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RESEARCH ARTICLE

Effects of temperature on responses to anoxia and oxygen reperfusion in *Drosophila melanogaster*

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SUMMARY

Insects in general, and *Drosophila* in particular, are much more capable of surviving anoxia than vertebrates, and the mechanisms involved are of considerable biomedical and ecological interest. Temperature is likely to strongly affect both the rates of damage occurring in anoxia and the recovery processes in normoxia, but as yet there is no information on the effect of this crucial variable on recovery rates from anoxia in any animal. We studied the effects of temperature, and thus indirectly of metabolic flux rates, on survival and recovery times of individual male *Drosophila melanogaster* following anoxia and O_2 reperfusion. Individual flies were reared at 25°C and exposed to an anoxic period of 7.5, 25, 42.5 or 60 min at 20, 25 or 30°C. Before, during and after anoxic exposure the flies' metabolic rates (MRs), rates of water loss and activity indices were recorded. Temperature strongly affected the MR of the flies, with a Q_{10} of 2.21. Temperature did not affect the slope of the relationship between time to recovery and duration of anoxic exposure, suggesting that thermal effects on damage and repair rates were similar. However, the intercept of that relationship was significantly lower (i.e. recovery was most rapid) at 25°C, which was the rearing temperature. When temperatures during exposure to anoxia and during recovery were switched, recovery times matched those predicted from a model in which the accumulation and clearance of metabolic end-products share a similar dependence on temperature.

Key words: temperature, ischemia, reperfusion damage, insect, CO_2 production.

INTRODUCTION

The deleterious effects of anoxia in humans (e.g. caused by ischemia) followed by reperfusion with O_2, are well known and are a key topic in biomedical studies. As a model organism *Drosophila melanogaster* has the advantage of ease of culture, a well-characterized genome, ample availability of mutants and ease of genetic manipulation, making it the principal invertebrate model animal in this field (Azad et al., 2009; Kanaan et al., 2006; Lighton and Schilman, 2007). As discussed elsewhere (Lighton and Schilman, 2007), the respiratory system of tracheate arthropods makes them ideal for monitoring the effects of changing O_2 concentration at the subcellular level, i.e. in the mitochondria, because their tissue partial pressure of oxygen (P_{O_2}) can be manipulated *via* changes in air P_{O_2} even after anoxic paralysis, because such animals do not rely on a circulatory system to exchange respiratory gases. Thus, the effects of anoxia and reperfusion on their tissues are largely decoupled from the effects of these parameters on the motor effectors of their circulatory systems. In addition, insects, and invertebrates in general, are ectothermic animals. Their body temperature tracks their environmental temperature, so that the researcher can alter the metabolic rate (MR) of the intact animal simply by changing the temperature of its surroundings. These two facts make tracheate arthropods, such as insects, excellent models to study the modulatory effect, if any, of temperature, and therefore MR, on recovery from anoxia.

It has been known for years that *D. melanogaster*, like many insects, shows excellent recovery from short bouts of anoxia lasting less than 4 h (Krishnan et al., 1997). Adult flies do not encounter anoxia in the wild, so it is probable that their resistance to anoxic exposure is an exaptation derived from larval exposure to hypoxic or even anoxic conditions. Notwithstanding *Drosophila*'s impressive resistance to anoxia, Lighton and Schilman have recently demonstrated that they are damaged by repeated short bouts (60 s) of normoxia during prolonged anoxia; with CO_2 emission rates and spiracular control reduced with higher numbers of exposures to O_2 (Lighton and Schilman, 2007). This was the first evidence of physiological damage caused by O_2 reperfusion in any invertebrate. However, multiple (i.e. 5 or 10) 15–20 s anoxic bouts interspersed with longer duration normoxia that allowed recovery shows no negative effects on the MR of flies after recovery (Van Voorhies, 2009). Although the two studies were methodologically a little different, i.e. Lighton and Schilman used single fly, flow-through respirometry with good time resolution, as well as measuring water loss rates, whereas Van Voorhies pooled groups of ~100 flies, which may have obscured some subtle effects, we can conclude that short cycles of normoxia interspersed in prolonged anoxia is more damaging than short anoxic exposures during prolong normoxia (Lighton and Schilman, 2007; Van Voorhies, 2009).

