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REPORT

Flexibility in energy metabolism supports hypoxia tolerance in *Drosophila* flight muscle: metabolomic and computational systems analysis

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The fruitfly *Drosophila melanogaster* offers promise as a genetically tractable model for studying adaptation to hypoxia at the cellular level, but the metabolic basis for extreme hypoxia tolerance in flies is not well known. Using ^{1}H NMR spectroscopy, metabolomic profiles were collected under hypoxia. Accumulation of lactate, alanine, and acetate suggested that these are the major end products of anaerobic metabolism in the fly. A constraint-based model of ATP-producing pathways was built using the annotated genome, existing models, and the literature. Multiple redundant pathways for producing acetate and alanine were added and simulations were run in order to find a single optimal strategy for producing each end product. System-wide adaptation to hypoxia was then investigated *in silico* using the refined model. Simulations supported the hypothesis that the ability to flexibly convert pyruvate to these three by-products might convey hypoxia tolerance by improving the ATP/H^{+} ratio and efficiency of glucose utilization.
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Introduction

Understanding cellular adaptation to hypoxia is central to the design of treatments for injury caused by ischemia–reperfusion, stroke, and myocardial infarction. Cell damage during acute hypoxia is thought to be caused by imbalances such as decreased pH, altered calcium homeostasis, increased intracellular osmotic pressure, and mitochondrial damage, resulting directly and indirectly from decreased ATP (Hochachka and Somero, 2002; Corbucci *et al*, 2005). Humans have complex physiological systems for regulating oxygen homeostasis that involve multiple spatial scales and cell types and have been delicately tuned during evolution. However, at the cellular level, hypoxia resistance mechanisms most likely evolved very early and appear to be highly conserved among species (O'Farrell, 2001).

Lending support to this hypothesis, several genes have been discovered in the fruitfly *Drosophila melanogaster* that are similar in sequence and function to human genes for regulation of metabolism, signaling, and transcription during hypoxia (Piacentini and Karliner, 1999; Wingrove and O'Farrell, 1999; Lavista-Llanos *et al*, 2002; Pan and Hardie, 2002). Although hypoxia defenses in flies and humans seem to be quite similar at the level of individual genes, stark contrasts exist at the phenotype level. *Drosophila* have a remarkable tolerance to hypoxia that is the subject of an increasing amount of investigation (O'Farrell, 2001; Haddad, 2006). In contrast to humans, who can only survive a few minutes without oxygen, flies can fully recover from up to 4 h in complete anoxia (Haddad *et al*, 1997). Differences in anaerobic generation of ATP are likely to be part of the reason for the disparity in hypoxia tolerance between humans and flies; however, *Drosophila* anaerobic metabolism is not well known.

Aerobic energy metabolism in insect flight muscle is similar to that of humans in most respects; however, there are some major differences that distinguish the species, such as the use of proline as an energy source, heavy reliance on the α-glycerol-3-phosphate shuttle, and the use of arginine as an alternative to creatine for ATP buffering (Gilmour, 1961). Anaerobic energy pathways in *Drosophila* are likely to deviate from those of humans as well. In human muscle, glycolysis is the major anaerobic energy pathway and lactate is the only end product of anaerobic metabolism (Nelson, 2000; Wadley *et al*, 2006). Many terrestrial insects yield lactate and alanine as anaerobic end products, but other species have been known to produce a wide array of other products during hypoxia, including sorbitol, succinate, glycerol, α-glycerol-3-phosphate, pyruvic acid, and fatty acids (Hoback and Stanley, 2001). The specific end products for *Drosophila* are not known; however, the wide diversity of insect biochemistry suggests that exotic

pathways for anaerobic energy production may also exist in flies (Gilmour, 1961; Hoback and Stanley, 2001).

Regardless of the pathways used, anaerobic metabolism must be regulated over the long term to balance pH, ATP production, redox potential (most importantly, NADH/NAD^+), and coupling metabolites. Although strategies for maintaining these balances are known for many organisms (Hochachka, 1980), quantitative systems models can increase mechanistic understanding. A major advantage of a mathematical model is that conservation of mass is enforced; therefore, all elements and charges are balanced within the system, including electron transport, cofactor concentration, and protons (pH). The constraint-based method uncovers the space of all possible steady-state solutions under a set of physiochemical limitations imposed on the system (Palsson, 2004). These network models are useful both for performing detailed *in silico* experiments and for discovering more general systems-level properties (Almaas *et al*, 2004; Reed and Palsson, 2004).

