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SHORT COMMUNICATION

Chilling slows anaerobic metabolism to improve anoxia tolerance of insects

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Abstract
Background Insects are renowned for their ability to survive anoxia. Anoxia tolerance may be enhanced during chilling through metabolic suppression.
Aims Here, the metabolomic response of insects to anoxia, both with and without chilling, for different durations (12–36 h) was examined to assess the potential cross-tolerance mechanisms.
Results Chilling during anoxia (cold anoxia) significantly improved survival relative to anoxia at warmer temperatures. Reduced intermediate metabolites and increased lactic acid, indicating a switch to anaerobic metabolism, were characteristic of larvae in anoxia.
Conclusions Anoxia tolerance was correlated survival improvements after cold anoxia were correlated with a reduction in anaerobic metabolism.

Keywords Anoxia · Hypoxia · Cold · Lactic acid · False codling moth

1 Introduction

Periods of hypoxia (e.g. ischemia) followed by oxygen reperfusion are detrimental for humans and many mammalian cells. However, insects are well-adapted to survive anoxia exposures, and may serve as models for studying and testing mechanisms proposed to underlie diseases or processes that are thought to be related to hypoxia or oxidative damage (e.g. ageing, cancer, sleep apnoea, ischemia) (Haddad 2006). Before insect models can become widely accepted, a thorough understanding of hypoxia tolerance, and the factors that influence hypoxia- or reperfusion-induced damage are needed. Overlapping biochemical pathways between diverse taxa rapidly expands the range of model organisms that can be studied, and can generate novel insights into classic research problems such as hypoxia tolerance and adaptations thereof.

In nature, insects are routinely exposed to a wide range of stressors including temperature and hypoxia. Hypoxia can occur while living underground, at high altitudes, or frozen under ice; during different stages of development; and during periodic flooding or submersion events. Hypoxia is particularly pertinent to aquatic insects, and can have detrimental effects on growth and development for insects exposed to hypoxia during development (Harrison et al. 2006; Verberk et al. 2016). In its most extreme form, hypoxia can also be referred to as anoxia, with many insects surviving hours of 0 % oxygen, some even surviving for several days (Hoback 2012). Survival after hypoxic conditions is variable depending on several factors

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including gas concentrations, temperature, humidity and developmental stage. The mechanisms of extreme hypoxia or anoxia tolerance in terrestrial insects are not typically well studied, with the notable exception of the vinegar fly *Drosophila melanogaster*, and therefore the mechanistic basis underlying differences in tolerance between species are largely unknown (Feala et al. 2007; Schilman et al. 2011). While metabolism is typically downregulated during hypoxia, ATP levels during anoxia are actively maintained in some insects, while other species allow ATP to decline rapidly during hypoxia (Hoback et al. 2000; Weyel and Wegener 1996). Anaerobic metabolism in insects facilitates exposure to environmental hypoxia, and cellular hypoxia (as in insect flight) (Gäde 1985). In insects, a switch to anaerobic metabolism during anoxia is typically associated with increases in levels of lactate, succinate and/or alanine (Feala et al. 2007; Hoback et al. 2000; Meidell 1983; Price 1963).

In ectothermic organisms, cooling can negate the detrimental effects of anoxia at higher temperatures (including optimal rearing temperatures). Recently, the interactive effect between cold and anoxia has been demonstrated in *D. melanogaster* with anoxia at 3 °C significantly reducing post-anoxia recovery time in comparison to anoxia at 23 °C (Benasayag-Meszaros et al. 2015). While whole-animal metabolic responses to cold are relatively well documented, fewer metabolite responses to both anoxia and cold have been documented (Hoback et al. 1998; Meidell 1983; Storey and Storey 2010), thus it remains unclear what biochemical pathways are being employed under these conditions or if overlapping cross-tolerance mechanisms aid survival.

Cross tolerance between hypoxia and cold has been documented in false codling moth larvae (*Thaumatotibia leucotreta*), an agricultural pest of quarantine concern (Boardman et al. 2015). Additional studies on this system showed that the critical oxygen partial pressure of these larvae is 4.5 kPa, below which metabolic rate is suppressed. Further, we have previously shown that these larvae have some scope for anaerobic metabolism (increased concentrations of lactate and alanine) during short-term cold exposure in both normoxic and hypoxic (2.5 kPa) exposure (Boardman et al. 2016). Consequently, we were interested in studying the metabolomic responses to longer-term anoxia, and the potential for cold temperatures to negate detrimental anoxic effects.