The duration of anoxic exposure directly and linearly affects the time required to recover from such exposure (Krishnan et al., 1997).

Presumably this occurs because longer durations of anoxia lead to greater damage, including such processes as accumulation of anaerobic end-products, loss of ion gradients and protein unfolding (Harrison and Haddad, 2011). Longer anoxic exposure may also cause mitochondrial changes that increase reactive oxygen species (ROS) production during re-exposure to O_2. The processes that occur during recovery are less understood, but probably include removal of anaerobic end-products, restoration of ion and water gradients, and repair and/or restoration of protein function (Harrison and Haddad, 2011).

Temperature is a ubiquitous environmental variable for ectotherms, affecting virtually every aspect of metabolic function. Thermal effects on recovery time from anoxic exposure are likely to depend on the relative temperature sensitivity of damage rates during anoxia and repair rates during subsequent re-exposure to normoxia. Higher body temperatures will increase metabolic rates of ectotherms, probably leading to increased production rates of anaerobic end-products and ROS during re-exposure to O_2. Higher metabolic rates will increase the rate of formation of anaerobic end-products: lactate, alanine and acetate in *Drosophila* (Feala et al., 2007). Higher temperatures probably also increase ion leakages that disrupt membrane electrical function. During recovery, higher temperatures would be expected to increase the activity of ion pumps restoring membrane ion gradients, catabolic ATP production and enzymatic repair. Increased temperature will also increase MR, probably increasing the rate of reperfusive damage.

The prediction, which to our knowledge has never been experimentally tested, is that if the thermal sensitivity of damage and recovery process are similar, then the recovery time at any given anoxic duration will be independent of temperature and thus indirectly of MR. Alternatively, correlations between temperature and recovery time could point to specific key processes. For example, anoxic damage could be primarily due to ROS production during re-exposure to O_2, with ROS production increasing exponentially with MR. In this case the slope of the regression line relating the duration of anoxic exposure to recovery time should increase as MR is increased. Another possibility is that recovery time may be primarily limited by the rates of recovery processes such as ion pumping and protein repair; if this is the case then recovery time from a given anoxic exposure should decrease at higher temperatures.

Another factor that may influence the effect of temperature on recovery times from anoxia is thermal adaptation or acclimation. There is abundant evidence that ectotherms, including *Drosophila*, demonstrate both acclimatory and adaptive responses that enhance performance at the habitat temperature (Gibbs et al., 1998; Overgaard et al., 2008). Such compensatory alteration of structure could result in the shortest recovery times occurring at the rearing temperature for these flies.

MATERIALS AND METHODS

Animals

Male *Drosophila melanogaster* Meigen, between 4 and 10 days of age were used in the experiments. All flies were of the Canton-S strain, reared at 25°C at the University of Nevada at Las Vegas, NV, USA, and kept at Sable Systems International (SSI, Las Vegas, NV, USA) at the ambient light:dark cycle at a controlled temperature of 25±0.2°C.

Respirometry

Flow-through respirometry with automated switching between anoxia and normoxia was performed as described by Lighton and Schilman (Lighton and Schilman, 2007). Briefly, we used a Sable Systems International SI-1 CO_2-based flow-through respirometry system, capable of measuring the CO_2 and water vapor output of individual flies in real time at a mass flow rate of 50 ml min^{-1}. The respirometry chamber (interior volume <1 ml) was constructed of metal, with sapphire windows at each end to allow photoelectric detection of activity while maximizing thermal homogeneity (Drosophilator; SSI). The fly was therefore surrounded by a highly thermally conductive environment. Air entering the chamber for flow-through respirometry was first equilibrated to the chamber's temperature by traveling through a serpentine path milled into the upper metal surface of the metal block from which the chamber was machined. The chamber was in tight thermal contact with a temperature-controlled plate (PELT-5; SSI), which controlled the chamber's temperature with a precision of ±0.1°C (Fig. 1). The input gas stream for the system could be switched between ambient air scrubbed of CO_2 and pure N_2. Any residual leakage of CO_2 into the inactive side of the solenoid valve was eliminated by a small downstream CO_2 scrubber. We experimentally verified that switching between gas streams took place without affecting the CO_2 readings. Essentially complete transition between normoxia and anoxia took place in <30 s. Switching of the incurrent airstream and periodic baselining of the system took place automatically under the control of the data acquisition system (ExpeData, SSI). Data were sampled at 1 Hz, using intra-sample finite impulse response digital filtration to reduce analyzer noise (Lighton, 2008).

