowercase letters indicate differences in means for anoxic exposure (orange circles), and dissimilar capital letters indicate differences in means for anoxic recovery (blue circles) (Tukey's HSD). N=8–10 locusts per sample point. All values are means ±s.e.m. (non-visible error bars are obstructed by symbols).

The pH plateau observed after 2–4 h of anoxia could indicate depletion of anaerobic substrate or be a consequence of depressed ATP turnover following cellular death as many of the animals died and were injured during this time interval (Fig. 1). Clearly, ATP production by anaerobic pathways was not sufficient to sustain ATP levels, and ion or water balance, as all these parameters changed substantially during anoxia (Figs 2, 3 and 4).

The reduction of energy state probably disrupted ion homeostasis through the decreased activity of ion-motive ATPases, leading to a 5-fold increase in $[K^+]_o$ (Fig. 3D), a 2-fold decrease in $[Na^+]_o$ (Fig. 3C) and a 2-fold decrease in hemolymph volume (Fig. 4A). Water and ion balance are tightly linked, and conservation of hemolymph volume requires active reabsorption of water and ions to balance the passive leakage (Gerber and Overgaard, 2018). The large increase in hemolymph $[K^+]_o$ observed in our experiments is similar to previous findings in *Drosophila* (Campbell et al., 2018). This increase in $[K^+]_o$ could not be ascribed to intracellular leakage from muscle cells as these had a nearly constant $[K^+]_i$ (Fig. 3B) and water volume (Fig. 4B). Part of the increase in $[K^+]_o$ is linked to a

decrease in hemolymph volume (Fig. 4A), which concentrates K^+ ions, and this effect is quite similar to that found in many insects exposed to low temperature (MacMillan and Sinclair, 2011; Koštál et al., 2004). The reduction in hemolymph volume cannot, however, explain the 5-fold increase in $[K^+]_o$ as the hemolymph volume was only halved. Another possible source of the increased K^+ could be a leakage from the gut, but this was not tested in the present study. Nevertheless, there is a strong K^+ gradient from both the midgut and the hindgut towards the hemolymph (Dow, 1981), suggesting that inhibition of ATPases in the gut and Malpighian tubule may contribute to the rise in $[K^+]_o$. Interestingly, the decrease in hemolymph water content could not be explained by water movement towards muscle tissue and gut lumen as these only showed small and non-significant increases during anoxia. The loss of hemolymph volume may therefore involve regurgitation or urination, or possibly water is relocated to other tissues.

The increase in $[K^+]_o$ in locust hemolymph is qualitatively similar to the fast anoxic $[K^+]_o$ surges that have been observed in the brains of locusts (Rodgers et al., 2007; Armstrong et al., 2009) and *Drosophila* (Armstrong et al., 2011). These surges occur much faster in the CNS, where the extracellular space is very small, and the $[K^+]_o$ surges cause rapid depolarization within the CNS that lead to coma (Rodgers et al., 2010). Thus, chronic depolarization of either neuronal or muscular tissue will result in a loss of excitability (Hosler et al., 2000; Rodgers et al., 2010) and because these events occur much faster in the CNS, it seems clear that depolarization of neurons during anoxia is the initiating cause of anoxic coma (Hosler et al., 2000; Rodgers et al., 2010; Hou et al., 2014). Depolarization caused by hyperkalemia may also cause cell injury and it is therefore plausible that anoxic damage is partially linked to the massive increase in $[K^+]_o$ that develops during anoxia (see also discussion below).

We observed that $[Na^+]_i$ increased by ~5–8 mmol l⁻¹ (Fig. 3A) and $[Na^+]_o$ decreased substantially (~40 mmol l⁻¹) (Fig. 3C) during anoxia. This suggests that some of the extracellular Na^+ leaked into the muscle cells. Hemolymph volume is substantially smaller than intracellular water volume (Harrison, 1989), so a smaller increase in intracellular Na^+ is expected when Na^+ moves from the hemolymph
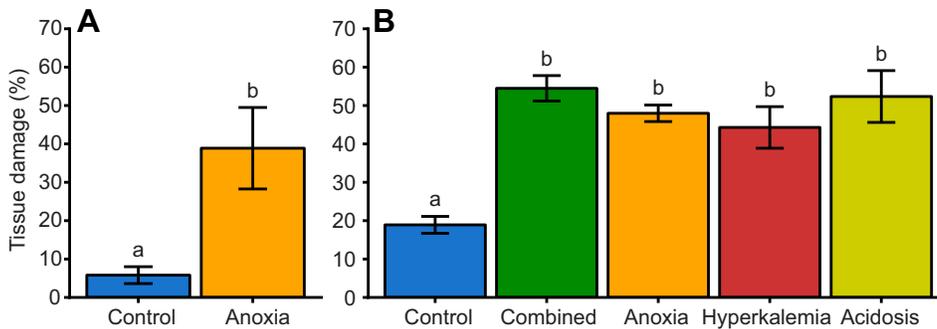
Fig. 6. Muscle tissue damage of _L. migratoria_. (A) _In vivo_ muscle tissue damage of intact locusts. Treatments: control or exposure to anoxia for 4 h. N=5–6 locusts per treatment. Dissimilar letters above bars indicate differences in means (ANOVA). (B) _In vitro_ muscle tissue damage of semi-intact locusts treated for 4 h. Treatments: control, exposure to anoxia only, hyperkalemia only, acidosis only or all three combined. N=6–8 locusts per treatment. Dissimilar letters above bars indicate differences in means (Tukey's HSD). All values are means±s.e.m.

