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Developmental and metabolic changes induced by anoxia in diapausing and non-diapausing flesh fly pupae

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Summary. Anaerobic metabolism was compared in non-diapausing (ND) and diapausing (D) pupae of the flesh fly, *Sarcophaga crassipalpis* using in vivo ^{13}C NMR spectroscopy. Anoxia-induced changes in the development of ND and D pupae were correlated with oxidative metabolism and mitochondrial integrity. ND pupae tolerated 1 day of anoxia without any obvious developmental effect, while D pupae tolerated up to 6 days of anoxia. Longer exposure to anoxia (3 days in ND pupae and up to 14 days in D pupae) allowed development to the pharate adult stage but precluded eclosion. Four-day anoxia applied to ND pupae during 4–6 days post-pupariation arrested development in a stage indistinguishable from diapause. This morphological stasis was accompanied by 80% suppression of oxidative metabolism and a 100% increase in glycerol concentration. However, unlike a true diapause, this arrest could not be terminated with 20-hydroxyecdysone or hexane. Four-day anoxia treatment applied to D pupae stimulated development and raised their oxygen consumption. The anoxia-induced changes in oxidative metabolism were not accompanied by mitochondrial changes. Exposure to 95% PO_2 atmosphere had no apparent developmental or metabolic effects on ND or D pupae. Major metabolites (lipids, trehalose, glycogen, glycerol, glutamine, and alanine) were detected in the ND and D pupae but their rates of turnover differed. Anoxia induced synthesis of glycerol and alanine in both D and ND pupae. Injected labeled glucose was incorporated primarily into trehalose and glycogen by both D and ND pupae. The rate of incorporation in ND pupae was approximately twice that observed in D pupae. Anoxia resulted in glycerol and alanine synthesis in both groups of pupae, but more glycerol was labeled in ND pupae and more alanine in D pupae. Glycogen and trehalose were depleted in the D pupae under anoxia. Cold acclimation had no effect on the steady-state or rate of synthesis of metabolites.

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Introduction

Insect diapause is a state of arrested development (Tauber et al. 1986; Danks 1987), during which oxygen consumption is suppressed by 30–90% (Keister and Buck 1964). One major distinguishing feature between diapausing and non-diapausing insects is the depressed oxidative metabolism. Oxygen consumption is usually suppressed during the early phase of metamorphosis (Lees 1955) and a further decrease of oxygen uptake occurs when metamorphosis is interrupted by diapause (Schneiderman and Williams 1953; Denlinger et al. 1972). Reduced oxygen consumption in diapausing pupae of *Hyalophora cecropia* is associated with the reduction of mitochondrial cytochromes $a+a_3$ and c (Shappirio and Williams 1957a, b). Cold acclimation also led to mitochondrial degradation, reversible upon warm acclimation, in the larvae of *Gynaephora groenlandica* (Kukal et al. 1989). Diapause induction can be averted in *Bombyx mori* eggs by exposing them to an oxygen-saturated atmosphere (Sonobe et al. 1979). Could suppression of oxidative phosphorylation by anoxia provide a metabolic trigger for induction of the diapause program? This study was designed to test whether restricted oxygen supply can alter the developmental program and metabolism in non-diapausing vs diapausing pupae of the flesh fly.

In diapausing pupae of the flesh fly, *Sarcophaga crassipalpis* Macquart (Diptera: Sarcophagidae), oxygen consumption is only 5–10% of the rate found in non-diapausing pupae (Denlinger et al. 1972). Moreover, the diapausing pupae experience hormonally-driven cycles of oxygen consumption that have a periodicity of about 4 days at 25 °C (Denlinger et al. 1984). Days of relatively high activity (40–60 $\mu l \cdot g^{-1} \cdot h^{-1}$) cycle with days in which no oxygen consumption can be detected. Suppressed oxidative metabolism has previously been cor-

related with the accumulation of anaerobic by-products in insects (Wilhelm et al. 1961; Meyer 1978, 1980; English et al. 1982; Redecker and Zebe 1988; Kukal et al. 1989). By-products of anaerobiosis, polyols, carbohydrates, and some amino acids, may also function as antifreezes/cryoprotectants in enhancing insects' cold tolerance (Chino 1957; Salt 1958; Conradi-Larsen and Sømme 1973; Sømme 1974; Kukal et al. 1989; Loomis et al. 1989). Both diapausing and non-diapausing pupae of *S. crassipalpis* synthesize glycerol in response to short- or long-term exposure to low temperature, and this increase in glycerol concentration is correlated with an increase in cold tolerance (Lee and Denlinger 1985; Lee et al. 1987a, b; Chen et al. 1987). Can pupal cold tolerance be enhanced by anoxia and does the response to oxygen deprivation depend on developmental programming?

