Metabolic effects of CO_2 anaesthesia in *Drosophila melanogaster*

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Immobilization of insects is necessary for various experimental purposes, and CO_2 exposure remains the most popular anaesthetic method in entomological research. A number of negative side effects of CO_2 anaesthesia have been reported, but CO_2 probably brings about metabolic modifications that are poorly known. In this work, we used GC/MS-based metabolic fingerprinting to assess the effect of CO_2 anaesthesia in *Drosophila melanogaster* adults. We analysed metabolic variation of flies submitted to acute CO_2 exposure and assessed the temporal metabolic changes during short- and long-term recovery. We found that *D. melanogaster* metabotypes were significantly affected by the anaesthetic treatment. Metabolic changes caused by acute CO_2 exposure were still manifested after 14 h of recovery. However, we found no evidence of metabolic alterations when a long recovery period was allowed (more than 24 h). This study points to some metabolic pathways altered during CO_2 anaesthesia (e.g. energetic metabolism). Evidence of short-term metabolic changes indicates that CO_2 anaesthesia should be used with utmost caution in physiological studies when a short recovery is allowed. In spite of this, CO_2 treatment seems to be an acceptable anaesthetic method provided that a long recovery period is allowed (more than 24 h).

1. INTRODUCTION

To paralyse insects for various purposes, several anaesthetic methods are used, including chilling, etherization and carbon dioxide (CO_2). Among these, CO_2 anaesthesia is routinely used for colony maintenance, identification of specimens, surgery, dsRNA injection and sexing of flying insects [1,2]. A number of negative side effects of CO_2 have been reported on thermal, reproductive and behavioural traits, some being manifested several hours or days after the CO_2 exposure [2–4]. However, the impact of this anaesthetic method on physiological traits remains poorly known [2,5].

CO_2 anaesthesia constitutes a fairly stressful treatment because it increases haemolymph acidity and

causes the heartbeat to stop [2,6]. Exposure to pure CO_2 thus impairs oxygen delivery to the tissues, compromising oxidative phosphorylation and ATP production in cell mitochondria [7]. During the recovery, the heart rate accelerates rapidly to a maximum value above that measured before anaesthesia [2]. Rapid paralysis results from a failure in synaptic transmission at the skeletal neuromuscular junction due to CO_2 [6]. In addition, acute CO_2 exposure likely brings about modifications in metabolic pathways whose interactions are poorly known [2]. Compared with the amount of work describing the negative side effects of CO_2 on insect's performance and behaviour, little effort has been made to determine the metabolic response during acute CO_2 exposure. In addition, it is of paramount importance to determine whether CO_2 anaesthesia has persistent metabolic effects, as this may confound studies of physiological responses if unaccounted for.

In the present study, we used GC/MS-based metabolic fingerprinting to assess the effect of CO_2 anaesthesia on *Drosophila melanogaster* females. We analysed metabolic trajectories in order to understand (i) the degree to which the metabolome is altered by an acute CO_2 exposure and (ii) the pattern of homeostatic response during short- and long-term recovery.

2. MATERIAL AND METHODS

(a) Fly culture

We conducted our experiments on a mass-bred *D. melanogaster* line derived from mixing two wild populations collected in October 2010 at Plancoët and Rennes (Brittany, France). The flies were maintained in the laboratory for 15 generations when tested, using a previously described rearing method [8].