Focusing on *Drosophila* flight muscle, we used NMR metabolomic analysis to discover end products of anaerobic energy metabolism. We then added all pathways that might produce these compounds, linked them to existing *Drosophila* genes, and built them into a constraint-based model of fly energy metabolism. Simulations were used to select specific anaerobic pathways from a number of alternatives by optimizing for ATP production. Metabolite fluxes measured by NMR were integrated into the model and simulations were conducted to investigate production of ATP, H^+, and glucose during hypoxia. Simulations were compared with those of classical anaerobic energy pathways in mammals to generate hypotheses for mechanisms of hypoxia tolerance in flies.

Results and discussion

Global metabolite profiles under hypoxia

Of the 21 compounds with at least one sample measurement greater than 0.05 mM, six were found to change significantly according to one-way analysis of variance (ANOVA): acetate, alanine, arginine, glucose, lactate, and threonine. All six compounds had a statistically significant linearly increasing trend. Three compounds (acetate, alanine, and lactate) had high R^2 goodness-of-fit values and significant changes among several time points. In the remaining three (arginine, glucose, and threonine), only the 4-h hypoxia group was significantly different from the rest. Figure 1 displays the statistically significant subset.

One common behavior was that the concentration of a metabolite remains stable for the first hour of hypoxia, but shows a large increase at 4 h. Glucose, threonine, and arginine show a statistically significant difference from early time points to the 4 h concentration. As the survival rate for anoxic flies at 25°C is very high for the first few hours and then starts to decline at 6 h (Haddad *et al*, 1997), it is reasonable to suggest that these metabolites are an early indication of the loss of homeostasis, that is, the breakdown of the system under stress. Mechanisms for protecting the cell and balancing metabolic requirements may begin to lose effectiveness at some threshold period of time. For example, a likely explana-

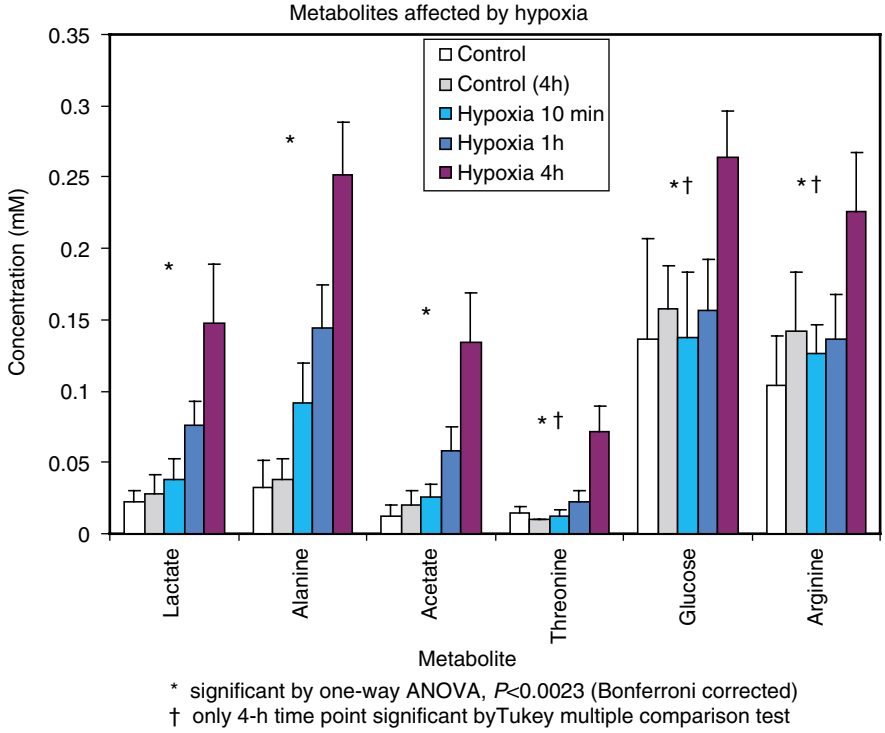

Figure 1 Metabolites significantly altered during hypoxia, measured by ^{1}H NMR spectroscopy. Lactate, alanine, and acetate accumulate over the time course of the experiment and are by-products of anaerobic metabolism, whereas glucose, threonine, and arginine concentrations only peak at 4 h and are possible indications of a loss in homeostasis.