The objectives of our study were: (1) to investigate tolerance of anoxia in non-diapausing vs diapausing pupae of *S. crassipalpis*, (2) to determine the developmental phase(s) sensitive to anoxia, (3) to evaluate the similarity between diapause and anoxia-induced arrest of development, (4) to test the influence of an oxygen-saturated atmosphere on the decision to enter diapause, (5) to examine the mitochondria in non-diapausing and diapausing pupae before and after anoxic treatments, (6) to compare oxidative metabolism in non-diapausing and diapausing pupae and determine which compounds accumulate during and following anoxic treatment, and (7) to test the cryoprotective function of the anaerobic by-products.

Materials and methods

Insect rearing. Flesh flies, *Sarcophaga crassipalpis* Macquart (Diptera: Sarcophagidae), were reared in the laboratory as described in Denlinger (1972). Adults were held at 25 °C and short (12 h light: 12 h dark) or long (15 h light: 9 h dark) photoperiods. Females larviposited on liver and ca. 80 larvae were placed in individual packets of liver held at 20 °C or 25 °C. Diapausing (D) pupae reared in short photoperiod and non-diapausing (ND) pupae from the long photoperiod regimen were used in all the experiments.

Anoxia treatments of diapausing and non-diapausing pupae. Tolerance of D and ND pupae to anoxia was determined by placing pupae in 500-ml Erlenmeyer flasks continously flushed with N_2 gas previously bubbled through water. Thirty pupae were exposed to anoxia for different periods of time ranging from 1h to 30 days and also at different stages post-pupariation. Response to anoxia was scored by following pupal development (Fraenkel and Hsiao 1968) in anoxia-treated pupae in comparison to a control group. Time required for development of the antennae, pigmentation of the eyes and bristles, and the eclosion of adults was recorded. These initial experiments helped to establish the developmental phase (4–5 days post pupariation in ND pupae and 10 days in D pupae) and duration of exposure to anoxia (4 days) which altered the diapause program. This information was subsequently used in the majority of experiments comparing D with ND pupae.

The response of D and ND pupae to an oxygen-saturated atmosphere was tested by exposing 50 pupae to 95% O_2 (5% CO_2) in a sealed container for 4 days at 4 days post-pupariation and at the red-eye stage of the pharate adult. Results were assessed in the same manner as for anoxia-treated pupae.

The concentration of glycerol was determined following the methods of Lee et al. (1983) by HPLC (Waters Associates) in ND pupae, at 4 days post-pupariation, exposed to anoxia treatments for 1–7 days ($n = 10$ pupae /treatment). Stimulation of development by 20-hydroxyecdysone and hexane (Zdarek and Denlinger 1975; Denlinger et al. 1980) was tested in ND pupae following the conditions of anoxia which appeared to induce a diapause-like state (i.e., 4 days of anoxia on the 4th day after pupariation). The pupal caps were removed and 5 $\mu g \cdot \mu l^{-1}$ 20-hydroxyecdysone (Rohto Pharmaceutical, Osaka, Japan) was injected into each of 25 pupae after withdrawing an equivalent amount of hemolymph. Sixty pupae with intact puparia were submerged in hexane for 45 min following the anoxia treatment.

Cold acclimation and cold tolerance. Groups of 30 D pupae (10 days post-pupariation) and 30 ND pupae (4 days post-pupariation) were cold treated at 0 °C for a 4-day period. The effect of cold treatment on survival was determined by scoring the number of adults that emerged, compared to the influence of anoxia of similar duration on adult development.

Cold tolerance was assessed following the methods described in Chen et al. (1987). Thirty pupae were held in test tubes (10 × 1.5 cm) for 1.5 h and 2.0 h at −10 °C in a bath (Lauda RMT-20; Brinkmann). Non-diapausing pupae were held in N_2 for 24 h or for 4 days before groups of 30 individuals were exposed for either 1.5 or 2 h at −10 °C together with untreated control groups. Anoxic and untreated control groups were held at 25 °C. Differences in cold tolerance between treatment and control groups were assessed by the proportion of adults eclosed. Change in cold tolerance was similarly assessed in ND pupae, at 4 days after pupariation, treated with 95% oxygen atmosphere for 4 days.