Here, using *T. leucotreta*, we therefore aimed to investigate differences in longer-term (12–36 h) anoxia tolerance between anoxia at room temperature conditions (23 °C) (referred to hereafter as 'anoxia') and under low temperatures ('cold anoxia'), and identify metabolites correlated with responses to these conditions. We hypothesised that cold anoxia would result in lower mortality than anoxia or the control, and that this improvement in tolerance would be associated with decreased levels of anaerobic metabolites—indicating a suppression of anaerobic metabolism at low temperatures.

2 Materials and methods

2.1 Insects

Early instar larvae of false codling moth *T. leucotreta* (Lepidoptera: Tortricidae) were obtained from a mass-reared culture at Cedar Biocontrol Insectary, XSIT, Citrusdal, South Africa. Upon arrival, larvae were reared following established protocols (Hofmeyr et al. 2015) in an incubator at 25 ± 5 °C (~50–60 % RH) (L:D 12:12 h) on standard diet medium until they began searching for pupation sites. These non-feeding 5th instar larvae were used directly in our experiments. All larvae used for analyses were still in their larval phase at the end of the experiments. Larvae that pupated during the experiments were excluded. Additional samples were incorporated into treatments to account for these samples, and each group consisted of 30 randomly sampled larvae selected post-treatment.

2.2 Anoxia tolerance treatments

Anoxia tolerance was investigated by exposing larvae to pure compressed N_2 (Air Products, South Africa) for 12, 24 or 36 h (flow rate: 100 mL min^{-1}) either at 23 °C or while kept cool (0 °C) in a programmable circulating and refrigeration bath filled with ethanol (CC410wl, Huber, Germany). Temperature was monitored using iButtons (DS1921G, accuracy ± 1 °C, Dallas Semiconductors, Dallas, Texas, USA). Durations were chosen to span from high survival (>90 %) regardless of temperature, to a duration that caused low survival (<20 %) in anoxia. Each set of duration experiments was performed on a different day, using individuals from the same rearing jar, and included a same-cohort control (kept at 23 °C under normal atmospheric air conditions). Larvae were placed in a 1.65 L (anoxia) or 0.6 L (cold anoxia) plastic container, plumbed to receive gas. Nitrogen was passed through a mass flow control valve (Sidetrak, 1 Sierra International, USA) connected to a mass flow control box (Sable Systems, Las Vegas, Nevada, USA) before entering the container. Containers were plumbed to have an exit tube open to the atmosphere to maintain N_2 flow and keep the containers under positive pressure (no atmospheric gas could enter). For the first 15 min, flow rate was 250 mL min^{-1} to rapidly flush the container with N_2. During this time, larvae

at 23 °C became inactive as a result of the N_2. Thereafter, flow rate was reduced to 100 mL min^{-1} for the duration of the experiment. Separate pilot trials showed that constant <0.5 % O_2 was readily achieved under these conditions. Each treatment group consisted of n = 30 larvae, and each group was replicated twice. Larvae in the control group were placed singly in tubes for the treatment duration to prevent cannibalism that may otherwise have occurred. As both cold and anoxia result in inactivity, cannibalism was not a problem for these treatments. After the treatment, larvae were placed individually into 1.5 m L microtubes, returned to normal rearing temperatures (25 °C) and monitored for pupation and emergence. No additional food was provided as these final instar larvae do not feed. Mortality rates were analysed using a GLZ in proc genmod with a binomial distribution and a logit link function, and corrected for overdispersion (SAS 9.4, SAS Institute Inc., Cary, NC, USA). Replicate was not a significant factor in the original models, and was thus removed from subsequent analyses. Significant differences between groups were identified using the *lsmeans* procedure in SAS.

2.3 Anaerobic metabolites

To determine whether *T. leucotreta* larvae used aerobic or anaerobic metabolism during the treatments, we measured the changes in 43 metabolites following methods outlined in Boardman et al. (2016). Samples for metabolomics were obtained by repeating the anoxia tolerance treatments. Larvae were immediately frozen in liquid nitrogen before reoxygenation took place and stored at −80 °C (n = 20 per treatment). Samples were weighed to determine fresh mass, extracted in 70 % ethanol, and analysed using a combination of GC/MS and liquid chromatography (LC) coupled to MS (LC/MS). Four individuals were pooled for each sample, and three samples were obtained for each treatment group.