(b) Treatments and CO_2 anaesthesia

The treatments consisted of (i) untreated control (C), (ii) CO_2 stress (S) and (iii) recovery post-CO_2 stress for 1, 4 and 14 h (R1, R4 and R14). In order to check for any circadian changes in metabolic concentrations [9], a set of time-of-day untreated controls was also tested at 0, 1, 4 and 14 h (C0, C1, C4 and C14), starting at 08.00. Potential persistent metabolic changes following CO_2 anaesthesia were also assessed through additional tests. For this, we compared metabolic profiles among the control (C0) and recovery periods of 24 and 48 h post-CO_2 treatment (R24 and R48). Only females were used in these experiments. They were sexed visually with an aspirator. For each condition, 10 biological replicates consisting of a pool of 15 females were used for metabolic fingerprinting. For the additional tests, four replicates were tested. The CO_2 treatment was conducted in a 42 ml glass vial, which was flushed with pure CO_2 (N45, Air Liquide, France) from a compressed canister (or air pump for control), using a regulator with a 1.5 mm diameter glass needle tip inserted into a foam plug. The vial containing flies was sealed with parafilm and left undisturbed for 7 min with continuous CO_2 flux. CO_2-stressed flies were sampled at the end of CO_2 exposure. For each treatment, females were directly snap-frozen in liquid nitrogen and stored at $-80°C$ until metabolites extraction. Flies took about 6–10 min to recover from 7 min CO_2 anaesthesia on food.

(c) Metabolic fingerprinting

We used a GC–MS platform to measure metabolites from the whole insect body as described by Colinet *et al.* [8]. Briefly, metabolites were extracted using methanol–chloroform (2:1) and then derivatized with methoxyamine HCl in pyridine followed by N-Methyl-N-trifluoroacetamide (MSTFA). MS detection was achieved using selective ion monitoring (SIM) mode. Quantification was based on calibration curves obtained from pure reference compounds. The system consisted of a CTC CombiPal autosampler (GERSTEL GmbH & Co. KG, Germany), a Trace GC Ultra chromatograph and a Trace DSQII quadrupole MS (Thermo Fischer Inc., USA).

(d) Statistical analyses

The levels of individual metabolites were compared with one-way ANOVAs. Dunnett's comparisons were used to compare treated
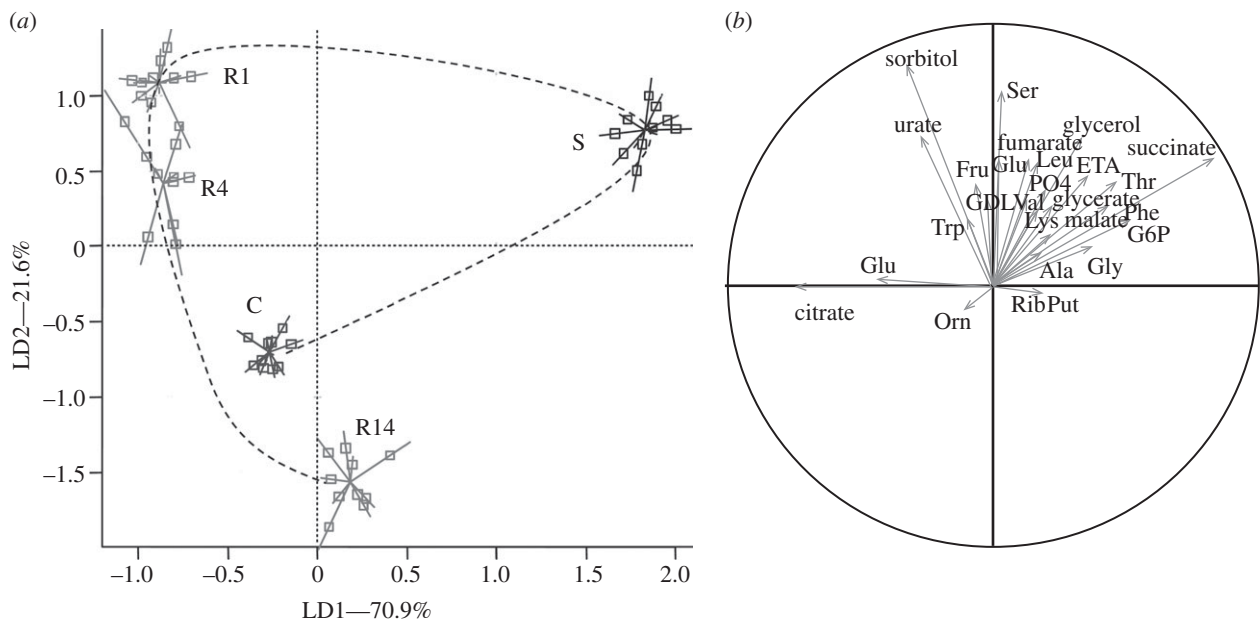
Figure 1. (*a*) Projection of samples onto the first discriminant plane of the LDA. Lines link samples to the centroid of their group (*n* = 10). Control flies (C), CO_2-stressed flies (S) and recovering flies assessed 1, 4 and 14 h following CO_2 treatment (R1, R4 and R14). The dotted line indicates the temporal succession of treatments. (*b*) Projection of variables on the correlation circle. Refer to electronic supplementary material, table S1 for compound abbreviations and electronic supplementary material, table S3 for correlation values.