Oxygen consumption. Oxygen consumption was determined on groups of 10 pupae at 25 °C using a Scholander respirometer (Mark Co., Brockton, MA). Diapausing (10 days post-pupariation) and non-diapausing (4 days post-pupariation) pupae were held in the respirometer continuously following 1–8 days of anoxia. Measurements of oxygen uptake were taken 0.5, 1, 2, 12, and 24 h after the anoxia treatment to check for metabolic compensation.

Mitochondrial numbers in diapausing and non-diapausing pupae. Numbers of mitochondria were compared in D and ND pupae exposed to a 4-day anoxia treatment. Mitochondrial state was correlated with differences in oxygen consumption between D and ND pupae and also in ND pupae with arrested development induced by anoxia. Mitochondrial counts were performed as in Kukal et al. (1989), using the fluorescent DNA stain DAPI (4,6-diamidino-2-phenylindole; Sigma Chemical Co., St. Louis) which binds to mitochondrial DNA and fluoresces under light of wavelength 365 nm (Wittekind 1972). The fluorescing mitochondria were viewed with an Olympus BH-2 microscope equipped with an epifluorescent light fixture. Photomicrographs were obtained with Ectachrome 160 ASA slide film exposed for 2–20 s. Controls and anoxia-treated D and ND pupae ($n = 10$ per treatment) were dissected under buffered insect saline and the individual fat bodies were stained in the dark at 5 °C for 20 min in DAPI ($2 \mu g \cdot ml^{-1}$). The fat bodies were placed in a droplet of Cargille B immersion oil on a slide and squashed gently with a cover slip to form a single layer of cells.

Instrumentation and materials. ^{1}H-Decoupled ^{13}C NMR spectra were obtained on a Bruker AM-500 Fourier-transform NMR spectrometer equipped with a pulse programmer and quadrature phase detection. Spectra were obtained with ~70° pulse widths and a recycle time (acquisition + relaxation delay) of 1 s; 16K–32K real points were obtained over a 0–190 ppm spectral width. Chemical shifts are reported in ppm (parts per million) relative to the C1 signal of deuterochloroform. D-[1-^{13}C]Glucose (99 atom-% ^{13}C) was obtained from Omicron Inc. (Chemistry Department, University of Notre Dame, Notre Dame).

Natural abundance ^{13}C NMR of live pupae and hemolymph. ^{13}C NMR spectra of individual, live pupae were obtained by inserting

them into a 5-mm NMR tube. Hemolymph was pooled from 10 pupae by puncturing their head with an insect pin after the pupal cap was removed. ^{13}C NMR-spectra of the hemolymph were obtained in a 5-mm NMR tube. All ^{13}C-spectra obtained were compared to standard chemical shifts determined previously (Kukal et al. 1988).

Comparisons were made between D and ND pupae at different stages of development. Different treatments included exposure to anoxia for different periods of time (4, 10, 20 days) and cold acclimation at 0 °C for a period of 1 and 4 days, followed by a 1- or 2-day warm acclimation. Steady-state abundance of ^{13}C compounds was also obtained for live pupae under anoxia for 24 h in a N_2-flooded and capped NMR tube, and for pupae after 4-day anoxia treatment. Another experiment was conducted with an NMR tube flooded with 95% O_2 with 5% CO_2, where steady-state spectra were obtained after 24 h and 4-day exposure to 95% O_2, then followed by 24 h in air.

^{13}C NMR of pupae injected with D-[1-^{13}C]glucose. The pupal cap was removed and individuals were injected with 1 µl 1 *M* 1-[^{13}C]glucose into their head region with a finely drawn micropipette. Each pupa was inserted into a 5-mm NMR tube and ^{13}C-spectra were obtained as a function of time using an automated NMR pulse program (KINET). The spectra were collected for 2 h each over a 15-h period. The lifetimes of metabolic intermediates were compared in D and ND pupae following a 4-day anoxia. In addition to the time-lapse spectra, steady-state NMR spectra were obtained for pupae subjected to ca. 24 h of anoxia following the injection of labeled glucose. Time-lapse spectra were also obtained for ND pupae exposed to 95% O_2 following injection of labeled glucose.