Whole-system metabolic changes were analysed in MetaboAnalyst 3.0 (Xia et al. 2015). Data were scaled using Pareto scaling (mean-centred and divided by the square root of the standard deviation of each variable). The entire dataset was analysed using two-way between subjects ANOVA with false discovery rate correction, and principal components analysis (PCA). Comparison of treatments within each duration were analysed independently using a Partial-Least Squares Discriminant Analysis (PLS-DA). Thereafter variance of importance scores (VIP) were used to identify the changes in metabolites responsible for the first principal component (PC1). Data from the 36 h duration were analysed using 2-way comparisons between treatments.

3 Results and discussion

3.1 Larval pupation and emergence

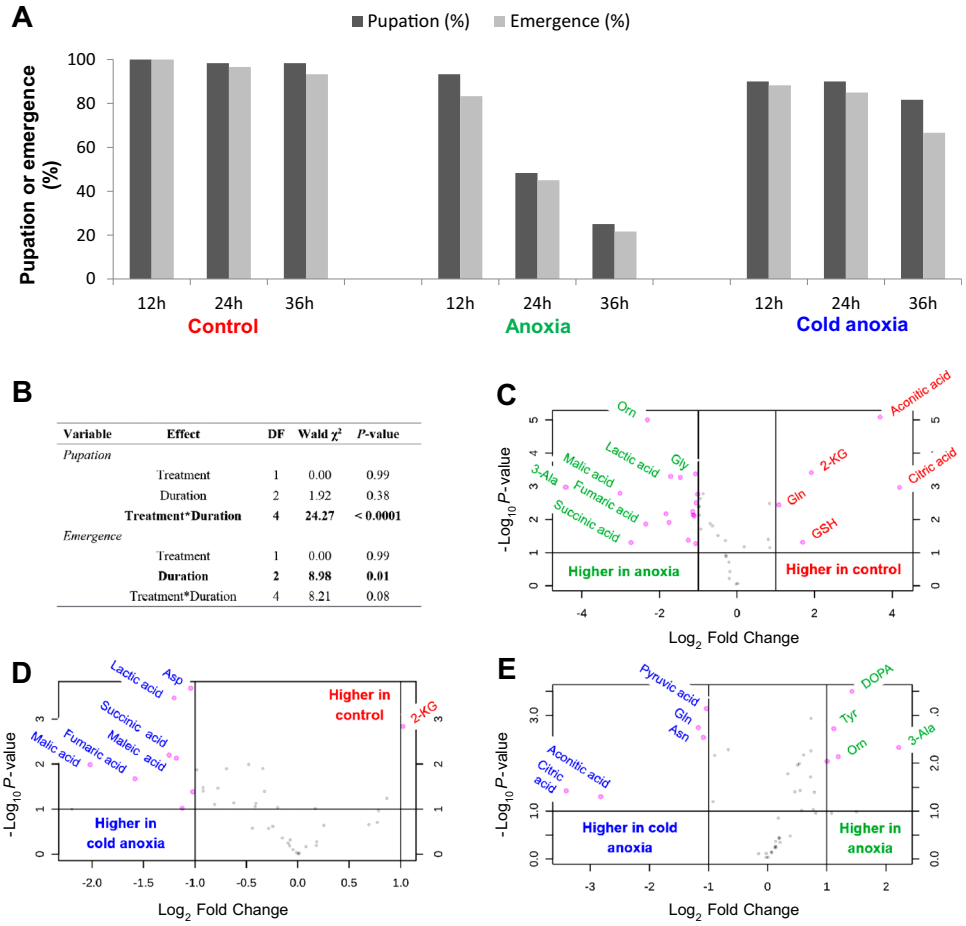
Larvae that did not pupate started turning black shortly after return to normoxia, indicating that irreparable damage had occurred during the exposure. Pupation was affected by an interaction between treatment (control, anoxia and cold anoxia) and duration, while emergence (indicative of survival of the pupal stage) was only affected by the duration of exposure (Fig. 1a, b). Larvae in the control groups were not significantly different from one another after exposure for different durations (12, 24, 36 h) indicating that the cohorts themselves were not different (Fig. 1a). Within the anoxia treatment, pupation was significantly reduced as duration increased. Cold alleviated the detrimental anoxia effect on survival and there were no significant differences in pupation success between 12, 24 or 36 h cold anoxia ($P > 0.1$).