Experimental procedure

In the first series of experiments, an individual male *Drosophila* was directly aspirated from its container and placed in the chamber. The recording began with a 3 min baseline segment to establish the zero points for the CO_2 and water vapor analyzers. After that, the CO_2 and H_2O released by the fly were measured for 24 min (plus another baseline after 12 or 17 min). The incurrent air was changed to pure N_2 for 7.5, 25, 42.5 or 60 min. After anoxia exposure, measurement continued for another 30–60 min (depending on the anoxia duration), and at the end another 3 min baseline was taken. The same procedure was repeated at 20, 25 and 30°C. When the

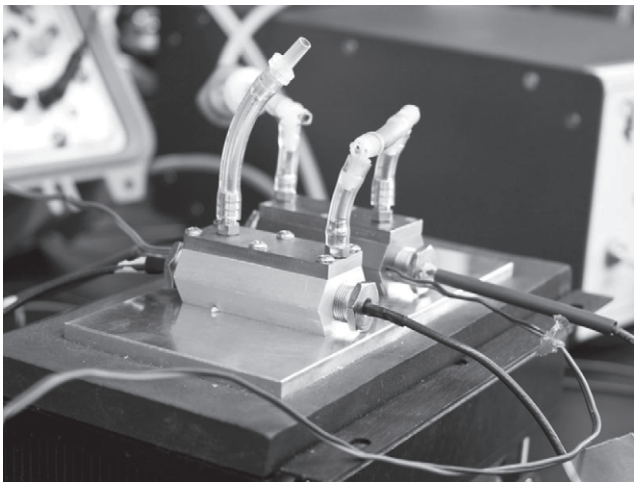

Fig. 1. Metal respirometry chambers (volume <1 ml) with sapphire windows at each end to allow photoelectric detection of activity used in all experiments. The chamber was in tight thermal contact with a temperature-controlled plate. Air entering the chamber was equilibrated to the chamber's temperature by traveling through a serpentine path milled into the upper metal surface of the metal block.

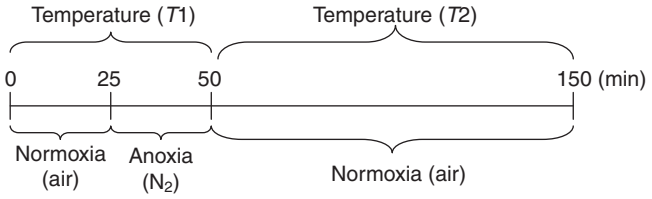

Fig. 2. Schema showing the protocol used in the second series of experiments. Time is in minutes. Temperature in the first 50 min (*T*1) was fixed at 20 or 30°C. For the first 25 min, flies were exposed to normoxic conditions. For the next 25 min flies were exposed to an anoxic atmosphere (pure nitrogen). Temperature in the last 100 min (*T*2) was also fixed at 20 or 30°C. *T*2 was the period allowed for recovery in normoxic conditions. Treatments were performed at 20/20; 20/30 and 30/20°C (*T*1/*T*2, respectively).