Results

Developmental changes induced by exposure to anoxia

Diapausing (D) pupae survived a longer exposure to anoxia than non-diapausing (ND) pupae. ND pupae exposed to 24 h or less of anoxia developed and emerged normally as adults. When exposed to anoxia for up to 3 days, flies developed into black-bristle stage pharate adults but failed to emerge. Four-day anoxia treatment of ND pupae arrested development in the phaneroce-phalic stage and such pupae remained in a morphological stasis that resembled diapause. These pupae, however, never resumed development and died within ca. 20 days. Hexane and 20-hydroxyecdysone, agents that readily terminate pupal diapause, were applied to the ND pupae 1 day after removal from 4 days of anoxia, but both treatments failed to stimulate development. As many as 6 days in anoxic conditions did not prevent eclosion in D pupae treated at 10 days after pupariation. D pupae exposed to 6–14 days of anoxia survived, terminated diapause, and eventually developed up to black-bristle pharate adults, but failed to eclose. Longer exposure was lethal.

When exposure of ND pupae to 4 days of anoxia was initiated during the first 5 days after pupariation further development was prevented. Four days of anoxic treatment initiated at 5–8 days post-pupariation allowed development to completion of the pharate adult stage, but prevented eclosion (Fig. 1A). Four days of anoxia applied to D pupae at 0–8 days post-pupariation precluded adult eclosion and resulted in death except during day 4–6 after pupariation. During this narrow developmental interval, anoxia averted diapause and development was initiated (Fig. 1B).

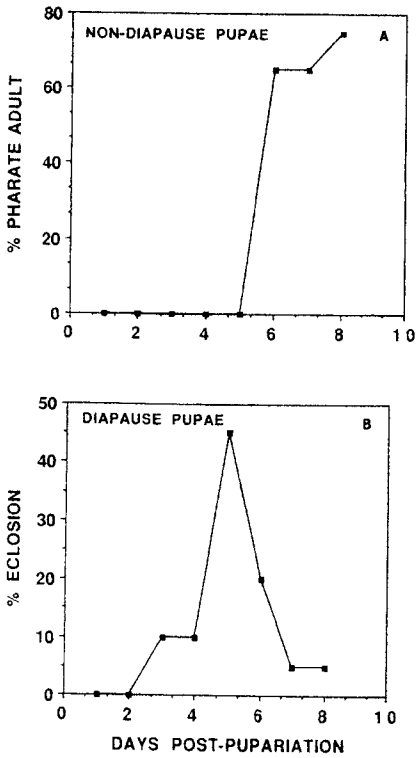

Fig. 1. Developmental success in (**A**) non-diapause and (**B**) diapause pupae of *S. crassipalpis* following a 4-day anoxia treatment started on days 1–8 after pupariation. The non-diapausing pupae developed but never eclosed, and therefore developmental success is expressed in terms of % reaching the black-bristle pharate adult stage, as opposed to % adult eclosion from the diapausing pupae

Exposure to 95% oxygen-saturated atmosphere had no influence on the development of ND pupae and did not interrupt diapause in the D pupae.

Anoxia and cold tolerance

Anoxic treatment for 1 or 4 days had no effect on the cold tolerance of ND pupae. Although brief exposure (1–2 h) of *S. crassipalpis* to 0 °C enhances survival at −10 °C (Chen et al. 1987), none of the flies ($n = 30$ per group) emerged as adults when exposed to 1 or 4 days of anoxia before being cold-shocked at −10 °C for 1.5 or 2 h. Similarly, 95% O_2 atmosphere did not change the cold tolerance of ND pupae. All individuals from control groups ($n = 30$ per group) held at 25 °C with or without anoxia eclosed.

Influence of anoxia on oxidative metabolism

Anoxia affected the subsequent metabolic rate in both ND and D pupae only after a 4-day exposure to nitrogen (Fig. 2). This change in oxygen uptake was induced only during a specific phase of development; 4 days post-pupariation in the ND pupae and 10 days in the D pupae.

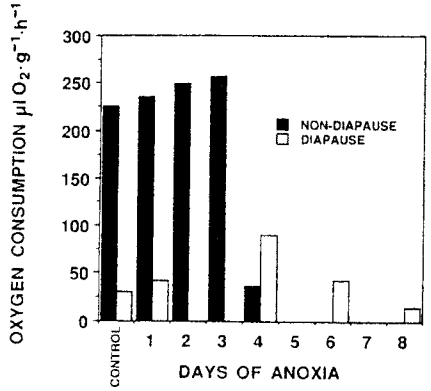

Fig. 2. The effect of anoxia on metabolic rate in non-diapausing and diapausing pupae of *S. crassipalpis*. Groups of 10 diapausing or non-diapausing pupae were exposed to anoxia for a period of 1–8 days. The anoxia treatment was initiated in the 2 groups during comparable developmental phases: at 4 days post-pupariation in non-diapausing pupae and at 10 days post-pupariation in diapausing pupae. Metabolic rates at day 0 represent the controls