There were no significant differences between treatments at 12 h. At 24 and 36 h, pupation success after anoxia was significantly lower than control or cold anoxia ($P < 0.001$). Pupation after cold anoxia was also significantly less than control levels (24 h; $P = 0.03$; 36 h; $P = 0.002$). In general, emergence rates closely followed pupation rates, indicating that mortality during the pupal phase was low and larvae that pupated generally emerged successfully as adults. The exception was 36 h cold anoxia with high pupation and lower emergence, which indicated that some damage could not be repaired after the longer cold anoxia treatment.

These results demonstrate that cold improved anoxia tolerance in *T. leucotreta* larvae scored in different functional assays. This confirmed our hypothesis based on literature from other insect studies, and matches the limited evidence available for other Lepidoptera (Soderstrom et al. 1990, 1991; Whiting et al. 1992). The dry air and lowered relative humidity caused by anoxia is not responsible for the decrease in pupation and emergence, as 36 h of dry conditions is easily tolerated by *T. leucotreta* larvae (Boardman et al. 2013).

3.2 Metabolomics

Forty-three metabolites were measured in *T. leucotreta* larvae (Fig. 2a, Table S1). Fifteen metabolites were significantly altered by experimental treatment alone, 22 were significantly changed as a result of both treatment and duration independently, and two metabolites were affected by the treatment, duration and the interaction between them (Fig. 2a, Table S1). Arginine, histidine, threonine and glutathione were not significantly altered by either treatment or duration. The heatmap illustrates that anoxia was



Variable	Effect	DF	Wald χ^2	*P*-value
Pupation				
	Treatment	1	0.00	0.99
	Duration	2	1.92	0.38
	Treatment*Duration	**4**	**24.27**	**< 0.0001**
Emergence				
	Treatment	1	0.00	0.99
	Duration	**2**	**8.98**	**0.01**
	Treatment*Duration	4	8.21	0.08