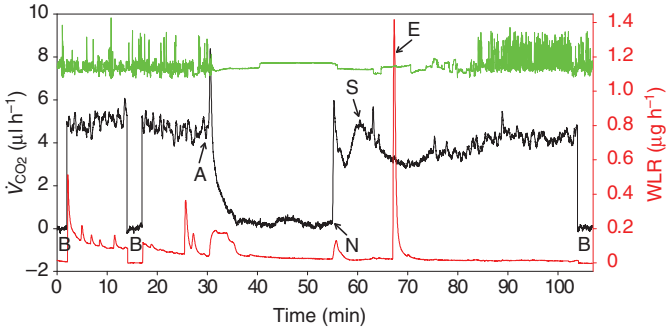

Fig. 3. Typical traces of a 25 min anoxia exposure at 25°C (single male fly, mass 0.793 mg). Black trace: rate of CO_2 emission ($\dot{V}_{CO_2}$); red trace: rate of water loss (WLR); green trace: activity (arbitrary units). A, initiation of anoxia; B, baseline (respirometer chamber switched out of circuit under program control); E, excretion event (which demonstrates the rapid response time for the water vapor signal); N, re-initiation of normoxia; S, secondary peak of CO_2 output.

recording was complete, the fly was immobilized with N_2, removed from the chamber and weighed to the nearest 1 µg using a Cahn C-32 ultramicrobalance (Cahn Instruments Inc., Cerritos, CA, USA). The mass of water lost during the recording, as determined from the respirometry data, was added to its post-recording mass (Lighton et al., 2004).

In order to uncouple the effect of temperature, and thus MR, on metabolic end-product production and clearance, a second series of experiments was performed. In this series, only the activity was monitored because it has been established that no significant differences exist between critical P_{O_2} values calculated from activity-based and CO_2 production-based methods (Klok et al., 2010). The second series of experiments consisted of two periods with temperatures *T*1 and *T*2. *T*1 lasted for 50 min. During half of that time, i.e. 25 min, the flies were exposed to normoxic conditions, and during the other 25 min flies were exposed to an anoxic atmosphere. *T*2 lasted for the 100 min of the recovery period in normoxic conditions at temperature 2 (see schema in Fig. 2). *T*1 and *T*2 were fixed at 20 or 30°C giving, as a result, the following treatments: 20/20, 20/30 and 30/20°C (*T*1/*T*2, respectively).

Resumption of activity after anoxic exposure was clearly evident in the photoelectric activity record, and was objectively quantified using the absolute difference sum breakpoint method (Lighton and Turner, 2004). Recovery times were measured, analyzed and compared across groups.

Statistics

CO_2 and water vapor signals were converted and expressed in µl h^{-1} and µg h^{-1}, respectively [for a complete set of equations see Lighton (Lighton, 2008)]. Values are means ± standard errors (s.e.m.) and the sample sizes (*N*) are given. Regressions were computed by the least squares method, with significance testing by ANOVA. Regressions were compared using analysis of covariance (ANCOVA) using the RudeStat statistical package within ExpeData. For the second experimental series, the effect of temperature, i.e. MR, on the proportion of recovered flies during the assay time (i.e. 100 min) was tested using the χ^2-test of independence, and the effect of temperature on recovery times was tested using one-way ANOVA and *a posteriori* Tukey's tests (Zar, 1984); these tests were carried out within InStat (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

The mean body mass of the flies was 0.878±0.006 mg (*N*=101). Basic statistics can be found in Table 1. In the first series of experiments to assay the effects of body mass and temperature on the $\dot{V}_{CO_2}$ of the flies, we performed a multiple regression analysis of log-transformed $\dot{V}_{CO_2}$ against log-transformed body mass and measurement temperature. As expected, given the limited variance of body mass, no significant effect of body mass on $\dot{V}_{CO_2}$ was found (t_{98}=0.13, P>0.4). Thus, we expressed $\dot{V}_{CO_2}$ on a whole-animal rather than a mass-specific basis. Expressed as the relationship between log-transformed $\dot{V}_{CO_2}$ and temperature, the effect of temperature on $\dot{V}_{CO_2}$ was profound (t_{98}=10.77, $P<10^{-6}$), with a slope of 0.0343, corresponding to a Q_{10} of $10^{0.343}$ or 2.21 (overall significance: $F_{2,98}$=59.77, $P<10^{-6}$).