to the cytoplasm. MacMillan and Sinclair (2011), MacMillan et al. (2015b) and Gerber and Overgaard (2018) investigated ion and water balance in _Drosophila_, _Gryllus_ and _Locusta_ during cold exposure where activity of ion motive ATPases is also reduced. These studies found that a cold-induced reduction in Na^+ reabsorption led to the passive transport of Na^+ from the hemolymph to the guts (and possibly also to other tissues) and these studies suggested that hemolymph water would follow Na^+ osmotically. A similar process is also likely to occur in locusts during anoxia as there is a strong gradient for Na^+ diffusion from the hemolymph towards the gut lumen (Dow, 1981). We can therefore conclude that prolonged anoxia leads to massive perturbations of extracellular ion and water balance, but also that further studies are needed to describe the exact nature of these ion and water movements.

Anoxic tissue damage

Prolonged anoxic bouts lead to cell damage and eventually death. This was also observed in the present study, where anoxic exposure led to cellular injury _in vivo_ and _in vitro_ (Fig. 6). We hypothesized that injury would be caused by the additive effects of anoxia, acidosis and hyperkalemia; however, to our surprise, we found that each of these factors in isolation caused the same level of injury as when they were combined (also discussed below). In theory, a lack of ATP can lead to cell damage via the associated effects linked to hyperkalemia and acidosis. High $[K^+]_o$ causes depolarization of cell membranes, and a sufficiently large depolarization activates voltage-dependent Ca^{2+} channels, creating a large Ca^{2+} influx into the cells (Hochachka, 1986; Boutilier, 2001; Bayley et al., 2018). The ensuing increase in $[Ca^{2+}]_i$ may then lead to activation of Ca^{2+}-dependent phospholipases and proteases that cause cell damage and death (Hochachka, 1986; Boutilier, 2001; Bayley et al., 2018). The challenges of defending membrane potential during anoxia are therefore very similar to those experienced by cold-exposed insects as both anoxia and hypothermia cause hyperkalemia (Overgaard and MacMillan, 2017). Furthermore, both cold and anoxia will also reduce the activity of electrogenic ion pumps (e.g. the Na^+/K^+-ATPase) that contribute considerably to insect membrane potential (Huddart and Wood, 1966; Rheuben, 1972). It is therefore likely that part of the cellular injury observed is linked to the gradual accumulation of $[K^+]$, but as evident from Fig. 6B there are also other factors that contribute to cell injury.

Acid–base status has been shown to impact many behavioral and physiological aspects in insects (Harrison, 2001), and low pH values have been demonstrated to directly impact insect survival (see e.g. Bell and Nebeker, 1969; Bell, 1971). All proteins have pH optima for activity and stability, and a large shift to more acidic conditions can retard the proteins' normal activities – possibly resulting in cell damage. Further, acidosis has been shown to depolarize muscle fiber potentials in the moth _Antherea polyphemus_

(Rheuben, 1972) and the butterfly _Pieris brassicae_ (Piek et al., 1977). In the moth, depolarization appeared to be partly due to a decrease in membrane resistance and therefore a decrease of the electrogenic effect (Rheuben, 1972). Thus, hemolymph acidosis might also be involved in depolarization-induced cell damage.

Besides hyperkalemia and acidosis, the lack of ATP may lead to a general loss of organismal homeostasis, which potentially could result in damage (Harrison et al., 2018). Damage to macromolecules, protein aggregation and unfolding of proteins during anoxia are some hypothesized causes of damage, and they are supported by the findings that accumulation of heat shock proteins and trehalose is protective during anoxic exposure of _D. melanogaster_ (Chen et al., 2002; Azad et al., 2009). Another potential indirect effect of low ATP is depolarization of membrane potentials by elimination of the electrogenic effect (see discussion above).