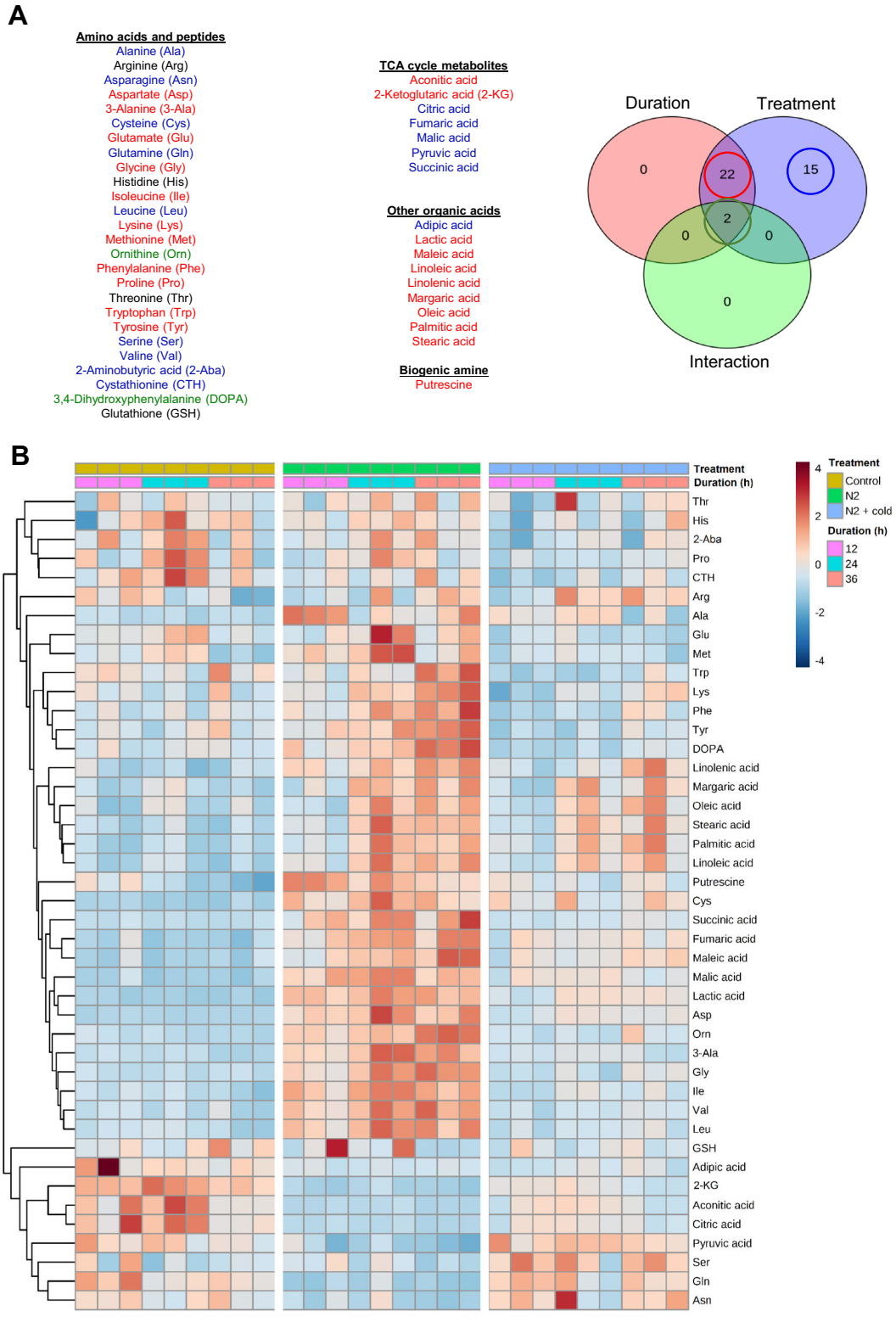
Fig. 1 Survival results after all treatments (**a**, **b**) and 2-way comparisons of analytes between different 36 h experimental treatments (**c**–**e**). **a** Barplots of pupation (*black*) and emergence (*grey*) after the experimental treatments. As replicates were not significantly different ($P > 0.4$) these data were combined. N = 60 per treatment. **b** Summary of results of generalized linear model (GLZ) for the effects of treatment and duration on the variables measured. Significant effects are highlighted in bold font. **c**–**e** Volcano plots based on *P*-values and fold changes highlighting the metabolites that were significantly different (after 36 h) between: control and anoxia (**c**), control and cold anoxia (**d**), anoxia and cold anoxia (**e**). *2-KG* 2-ketoglutaric acid, *Asn* asparagine, *Asp* aspartate, *3-Ala* 3-alanine, *Gln* glutamine, *GSH* glutathione, *Gly* glycine, *DOPA* 3,4-dihydroxyphenylalanine, *Orn* ornithine, *Tyr* tyrosine

correlated with increases in numerous metabolites, and was clearly different to control, with cold anoxia as an intermediate (Fig. 2b).

Analysing the differences between experimental treatments within each duration shows that anoxia forms a separate group to control and cold anoxia at each duration (Figure S2). Control treatments showed high aerobic metabolism, indicated by an active TCA cycle (high ketoglutaric, citric and aconitic acid) (Fig. 2b). Anoxia treatments showed an increase in anaerobic metabolism (e.g. increase in lactic acid) (Fig. 2b). Cold anoxia responses were more similar to control than anoxia-responses, but did show some accumulation of anaerobic metabolites at longer durations (Fig. 2b).

In all three durations, the PLS-DA could completely differentiate between the experimental treatments, with PC1 explaining at least 83 % of the variance (Figure S2). At 12 and 24 h, ketoglutaric acid, lactic acid, citric acid and alanine were responsible for separating the treatments on PC1 (Figure S2B, S2D). After 36 h, ketoglutaric acid, lactic acid and margaric acid were the biggest contributors to PC1 (Figure S2F). At all durations, ketoglutaric and citric acid were highest in the control, indicating aerobic metabolism, while lactic acid was highest during anoxia, indicating reliance upon anaerobic metabolism. After 36 h, two-way comparison between treatments showed that five metabolites were significantly higher in control, compared to anoxia. Seven metabolites were higher in anoxia (Fig. 1c). When the control was compared to cold anoxia, only ketoglutaric acid was higher in control; while aspartate, lactic acid, succinic acid, malic, maleic and fumaric acid were higher in cold anoxia (Fig. 1d). Comparing anoxia with cold anoxia revealed that anoxia has higher 3,4-dihydroxyphenylalanine (DOPA), tyrosine, ornithine and 3-alanine while cold anoxia had higher pyruvic acid, glutamine, asparagine, aconitic acid and citric acid (Fig. 1e).