Table I Approximation of end-product accumulation from NMR data

Product	Goodness-of-fit (R^2)	Slope of trendline (μM/min · sample)	Accumulation (nmol/min · fly)
Lactate	0.81	495	12.4
Alanine	0.82	798	20.0
Acetate	0.86	485	12.1

Accumulation of substrate was calculated using 500 μl sample volume and 20 flies per sample.

tion for the time course of glucose is that this substrate is replenished by a steady depletion of trehalose and glycogen supplies during the manageable first hour of hypoxia. At 4 h, glucose supply undergoes a large increase, which suggests that the system has experienced a drastic reduction in its ability to utilize carbohydrate substrates.

Steady accumulation of lactate, alanine, and acetate is the second phenomenon that can be seen in the NMR spectra. The end products of anaerobic metabolism in *Drosophila* were not previously known. However, the discovery of lactate and alanine accumulation is consistent with the fact that these compounds, which do not accumulate under normal conditions, are known to be the by-products of anaerobic metabolism in other terrestrial insects (Hoback and Stanley, 2001). During hypoxia, lactate fermentation regenerates NAD^+ for glycolysis, with the trade-off of decreasing pH (Nelson, 2000), and certain other organisms have alanine and acetate fermentation pathways that perform a similar function (Gade, 1984; Hochachka and Somero, 2002). Table I contains calculated results of accumulation during the hypoxia experiment.

Reconstruction and expansion of *Drosophila* metabolic network

We built and validated a constraint-based model of known ATP-producing pathways in *Drosophila*. Results from the NMR metabolomics experiment were then used to refine and expand the model, as well as incorporate quantitative flux data. These results suggest that the major metabolites that accumulate under hypoxic conditions are lactate, alanine, and acetate. We expanded the scope of the model to include several alternative pathways for generating alanine and acetate from pyruvate, and then integrated NMR measurements by placing constraints on the steady-state flux out of the system.

In other organisms, alanine is produced during hypoxia by transamination to pyruvate from another amino acid. In mammals, this pathway involves the cycling of α-oxoglutarate (also known as α-ketoglutarate) and glutamate (Nelson, 2000) through enzymes that are also present in flies. We added two transaminations to the model based on genetic evidence in the KEGG pathway database (Ogata *et al*, 1999): alanine-α-oxoglutarate transaminase (using glutamate as in mammals) and alanine-glyoxylate transaminase (using glycine and pyruvate as substrates).

Acetate production has been previously hypothesized as a possible mechanism for dealing with mitochondrial acetyl-CoA that cannot be catabolized further in the absence of oxygen (Hochachka, 1980). The benefits of this pathway are two-fold. Acetate is a weaker acid than lactate (p*K* is 4.8 versus 3.7); also hydrolysis of acetyl-CoA to acetate by acetate-CoA ligase is ADP linked, offering the additional benefit of ATP production. Genetic evidence in flies (from KEGG) also suggests alternative pathways for acetate production from pyruvate via acetyl-phosphate or acetaldehyde. These reactions were added along with the acyl-carnitine shuttle for transport of mitochondrial acetyl-CoA to the cytosol.

Hypoxia simulation

There are several benefits of using a quantitative model to test and generate hypotheses about hypoxic energy metabolism. First, although much is known about the individual pathways, it becomes very hard to manually calculate and predict behavior when pathways are combined into an integrated network. With a few reasonable assumptions, steady state in this study for example, some of the vast knowledge contained in the literature and public databases can be compiled into a comprehensive, predictive model into which individual experiments, such as our metabolomic data, can be integrated. In this mathematical model, the complicated problems of balancing redox potential and accounting for proton production are solved intrinsically during flux-balance analysis.