The effects of anoxic exposure were similar to those previously reported for *D. melanogaster* strain Oregon-R at 25°C (Lighton and Schilman, 2007). Fig. 3 shows a typical example. Briefly, a short peak of CO_2 emission occurred at the onset of anoxia, followed by a rapid decline to values close to the system baseline of zero CO_2. Following resumption of normoxia, mitochondrial CO_2 production resumed and recovery, as assayed by resumption of voluntary motor activity, resumed in due course.

Our primary aim was to assess the effects of changing MR on the relationship between the duration of anoxic exposure and the time taken for recovery, i.e. restoration of voluntary motor activity. The slope of this line was not affected by measurement temperature and thus was unaffected by MR (Fig. 4). The common, dimensionless slope was 0.583±0.028.

Table 1. Rate of CO_2 emission and water loss of male *Drosophila melanogaster* at 20, 25 and 30°C

Temperature (°C)	$\dot{V}_{CO_2}$ (µl h^{-1})	P$\dot{V}_{CO_2}$ (µl h^{-1})	WLR (µg h^{-1})	*N*
20	2.58±0.14	4.59±0.15	0.066±0.007	34
25	3.73±0.20	6.61±0.21	0.078±0.009	31
30	5.47±0.16	8.72±0.23	0.124±0.016	36

$\dot{V}_{CO_2}$, rate of CO_2 emission per fly; P$\dot{V}_{CO_2}$, peak rate of CO_2 emission per fly at the start of anoxia exposure; WLR, rate of water loss per fly; *N*, sample size.

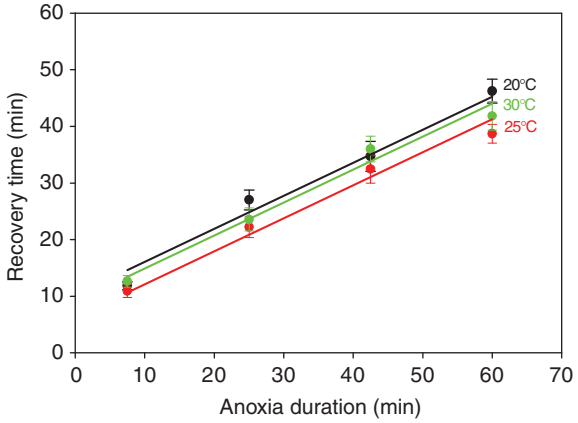

Fig. 4. The relationship between anoxia exposure duration and recovery time (the point at which the photoelectric activity recording revealed that voluntary activity had resumed) at 20, 25 and 30°C (black, red and green traces, respectively). Temperature, and thus metabolic rate, had no significant effect on the slope of recovery time *vs* duration of anoxic exposure (ANCOVA: $F_{2,81}$=1.12, P=0.3). Slopes measured at 20, 25 and 30°C shared a common dimensionless value of 0.583±0.028. However, intercepts differed significantly across temperatures, being significantly lower at the raising temperature of 25°C than at either 20 or 30°C ($F_{2,83}$=4.69, P=0.01).