The TCA cycle should be considered segmentally under conditions of hypoxia, not just continuing in a cycle, but with different segments operating in sometimes opposing directions (reviewed in Chinopoulos 2013). These three segments are: (1) oxaloacetic acid + acetyl-CoA → citric acid ↔ isocitric acid ↔ 2-ketoglutaric acid; (2) 2-ketoglutaric acid → succinyl-CoA ↔ succinic acid; and (3) oxaloacetic acid ↔ malic acid ↔ fumaric acid ↔ succinic acid (sensu Chinopoulos 2013). Using our data within this


A
Amino acids and peptides
Alanine (Ala)
Arginine (Arg)
Asparagine (Asn)
Aspartate (Asp)
3-Alanine (3-Ala)
Cysteine (Cys)
Glutamate (Glu)
Glutamine (Gln)
Glycine (Gly)
Histidine (His)
Isoleucine (Ile)
Leucine (Leu)
Lysine (Lys)
Methionine (Met)
Ornithine (Orn)
Phenylalanine (Phe)
Proline (Pro)
Threonine (Thr)
Tryptophan (Trp)
Tyrosine (Tyr)
Serine (Ser)
Valine (Val)
2-Aminobutyric acid (2-Aba)
Cystathionine (CTH)
3,4-Dihydroxyphenylalanine (DOPA)
Glutathione (GSH)
TCA cycle metabolites
Aconitic acid
2-Ketoglutaric acid (2-KG)
Citric acid
Fumaric acid
Malic acid
Pyruvic acid
Succinic acid
Other organic acids
Adipic acid
Lactic acid
Maleic acid
Linoleic acid
Linolenic acid
Margaric acid
Oleic acid
Palmitic acid
Stearic acid
Biogenic amine
Putrescine
Duration
Treatment
Interaction
0
22
15
2
0
0
0
B
Treatment
Duration (h)
Thr
His
2-Aba
Pro
CTH
Arg
Ala
Glu
Met
Trp
Lys
Phe
Tyr
DOPA
Linolenic acid
Margaric acid
Oleic acid
Stearic acid
Palmitic acid
Linoleic acid
Putrescine
Cys
Succinic acid
Fumaric acid
Maleic acid
Malic acid
Lactic acid
Asp
Orn
3-Ala
Gly
Ile
Val
Leu
GSH
Adipic acid
2-KG
Aconitic acid
Citric acid
Pyruvic acid
Ser
Gln
Asn
4
2
0
-2
-4
Treatment
Control
N2
N2 + cold
Duration (h)
12
24
36

◀**Fig. 2** Metabolomics results for all the data. The table **a** shows the metabolites measured in our analytical setup. The colour of the metabolite refers to the Venn diagram illustrating the results of two-way between subjects ANOVA (see Table S1 for *P*-values). Metabolites significantly altered by treatment (control, anoxia or cold anoxia) only are shown in blue, metabolites significantly altered by both duration and treatment are in *red*, and metabolites altered by time, treatment and the interactions between the two are in *green*. Four metabolites that remained unchanged are shown in black. The heatmap **b** shows the overall data. Colour was used to highlight highest (*red*) and lowest (*blue*) relative metabolite values within each row. Durations were 12, 24 or 36 h. Experimental treatments were: control, N_2 (anoxia), or N_2 + cold (cold anoxia) (*Inset* on left hand side is the hierarchical clustering dendrogram showing metabolites which responded most similarly, irrespective of pathway or reaction node. Methods: Euclidean distance measure, ward clustering)