Every simulation of hypoxia produced a flux distribution that utilized only one pathway each for production of alanine and acetate, among multiple alternatives. Alanine formation used the glutamate-dependent alanine transaminase, as *Drosophila* lack the capability to further metabolize glyoxylate produced by the alternative transaminase reaction. To produce acetate, the model utilized the acetyl-CoA synthetase reaction in every simulation. Fluxes through these reactions were constrained to a maximum determined by the NMR data, but were not set explicitly; rather, the model utilized these pathways to optimize ATP production under hypoxic conditions (Figure 2).

Our model was used to simulate *Drosophila* ATP production ranging from normoxia to anoxia, using two different systems: the first allowing lactate, alanine, and acetate to accumulate through known and hypothesized pathways, and the second allowing only lactate to accumulate through the fermentation pathway also used by mammals ('pseudo-mammalian' in Figure 3). Figure 3 shows a clear advantage in converting pyruvate to acetate and alanine during all low-oxygen conditions. Figure 3A plots important fluxes under varying oxygen levels. Figure 3B compares these fluxes when alanine and acetate production are constrained to zero and the constraint on lactate accumulation is removed. Using pathways that generate alanine and acetate decreases proton production, increases ATP generation (Figure 3C), and decreases glucose uptake under all hypoxic and anoxic conditions simulated.

The ability to withstand hypoxia strongly depends on the ability to control pH within the system, while maintaining ATP production. The ATP/H^+ ratio is a simple indicator of the likelihood of hypoxia tolerance of an organism (Hochachka and Somero, 2002), as it directly quantifies the physiological tradeoff between generating energy for the system and maintaining pH levels required for homeostasis. Another