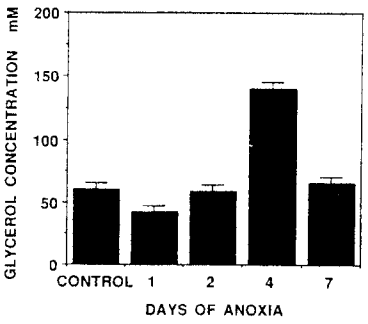

Fig. 3. Influence of anoxia on glycerol synthesis by non-diapausing pupae of *S. crassipalpis*. Groups of 10 pupae were exposed to different durations of anoxia (1–7 days) on the 4th day after pupariation. (Mean ± SE; $n = 3$ per treatment)

The effect was evident in an 80% reduction of oxygen consumption in ND pupae and a 50% increase in the D pupae. These changes in oxidative metabolism in anoxia-treated ND and D pupae were not correlated with changes in mitochondrial numbers or morphology (data not shown). There was no evidence of metabolic compensation during the initial period when pupae were removed from the nitrogen atmosphere.

Exposure to anoxia resulted in an increase in glycerol concentration in the ND pupae (Fig. 3). Approximately 100% increase was induced by a 4-day anoxia applied to ND pupae on the 4th day after pupariation.

Natural abundance of metabolites in non-diapausing and diapausing pupae

In vivo ^{13}C NMR spectra showed a similar set of metabolites in ND (Fig. 4A) and D (Fig. 4B) pupae: lipids (resonances at ~30, 130, and 175 ppm), trehalose (95, 74, 73, 71, and 62 ppm), glycerol (65 and 75 ppm), and glutamine (55 ppm). In addition, alanine (18 and 52 ppm) was a major component of D pupae. The presence of

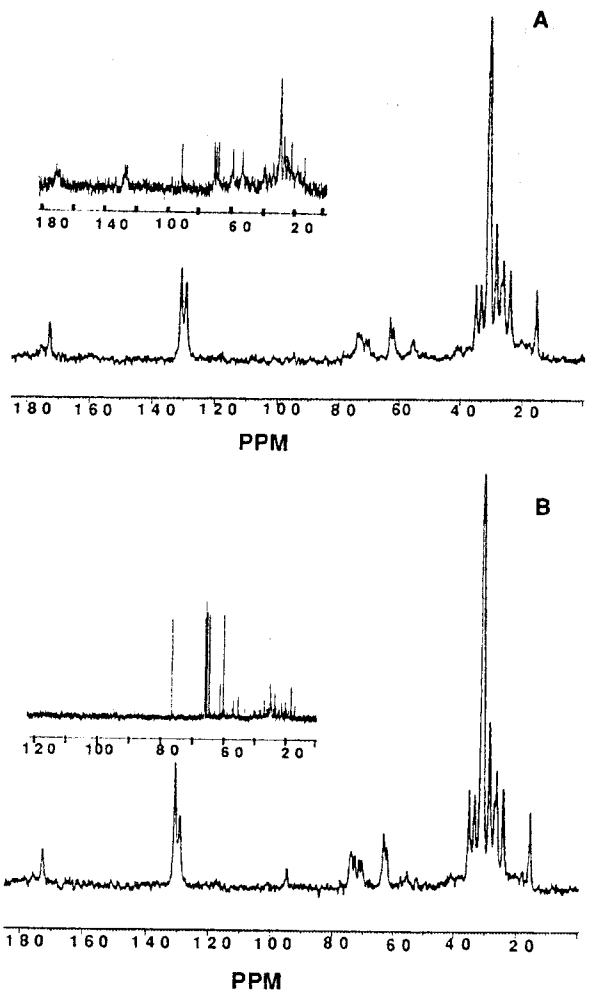

Fig. 4. ^{13}C NMR spectra representative of (**A**) an in vivo, non-diapausing pupa on the 4th day after pupariation and hemolymph (insert), and (**B**) an in vivo, diapausing pupa 10 days after pupariation and hemolymph (insert). The pupae were reared at 20 °C and spectra collected at 25 °C unless stated otherwise. The in vivo spectra **A** and **B** show prominent broad resonances at ca. 30, 130, and 175 ppm which correspond to lipids. Trehalose is represented by 6 carbon resonances between 62 and 95 ppm, glutamate at 55 ppm. The hemolymph spectra also indicate the presence of glycerol (65 and 75 ppm) and alanine (18 and 52 ppm). Peaks arising from resonances around the 30 ppm region show contamination of the centrifuged sample with unsaturated lipids

these compounds, particularly alanine and glycerol, was verified by the ^{13}C NMR spectra of hemolymph, where the compounds in solution gave rise to well-defined peaks (Fig. 4 inserts).