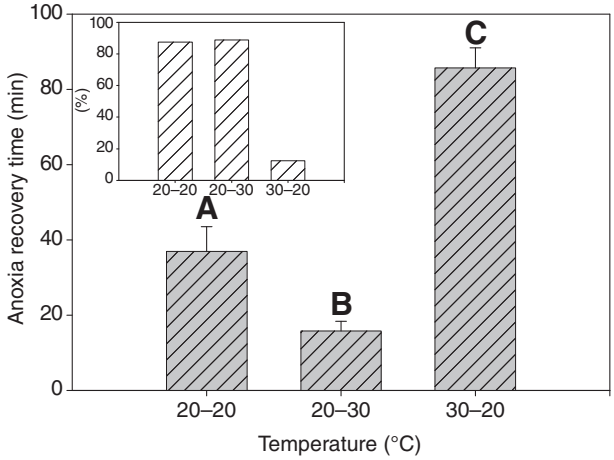

Fig. 5. Mean ± s.e.m. of Anoxia recovery times (the point at which the photoelectric activity recording revealed that voluntary activity had resumed) in minutes as a function of the temperature treatment (°C), which modulated the metabolic rates of the flies. Treatments were: anoxia at 20°C and recovery in normoxia at the same temperature (20/20; left bar) or at 30°C (20/30; middle bar) and anoxia at 30°C and recovery at 20°C (30/20; right bar; see Materials and methods for detailed explanation). Values are means ± s.e.m. Letters indicate significant differences between treatments – a, b: P<0.05; a, c and b, c: P<0.001 (*a posteriori* Tukey's test). Inset: percentages of insects that recovered in less than 100 min. Significant differences were found (χ^2-test of independence: χ^2_2=18.932, P<0.0001).

Temperature significantly affected the intercept of the regression relating recovery time to anoxia duration (Fig. 4). The intercept at 25°C, the rearing temperature, was lowest (6.29±8.41 min, adjusted mean 25.50), *vs* 20°C (10.24±8.70 min, adjusted mean 29.45) and 30°C (9.06±9.00 min, adjusted mean 28.29). The intercepts at 20 and 30°C did not differ significantly ($F_{1,53}$=0.56, P=0.4). The intercept at 20°C was significantly greater than at 25°C ($F_{1,57}$=8.72, P=0.004). The intercept at 30°C was also significantly greater than at 25°C ($F_{1,55}$=4.07, P<0.05).

In the second series of experiments we changed the temperature between the anoxic and recovery periods of the experiment while holding the anoxia duration constant at 25 min. From a total of 40 flies, 33 recordings were analyzed. One fly was discarded because it was a female, another because it was dead at the end of the experiment and the other five because they showed no activity during the pre-anoxic phase (first 25 min).

The statistical analysis revealed highly significant differences across treatments in the proportion of flies that recovered before the assay ended (χ^2-test of independence: χ^2_2=18.932, P<0.0001; Fig. 5 inset). The *a posteriori* comparison revealed a significantly smaller recovery for flies exposed to anoxia at 30°C and then allowed to recover at 20°C (only 2 out of 16, i.e. 12.5%), whereas no significant differences were found between treatments 20/20 (seven out of eight; i.e. 87.5%) and 20/30 (eight out of nine; i.e. 88.89%; χ^2-test of independence: χ^2_1=0.008, P<0.9293; Fig. 5 inset). Temperature, and thus MR, significantly affected recovery times ($F_{2,14}$=24.527, P>0.0001; one-way ANOVA; Fig. 5, Table 2). An *a posteriori* Tukey's test indicated significant differences across all treatments: 20/20 *vs* 20/30, q=4.515, P<0.05; 20/20 *vs* 30/20, q=6.723, P<0.001 and 20/30 *vs* 30/20, q=9.774, P<0.001 (Fig. 5). Recovery times from the first and second series of experiments in flies exposed to 25 min of anoxia at 20°C were not significantly different (t_{12}=1.446, P=0.174; unpaired t-test).

Table 2. Effects of ambient temperature on recovery time and survival of *Drosophila melanogaster* for four different anoxia exposure durations

Temperature (°C)	Anoxia duration (min)	Recovery time (min)	Survival
20	7.5	11.84±0.70	7/8 (87.5%)
20	25	27.00±1.75	7/8 (87.5%)
20	42.5	34.72±2.64	7/8 (87.5%)
20	60	46.22±2.09	8/10 (80%)
25	7.5	10.92±1.12	8/8 (100%)
25	25	22.21±1.84	8/8 (100%)
25	42.5	32.45±2.45	7/7 (100%)
25	60	38.69±1.65	8/8 (100%)
30	7.5	12.63±1.00	8/8 (100%)
30	25	23.55±2.04	7/8 (87.5%)
30	42.5	35.99±2.27	8/8 (100%)
30	60	41.84±2.52	4/12 (33%)

All flies were male and reared at 25°C.