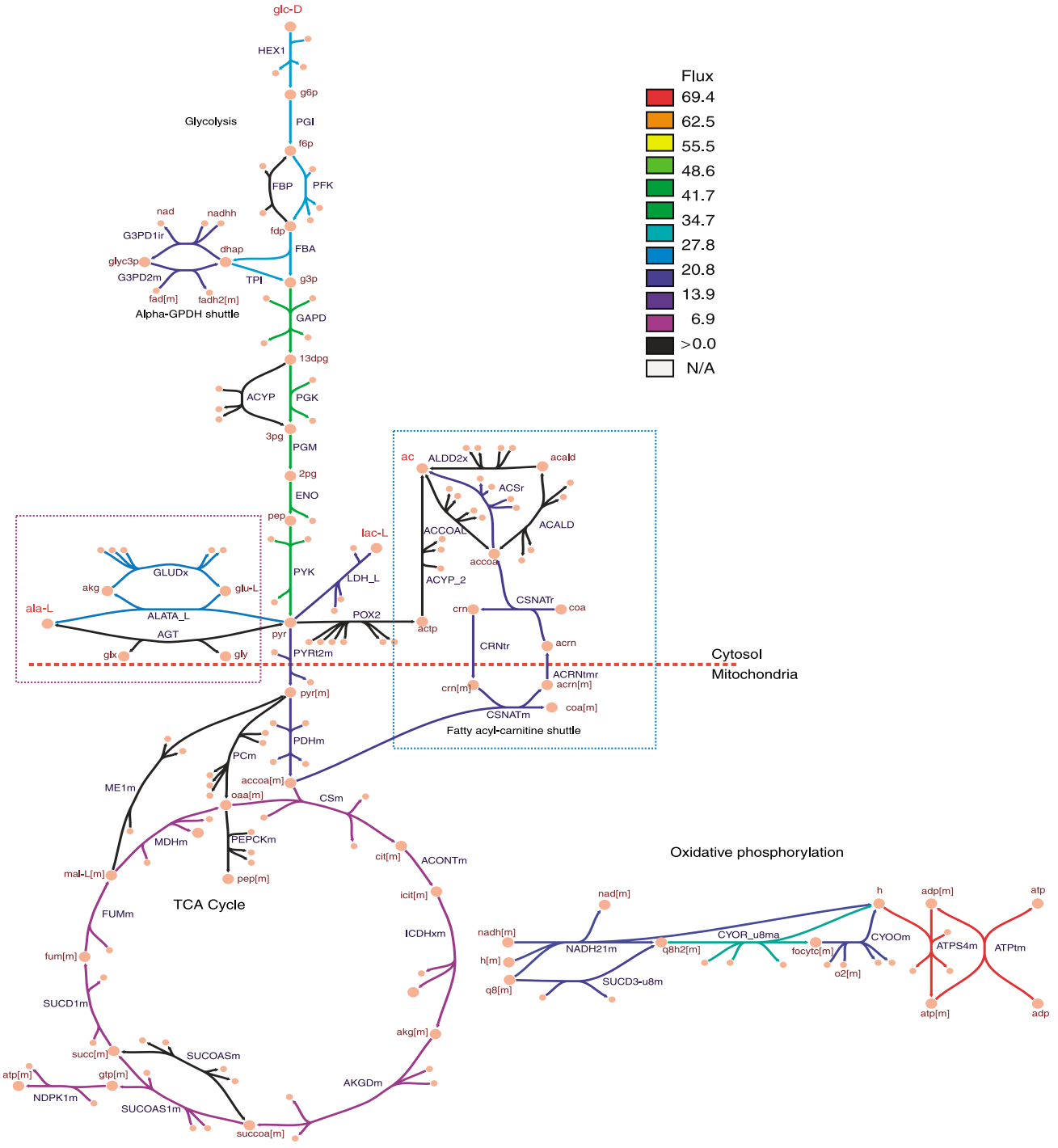

Figure 2 Pathways of ATP generation during hypoxia. The network includes glycolysis, TCA cycle, and oxidative phosphorylation, as well as reactions for generating lactate, alanine, and acetate (in red) during hypoxia. Of the two pathways for creating alanine (purple box) and four options for creating acetate (blue box) from pyruvate, optimization of the model selected one optimal route for generating each end product (non-zero fluxes in color).

suggested indicator, the ATP/substrate ratio, is an obvious way of quantifying the decreased efficiency of fuel utilization as respiration, the most efficient pathway, is restricted. As all simulations of restricted oxygen produce more ATP, use less glucose, and accumulate fewer protons in the acetate- and alanine-producing system, the model suggests that these mechanisms contribute to hypoxia tolerance in flies.

Both NMR metabolomic profiling and quantitative systems modeling suggest that having more possibilities for the fermentation products of pyruvate may contribute to hypoxia tolerance in fruitflies. Our approach can be used to generate and test new hypotheses about the metabolic basis for hypoxia tolerance. Further experiments using 13-carbon-labeled isotopomers can add sensitivity to NMR measurements and

directly trace intrasystem fluxes (Sauer, 2006). In addition, enzyme mutations can be simulated by adding flux constraints, and a system-wide perturbation analysis can be performed *in silico*. Future work will take advantage of the vast *Drosophila* gene deletion library (Spradling *et al*, 1999) by applying further NMR, phenotypic, and biochemical experiments on mutants suggested by the model.