values with the control ones. Bonferroni's corrections for multiple comparisons were applied. Metabolic differences among classes were also investigated using a linear discriminant analysis (LDA). Statistical significance of LDA was checked using the Monte Carlo test. Analyses were performed using the 'ade4' library in the statistical software R v. 2.13.0.

3. RESULTS

Thirty-three metabolites were detected and quantified in the samples (see the electronic supplementary material, table S1). Four metabolites showed a significant temporal variation among time-of-day controls (xylitol, proline, trehalose and sucrose; electronic supplementary material, table S2), and were thus discarded from further analyses because of their changes unrelated to CO_2 treatment. The distribution of the samples in the space formed by the two first linear discriminant axes (figure 1*a*) showed a significant clustering effect according to the different classes (Monte Carlo test, simulated *p*-value < 0.001). The first discriminant axis (LD1) accounted for 70.9 per cent of the total inertia. LD1 was characterized by a clear-cut opposition between treatment S and the other treatments. The compounds that contributed the most to the structure separation were succinate and glucose-6-phosphate (G6P) that were positively correlated with LD1, whereas citrate and glutamate were negatively correlated with LD1 (figure 1*b* and the electronic supplementary material, table S3). LD2 accounted for 21.6 per cent of the total inertia and was characterized by a temporal pattern opposing R1–R14. The compounds contributing the most to this separation were sorbitol, serine (Ser) and urate (figure 1*b* and electronic supplementary material, table S3). Treatment variations of individual metabolites are illustrated in figure 2. In addition, we

found no evidence of metabolic variations between control and stressed flies when a long recovery period was allowed (see the electronic supplementary material, table S4). This was further supported by LDA, which showed no clustering effect among classes (C0, R24, R48) (Monte Carlo test, simulated *p*-value = 0.329; figure 3).