DISCUSSION

The response of CO_2 emission and water loss rates during and after anoxia are complex. As previously observed, changes in the composition of the atmospheric air modulate the spiracular opening in insects, with anoxia promoting increased water loss, indicating increased spiracular opening (red trace in Fig. 3) (Lighton and Schilman, 2007; Lighton et al., 2004; Schilman et al., 2005). During anoxia, activity ceases within seconds, with full recovery of motion taking many minutes (green trace in Fig. 3). Both CO_2 and water emission rates spike after re-exposure to normoxia, with a secondary peak of CO_2 occurring a few minutes after normoxia resumes, which may be related to the repair of O_2 reperfusion damage (Fig. 3) (Lighton and Schilman, 2007). This post-anoxic increase of MR above normoxic values, or 'overshoot', has been observed in different species and it might be a consequence of a rapid compensation for the detrimental consequences of anoxia (Makarieva et al., 2006).

As expected from an ectothermic animal, the MR of *D. melanogaster* was strongly influenced by measurement temperature (Table 1 and Fig. 4); estimated Q_{10} was 2.21, which is within the range of 1.79 to 2.5 found for measurements made at 18 and 25°C (Berrigan and Partridge, 1997). Breeding temperature also has an effect on metabolism; flies evolving in the laboratory for more than 100 generations at 18°C have 5–7% higher mass-specific MRs at 25°C than flies evolving at 25°C (Berrigan and Partridge, 1997). Rearing flies at low atmospheric P_{O_2} (10 kPa; i.e. hypoxia) can limit their size (Klok and Harrison, 2009). However, the critical P_{O_2} values, the CO_2 emission rates and the maximal tracheal conductance values were independent of the rearing P_{O_2} (Klok et al., 2010). Also, rearing temperature and O_2 have interactive effects on size, eclosion times, growth rates and survival during the development of *D. melanogaster* (Frazier et al., 2001). In addition, total energy requirements during metamorphosis are dependent on temperature. Both low (18°C) and high temperatures (29°C) increase metabolic expenditure, with the optimal temperature for metamorphosis being 25°C (Merkey et al., 2008).

Temperature, and the correlated changes in MR, did not affect the slope of the regression line relating anoxic exposure duration to recovery time (Fig. 4). The thermal independence of the slope of this regression (Fig. 4) suggests that rates of anoxic damage and normoxic repair are equivalently sensitive to temperature and thus likely to affect metabolic flux rates. Flies recovered faster from anoxia and were less affected by O_2 reperfusion damage at the temperature at which they developed (Fig. 4). This suggests that the kinetics and pathway capacities of their enzymatic machinery are optimized to that temperature, perhaps as a result of adaptation or beneficial acclimation. These results are consistent with previous studies showing that optimal performance tends to occur at the intermediate developmental temperature of 25°C in *D. melanogaster* (Crill et al., 1996; Gibert et al., 2001).

Our second series of experiments, where we switched temperatures between exposure to anoxia and recovery time, clearly demonstrate that temperature strongly affects both recovery and damage rates, and suggests that both these processes are proportional to metabolic rates. As noted above, the estimated Q_{10} of the flies was ~2, i.e. metabolic rates of flies kept at 20°C were approximately half of those kept at 30°C (Table 1). Similarly, the recovery times for flies recovering from anoxia at 30°C were approximately half of the recovery times at 20°C (Fig. 5). These results suggest that higher temperatures directly accelerate enzymatic processes such as clearance of metabolic end-products, repair of damaged proteins and restoration of ion gradients. Damage rates also appear to be proportional to temperature/metabolic rates, as recovery times were approximately twice as long if anoxia occurred at 30°C and recovery occurred at 20°C (Fig. 5). These results suggest that anoxic damage is at least partly related to processes associated with metabolism such as production of ROS, accumulation of anaerobic end-products, etc. Flies that experienced anoxia at 30°C, followed by recovery at 20°C were the only group in which recovery was strongly impacted, i.e. most of the flies did not recover in the 100 min of normoxia after the anoxic period (Fig. 5 inset), further supporting the importance of temperature/metabolic rates on damage and recovery processes.

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