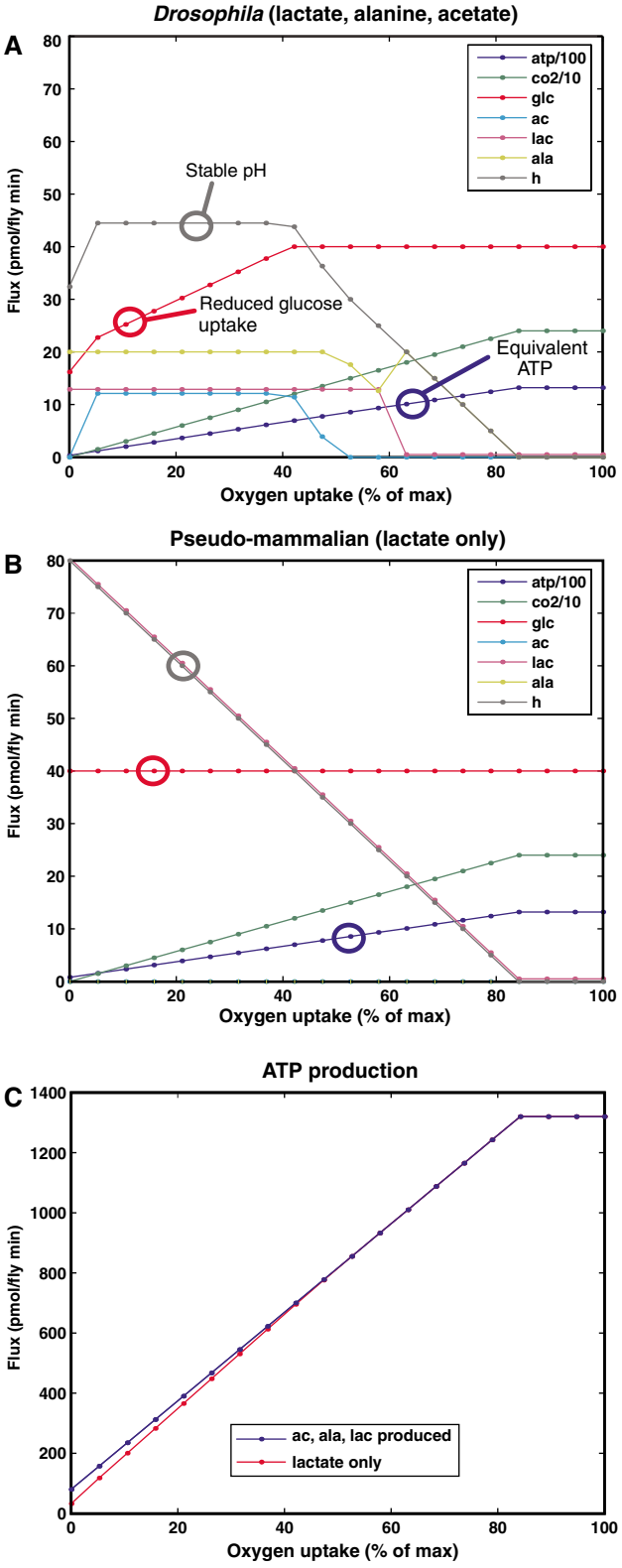

Figure 3 Results of flux-balance analysis on the model of *Drosophila* ATP-generating metabolism. (**A**) Proton production increases but then levels off at low oxygen levels as pyruvate begins to be fermented to alanine, acetate, and lactate. Glucose uptake is decreased during restricted oxygen. (**B**) When pyruvate is only allowed to be converted to lactate (pseudo-mammalian), proton production is much higher and glucose uptake remains constant during hypoxia, whereas (**C**) ATP production remains the same or better. Abbreviations: ac: acetate accumulation; ala: alanine accumulation; atp: ATP production; CO_2: CO_2 production; glc: glucose uptake; h: proton production and lac: lactate accumulation.

Materials and methods

Fly preparation

Oregon-R wild-type flies were reared in constant darkness at 25°C. When they were 3–5 days old, they were brought into light approximately 4 h before the experiment.

Hypoxia experiments

The hypoxia experiments included five samples for each of the five conditions: control, 10 min hypoxia, 1 h hypoxia, 4 h hypoxia, and 4 h control. As the vials used for experiment did not contain food, the 4-h control was included to offset the effects of starvation and dehydration over the same time period. Holes were drilled in the screw caps of Sarstedt 15-ml vials and rubber tubing was inserted into each cap and sealed airtight with silicone adhesive. At the time of experiment, filter paper was soaked in distilled water and placed in each vial to prevent drying. Approximately 50 flies were transferred and the caps were screwed loosely onto each vial to allow gas to flow out. A mixture of nitrogen and 0.5% oxygen was then bubbled through distilled water and passed through the tubing into the vials. After 5 min of application of hypoxia, the caps were sealed airtight, the gas pressure was cut off at the source, and tubing clamps were used to seal the rubber tube, in that order. Control flies were sealed in vials with wet filter paper, but without the application of gas. Vials were lightly shaken to increase spacing among immobile flies and stored on their side at 25°C. At the end of each time point, vials were snap frozen in liquid nitrogen and frozen flies were transferred for storage at −80°C.