4. DISCUSSION

The most important metabolic changes detected during CO_2 stress were the accumulation of succinate and G6P, whereas citrate was downregulated with respect to control flies. During CO_2 anaesthesia, the heartbeat stops, and oxygen delivery is markedly impaired generating anoxic conditions. Oxidative phosphorylation and ATP production are thus compromised [2,7]. Moreover, pure CO_2 directly inhibits several metabolic enzymes such as succinate dehydrogenase (SDH) [10] and NADPH-producing enzymes [11]. The accumulation of succinate thus likely relates to the inhibition of SDH. In the almond moth *Ephestia cautella*, hypoxia and CO_2 block the entrance of glycolysis products into tricarboxylic acid cycle (TCA) cycle, which results in reduction of citrate concentration [7]. Likewise, we found that citrate concentration decreased in parallel with the succinate increase during the CO_2 stress. A drastic reduction in ATP level with CO_2 treatment has been reported, suggesting that the phosphorylation system is affected in *E. cautella* [7]. The increase in G6P observed during CO_2 anaesthesia may suggest that cells are forced to depend on glycolysis for ATP production. It is not clear whether these metabolic alterations resulted from the direct effect of CO_2 or from anoxia or a combination thereof. Whatever the underlying causative mechanisms, it clearly appears
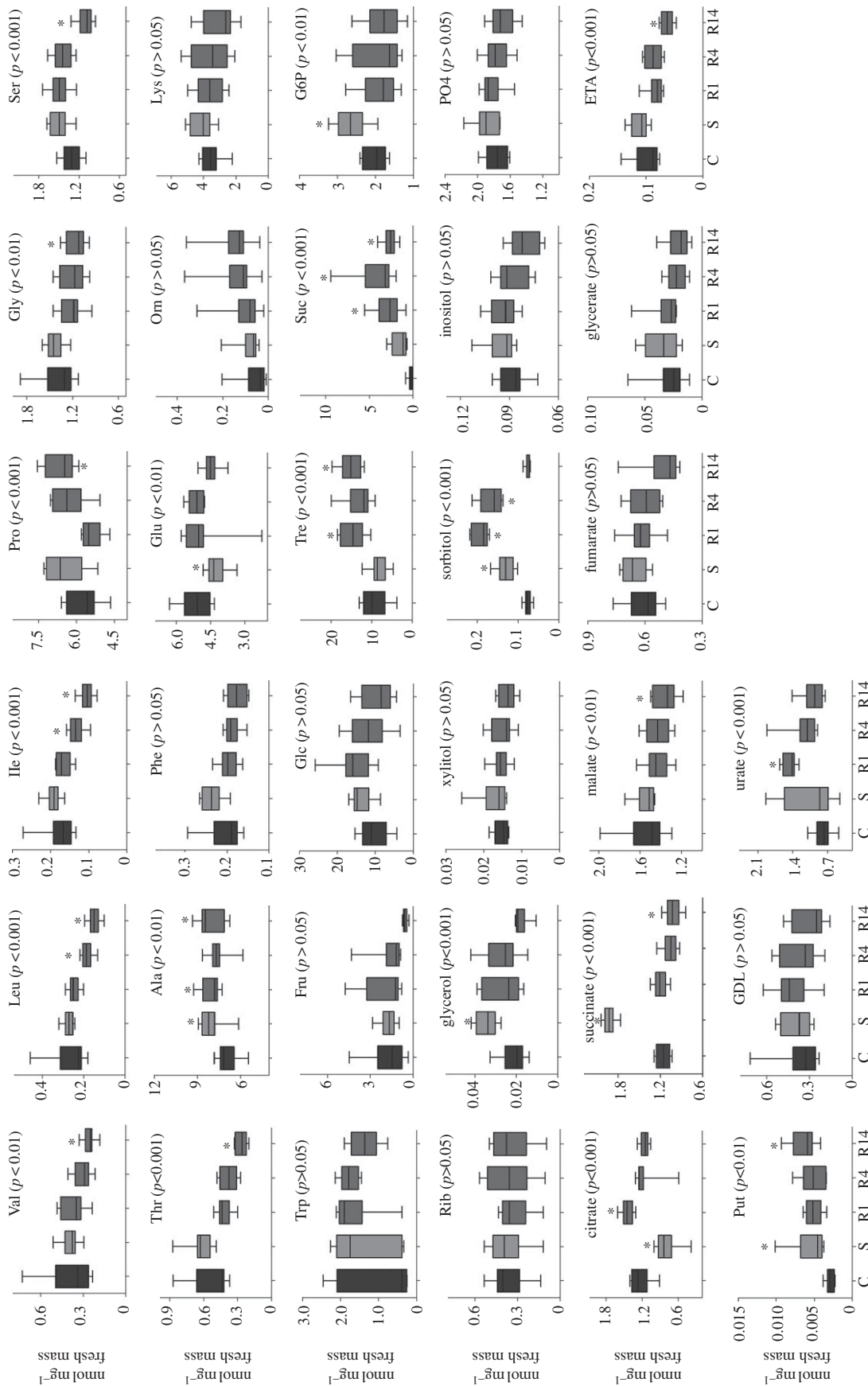
Figure 2. Box plots illustrating treatment and temporal variations of all individual metabolites detected in the *D. melanogaster* metabolome. A box consists of upper and lower hinges and a centre line corresponding to the 25th percentile, the 75th percentile and the median, respectively. Data are based on 10 replicates ($n = 10$). Control flies (C), CO_2-stressed flies (S) and recovering flies assessed 1, 4 and 14 h following CO_2 treatment (R1, R4 and R14). For each compound, statistical outcome is summarized within the figure (ANOVA1: p-value) and symbols (asterisks) indicate when a mean was significantly different from control (Dunnett's test). Bonferroni's correction for multiple comparisons was applied.
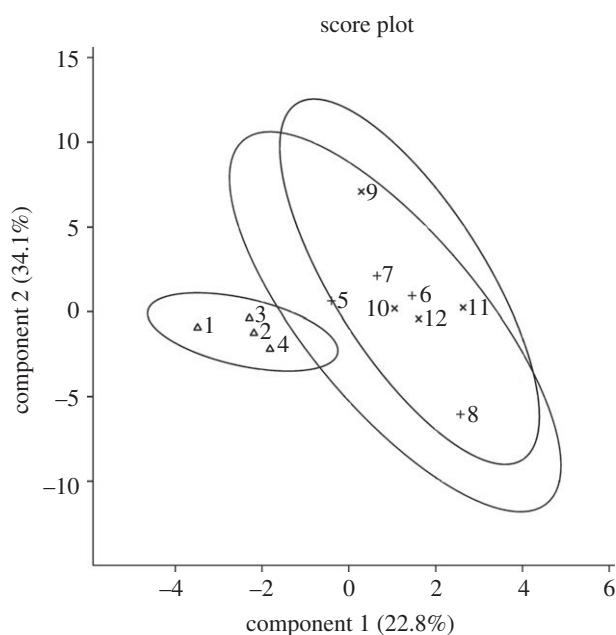
Figure 3. Projection of samples onto the first discriminant plane of the LDA. Control flies (C0, symbol Delta) and recovering flies assessed 24 and 48 h following CO_2 treatment (R24, symbol 'plus' and R48, symbol 'multiplication'; $n = 4$).