framework suggests several interesting areas for subsequent studies. First, the low pyruvic acid, citric acid and ketoglutaric acid under anoxia in our data indicates that the first segment of TCA slows down/stops as there is decreasing (but still continuing) influx of pyruvate from glycolysis. Part of this pyruvate is converted to lactate to regenerate NADH for continuing glycolysis, while another part goes to the third segment of TCA, and a third part is converted to alanine. Second, the high succinic acid during anoxia clearly shows that the second segment of TCA is actively converting ketoglutaric acid to succinic acid which greatly helps to continue production of ATP under anoxic conditions (Fig. 1c, d). This continued ATP production is likely the major reason underlying many of the changes we observed. Third, the NAD^+ required for the second segment is most probably generated by a third segment of TCA which works in a backflux. This interpretation of our data is well supported by the accumulated malic acid and fumaric acid after anoxia. Fourth, glutamine and glutamate could become very important sources of ketoglutaric acid for activity of the second TCA segment in anoxia. This is supported by the decrease in glutamine reserves during anoxia. Protein degradation during anoxia can also yield high amounts of glutamate, and other amino acids—which matches our data (Fig. 2b). Last, as the ketoglutaric acid is formed from glutamate, the amino-groups must be somehow deposited. Most probably they go to alanine and aspartate via alanine transaminase and aspartate transaminase, respectively, which may partially explain why alanine and aspartate accumulate during anoxia.

The differences between cold anoxia and anoxia may indicate early vs late stage anoxic effects (e.g. evidence from marine molluscs in Storey and Storey 2005) Nevertheless, there is another noteworthy feature in our results when comparing elevated metabolites at cold anoxia versus. anoxia (Fig. 1e). The higher citric acid and coupled aconitic acid indicate that cold anoxia may induce a flux in the other direction, i.e. from ketoglutaric acid over isocitric acid to citric acid, thus partly repairing the impairments of mitochondrial function. This alternative pathway is called reductive carboxylation (Wise et al. 2011). It is further interesting that glutamine that is also upregulated (Fig. 1e) is a source for ketoglutaric acid, perhaps further driving this bidirectional metabolism. There is still more evidence that this isocitrate-dehydrogenase (IDH) dependent carboxylation of ketoglutaric acid to citric acid is a part of the metabolic reprogramming associated with hypoxia-inducible factor 1 (HIF1) which is functional in all metazoans. This hypothesis is corroborated here by the observation that chilling improved the survival, and thus even anoxic cells are able to partly maintain cell proliferation (Wise et al. 2011). It is unclear how this works during anoxia versus hypoxia and in the context of cold adaptations, but the proposed hypothesis may lead to novel experiments for elucidation of this potentially adaptative mechanism.

The increase in asparagine and glutamine during cold anoxia may also suggest an accumulation of ammonia deposits. The general suppression of metabolic rate at cold temperatures is likely linked with a decreasing rate of proton production and, consequently, less acidification. Thus, cold may alleviate the acidosis typically associated with hypoxia. Interestingly, the metabolites that are higher in anoxia than cold anoxia can be linked to morphological changes during pupation. Both DOPA and tyrosine play a role in hardening and darkening of the cuticle, and increased DOPA has been linked to increased mortality and deformed pupae in bioassays on the southern army-worm *Prodenia eridania* (Rehr et al. 1973). The conversion of tyrosine to DOPA requires molecular oxygen as one of the co-factors, and while speculative, our data suggest that this correlation between these metabolites and anoxia warrants further exploration as the effects of anoxia on development are of interest to post-harvest control.

3.3 Conclusions and future directions

The data suggests that the accumulation of anaerobic metabolites is associated with mortality after 24 h anoxia and is linked to a substantial reduction in survivorship in comparison to control larvae. Cold anoxia was less detrimental than anoxia and was correlated with decreasing anaerobic metabolism, seemingly delaying the detrimental effects of anoxia. The need for energy (ATP) is much lower in the cold than under normothermia, and because anoxia severely impairs ATP production, the deleterious consequences of anoxia are much more pronounced under normothermia than during cold anoxia. The recent interactive effects shown between cold and anoxia in *D. melanogaster* (Benasayag-Meszaros et al. 2015) were attributed to the regulation of neuronal potassium homeostasis during

anoxia. Thus, it appears that numerous insect systems may be adapted to confer improved anoxia tolerance during cold temperatures. Further research is necessary to fully understand the interactive effects of cold and anoxia tolerance but this information may be especially useful to develop chemical-free phytosanitary treatments for pest management and in the development of insect models for hypoxia/anoxia associated diseases or pathologies.

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Compliance with ethical standards

Conflict of interest L Boardman, J. Sørensen, V. Kostal, P. Simek and J. Terblanche have no conflict of interest to declare.

Ethical Approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

Informed consent Informed consent and ethical approval was not required for this study.

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