Females were separated from males on dry ice under a dissecting microscope, then, using forceps and miniature spring scissors (Fine Science Tools Inc., 15003-08), 20 thoraces were separated from the head and abdomen and placed into Eppendorf vials in liquid nitrogen. Thoraces were homogenized in an ice bath for 10 min in 300 µl of cold 1:1 acetonitrile:water buffer, homogenates were centrifuged in a cold room (4°C) for 10 min at 13 000 r.p.m., and the supernatant was ultracentrifugated for 30 min at 8500 r.p.m. using Nanosep centrifugal devices (Pall Life Sciences, Ann Arbor, MI) with a 3 kDa molecular weight cutoff. To reduce the contamination of glycerol, a membrane preservative, to below 80 µM, all Nanosep devices were washed four times (by 5 min centrifugation at 13 000 r.p.m.) with 500 µl deionized water. Filtrate was lyophilized using a vacuum centrifuge for 2 h at 45°C. Dried samples were then dissolved in 500 µl D_2O buffered at pH 7.4 with monobasic/dibasic sodium phosphate. The NMR standard TSP (3-trimethylsilyl-$^2H_4$-propionic acid) was added to the samples at a ratio of 1:100 by volume, resulting in a concentration of 0.488 mM. Samples were stored at 4°C until measured.

NMR spectroscopy and data analysis

Analyses of samples were carried out by ^{1}H NMR spectroscopy on a Bruker Avance 500 operating at 500.13 MHz ^{1}H resonance frequency. The NMR probe used was the 5 mm TXI ^{1}H/^{2}H-^{13}C/^{15}N Z GRD. All NMR spectra were recorded at 30°C. Typically, ^{1}H were measured with

512 scans into 32 768 data points, resulting in an acquisition time of 1.36 s. A relaxation delay of 2 s additionally ensured T1 relaxation between successive scans. Solvent suppression was achieved via the Noesypresat pulse sequence (Bruker Spectrospin Ltd), in which the residual water peak is irradiated during the relaxation and mixing time of 80 μs. All ^{1}H spectra were manually corrected for phase and baseline distortions within XWINNMR™ (version 2.6, Bruker Spectrospin, Ltd). Two-dimensional (2D) NMR methods including homonuclear correlation spectroscopy (TOCSY) and heteronuclear single quantum correlation spectroscopy (HSQC) were carried out in order to identify and subsequently confirm the assessment of metabolites.

Peaks in the 1D spectra were identified, aligned, and quantified by 'targeted profiling' algorithms (Weljie *et al*, 2006) within the software Chenomix NMR Suite 4.5 (Chenomix Inc.). The list of metabolites discovered in the 2D spectra was used to guide quantification in one dimension. Metabolite concentrations were imported into Matlab (Mathworks, Cambridge, MA) and Excel (Microsoft, Redmond, CA) for plotting and curve fitting.

Statistical analysis

For every metabolite with at least one measurement above 0.05 mM, ANOVA was applied to the time course, with a Bonferroni correction applied to the *P*-value for the number of metabolites tested. Two *post hoc* tests were performed for metabolites, in which the null hypothesis of no change across time points was rejected by ANOVA, Tukey's test for cross-comparison of each time point, and a test for a linear trend in the data. Statistical analysis was performed using the Prism software package (GraphPad Software Inc., San Diego, CA).

Building the reconstruction

An *in silico* reconstruction of ATP-generating metabolism in *Drosophila* flight muscle was created, using the SimPheny biological database software (Genomatica, San Diego). These models have a high level of detailed manual curation. All genes and reactions in the reconstruction were individually inspected before inclusion into the model. Element- and charge-balanced reactions were included in the model based on evidence from sequence homology in the annotated genome, online enzyme databases, and the literature. Cellular compartment information was also included. Reed *et al* (2006) reviewed the reconstruction process in detail.

For the initial draft, we chose to use the human cardiac mitochondrial model (Vo *et al*, 2004) as a template, sifting through all ATP-producing pathways and then searching various resources (Flybase, KEGG, Brenda, MetaCyc, or PubMed) (FlyBase, 2003; Ogata *et al*, 1999; Schomburg *et al*, 2002; Caspi *et al*, 2006), to find evidence for a counterpart in *Drosophila* flight muscle. Reactions were entered into SimPheny and assigned a confidence level depending on whether evidence was derived from sequence, genetic, physiologic, or biochemical evidence. Gene–protein–reaction associations were made for each reaction, and, whenever necessary, changes were made to reflect subtle interspecies differences in cofactors and substrates.

The second phase of the reconstruction was a survey of *Drosophila* and insect biochemistry literature, with a focus on energy-producing pathways that occur exclusively in fruitflies or other species from the parent phylogenic order Diptera, especially the closely related blowfly, on which much flight muscle research has been carried out. Reactions were associated with genes whenever possible, but occasionally included in the model based on physiological or biochemical evidence even if the corresponding gene was not found.