In our experimental design, we tried to examine whether one of these physiological changes was particularly important for causing cellular damage during anoxia. Four hours of anoxia strongly increased cellular damage _in vivo_ and muscle damage _in vitro_ (Fig. 6). Interestingly, the isolated and combined effects of hyperkalemia, acidification and anoxia were equally injurious, and no single variable therefore seemed to cause most of the injury, and no additive effects of the variables were apparent. These results cautiously suggest that all three factors might be capable of triggering the same downstream process of injury, and that each of these perturbations is capable of doing so in isolation. However, further experiments are needed to verify this idea and it would also be interesting to examine to what extent the injury observed is linked to oxidative damage during re-oxygenation when ROS are formed. For example, it has been shown that hypoxia can induce modulations of the antioxidant defense system (Hermes-Lima et al., 2015), although this activation of defense systems is generally weaker in response to hypoxia/anoxia than it is towards other environmental stressors such as estivation, dehydration and freezing (Moreira et al., 2016).

Anoxic recovery

Locusts can physiologically and behaviorally recover surprisingly well after anoxia – if the anoxic bouts are not too long. Thus, locusts were able to completely recover their disrupted ATP levels, and ion and pH balance after a 2 h anoxic exposure, but the time courses of recovery varied dramatically among parameters. Muscle ATP was fully recovered after 15 min (Fig. 2), hemolymph $[K^+]_o$ and $[Na^+]_o$ followed shortly after and were fully recovered after 30 min (Fig. 3C,D), and hemolymph pH required 24 h to return to control values (Figs 2, 3 and 5). The rapid recovery of ATP levels matches findings in previous studies on insects including _L. migratoria_, and it has previously been ascribed to a low degradation of adenylates such that AMP accumulates during anoxia (Wegener, 1993; Weyel

and Wegener, 1996). The maintenance of a large adenylate pool is unlike most mammalian tissues (where AMP is further catabolized), and it allows insects, once re-exposed to normoxia, to rapidly re-synthesize ATP in order to restore cellular functions and recover from the coma (Weyel and Wegener, 1996). The observation that ion balance recovered slower than ATP balance supports the idea that ATP is necessary for restoring ion homeostasis through activation of ion-motive ATPases. pH balance took even longer to restore compared with ATP and ion balance, though it is unknown where in the interval between 2 and 24 h a full recovery took place. No previous studies have investigated recovery of anoxic acidosis in insects, but the notion that pH recovery in insects takes several hours has been demonstrated before. Harrison et al. (1992) showed that the locust *Schistocerca gregaria* had recovered from an extracellular acidification of 0.5 pH units (3.16-fold increase in $[H^+]$) after 8 h (but not after 4 h) at 21°C. Although Harrison et al.'s (1992) experiments were done with another locust species and with half as large a $[H^+]_o$ increase, this shows that pH regulation happens on a time scale that is much slower than recovery of ATP and ion homeostasis.

Interestingly, ATP and ion recovery happened faster than a behavioral recovery (defined as time to standing), which took ~87 min (Fig. 1A), while a complete pH recovery did not occur until after behavioral recovery. Consequently, a complete recovery of ATP, $[K^+]_o$ and $[Na^+]_o$ is not sufficient for behavioral recovery and a complete recovery of pH is not necessary for behavioral recovery. Further, ATP recovery occurred before locusts made their first movements (~29 min; Fig. 1A) while both $[K^+]_o$ and $[Na^+]_o$ recovery matched this time well. This indicates that recovery of ion gradients ($[K^+]$ in particular) is the determining factor in awakening from anoxic coma. Previous studies have shown that recovery from even larger anoxic $[K^+]_o$ surges in locust and *Drosophila* CNS occurs much more quickly (within a few minutes) than the recovery observed in locusts in our experiments (Rodgers et al., 2007; Armstrong et al., 2011), which suggests that recovered ion homeostasis in muscle tissues is the determining factor in coma awakening, which has also been observed to be a limiting factor for insects recovering from cold (MacMillan et al., 2012; Findsen et al., 2014).

Conclusions

In the present study, we have shown that locusts can survive several hours of anoxia at 30°C, although their performance worsens with longer anoxic bouts. Anoxic exposure causes gradual but dramatic disruption of ATP, ion, water and pH homeostasis, all of which are able to induce considerable cellular damage. Recovery of movement following anoxia was only observed after both ATP and ion balance were recovered but animals returned to a standing position before pH balance was recovered. We were unable to identify a single parameter (hyperkalemia, acidosis or anoxia) as the primary cause of cellular damage but showed that all these perturbations can cause cell injury in isolation. The absence of additive effects could indicate that some of the perturbations are acting through similar pathways (e.g. cellular depolarization) that are fully activated with a single or combined perturbation. However, further experiments are needed to verify this suggestion.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: M.V.R., J.B.C., J.F.H., J.O.; Formal analysis: M.V.R.; Investigation: M.V.R., J.B.C., L.G.; Writing - original draft: M.V.R., J.O.; Writing - review & editing: M.V.R., J.B.C., L.G., J.F.H., J.O.; Supervision: J.O.

Data availability
Data are available from the Dryad Digital Repository (Ravn et al., 2019): dryad. tt16kr6.

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