that a consequence of CO_2 anaesthesia is an alteration of energetic metabolism.

In this study, all flies quickly recovered from acute CO_2 exposure. This is true from a mobility standpoint; however, we found that metabolic profiles remained different from controls for several hours (up to 14 h). During the recovery period, concentrations of several metabolites were altered relative to controls. The metabolites contributing the most to the temporal pattern during the recovery were sorbitol, serine and urate. These metabolites had a high concentration in 1 h-recovering flies followed by a temporal reduction during the recovery. Upon reperfusion, the cells experience sudden oxygen influxes, which create a burst in reactive oxygen species (ROS) production [12,13]. Urate is an important scavenger of oxygen radicals [14], and the importance of urate in oxygen defence has been unambiguously demonstrated in D. melanogaster [15]. The occurrence of this biomarker of free radical damage indicates that oxidative damage probably occurred during the recovery post-CO_2 exposure. Likewise, polyols (such as sorbitol) are effective ROS scavengers in insects [16]. Sorbitol is also considered to be an anaerobic end product in insects [17]. The causative roles of these metabolic variations are not known, but these modulations clearly highlight that important metabolic changes take place during recovery from CO_2 anaesthesia.

This study brings evidence for detrimental effects of CO_2 anaesthesia on physiological traits. Metabolic disturbance caused by CO_2 was still manifested after 14 h of recovery. However, we found no evidence of system-wide metabolic changes when a long recovery period was allowed (more than 24 h). Thus, CO_2 anaesthesia should be used with caution in physiological studies,

especially if only short-term recovery is allowed. We suggest that CO_2 treatment is an acceptable anaesthetic method provided that a long recovery period (more than 24 h) is allowed. On the basis of the present metabolic profiling data, we corroborate the notion that at least 24 h recovery is necessary to avoid side effects of CO_2 anaesthesia [4]. Further comparisons with other types of anaesthetics such as chilling, nitrogen or ether would help us to elucidate whether the metabolic disturbances seen in this study are shared across several forms of anaesthesia or specific to CO_2.

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