In the third phase of the reconstruction, the model scope was expanded to account for the production of the end products (acetate and alanine) discovered in the NMR results, and to account for other metabolites and cofactors produced or used in these reactions. All pathways in the KEGG database converting pyruvate to alanine or acetate were manually inspected and then added to the model, if at least one enzyme in the pathway had sequence similarity to a *Drosophila* gene. Two pathways were added for the production of alanine and four pathways were added for acetate production.

The scope of the current *Drosophila* network spans only central, ATP-generating metabolism. Upon completion, this version of the reconstruction had a total of 162 genes, forming 143 proteins and catalyzing 158 reactions. The total number of metabolites represented in the model was 184. Reactions in the model can be grouped into four major pathways: glycolysis, TCA cycle, oxidative phosphorylation, and amino-acid (proline and glutamate) metabolism. The pentose phosphate pathway was not included in the current version owing to suggestions in the literature that in insects the pathway is used only for biosynthetic purposes (Hochachka and Somero, 2002). Fatty acid metabolism is not important for flight in Diptera, and is rarely used during hypoxic conditions (Gilmour, 1961; Hochachka, 1980).

Constraint-based modeling

The final product of the reconstruction process is a curated set of enzymatic reactions and metabolite transporters that define the complete metabolic network of interest. Additional constraints can be added to this stoichiometric matrix in the form of limits on uptake rates and transporter fluxes. Reactions and their associated biochemicals are represented mathematically as a matrix of stoichiometric relationships that can be manipulated with linear algebra to reveal the solution to the equation

$$d\mathbf{x}/dt = \mathbf{S} \cdot \mathbf{v} = 0,$$

where $\mathbf{x}$ is the vector of metabolites, $\mathbf{S}$ is the stoichiometric matrix, and $\mathbf{v}$ is the vector of reaction fluxes at steady state. The null space of $\mathbf{S}$ is the set of possible flux vectors that satisfy this steady-state condition. Linear programming methods within SimPheny were then used to maximize a desired subset of fluxes within this solution space to find a single optimum phenotype. See Kauffman *et al* (2003) and Papin *et al* (2003) for a review of the mathematics involved in flux-balance analysis.

Hypoxia simulation

In muscle cells, the objective of metabolism is primarily to provide ATP to the energy-consuming myosin cross-bridges and ion pumps. In all *in silico* experiments, ATP production was chosen as the single objective function for the linear programming algorithm. An ATP demand reaction was created in the cytosolic compartment, in which ATP and water are converted into ADP plus one phosphate ion plus one proton. The flux through this reaction was maximized subject to constraints given by the stoichiometry of the network, substrate uptake, and end product secretion. Flux information was exported into Matlab for plotting.

The *Drosophila* literature mentions three major energy substrates for flight muscle: glycogen, trehalose, and proline. Glycerol is also mentioned as a possible fuel (Martinez Agosto and McCabe, 2006), but as both the literature and our metabolomic data are inconclusive regarding this compound, it has been excluded as a substrate. For the purposes of this model, trehalose and glycogen are functionally equivalent as the predominant sources of glucose. The depletion of glycogen and trehalose are currently unknown; therefore, the amount of glucose entering glycolysis remains unknown as well. Initial simulations of hypoxia showed that proline uptake decreases as oxygen is constrained, reaching zero at oxygen levels higher than those at which lactate and acetate accumulation occurs. Therefore, it is reasonable to assume for our simulations that glucose is the only substrate during hypoxia, which reduces the degrees of freedom for substrates to two: glucose and oxygen. Supplementary Figure 4S shows all important energetic pathways included in the model.

In order to study changes in the model output using oxygen uptake as the independent variable, the system was saturated with glucose by setting the maximum uptake to a value much higher than the threshold required to produce the measured fluxes of end products under restricted oxygen. Constraints on O_2 influx in the model were then used to simulate low extracellular oxygen concentration in hypoxic experimental conditions. We reduced O_2 uptake, leaving all other fluxes free to vary up to their constrained maxima.

Model validation

Validation of the P/O and ATP/glucose ratios is described in Supplementary information.

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (www.nature.com/msb).

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