Natural abundance of metabolites in non-diapausing and diapausing pupae during and following anoxia

In vivo ^{13}C NMR of ND and D pupae following a 4-day anoxia showed increased concentration of alanine and glycerol, presumably anaerobic by-products (Fig. 5). The pupae contained glucose (α- and β-pyranose at ~62, 71, 73, 74, 76, 77, 93, 97 ppm with 4 doublet peaks) in addition to trehalose, glycerol, glutamate, and alanine

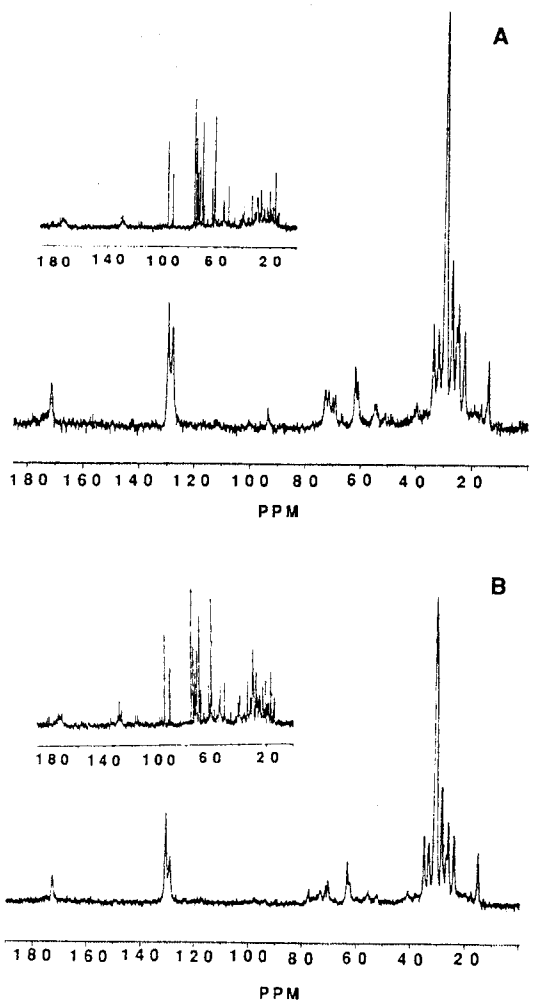

Fig. 5. ^{13}C NMR spectra following a 4-day anoxia of (**A**) non-diapausing pupa on 4th day post-pupariation, and (**B**) diapausing pupa at 10 days after pupariation. In vivo spectra are accompanied by spectra of hemolymph shown as inserts. All spectra show accumulation of glucose, in addition to the compounds also detected in non-diapausing and diapausing pupae not deprived of oxygen. Signals of glycerol and alanine show higher intensity than in the presence of oxygen, suggesting their accumulation under anoxia

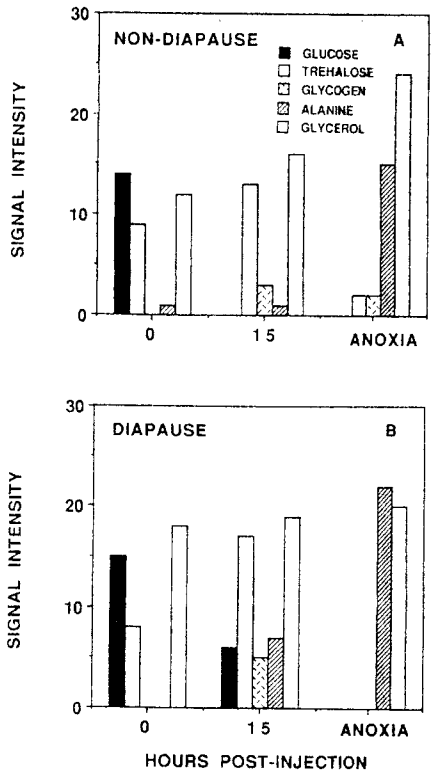

Fig. 6. Relative abundance of metabolites synthesized following an injection of 1-[^{13}C]glucose into (**A**) non-diapausing and (**B**) diapausing pupae. Time-lapse spectra were collected bi-hourly over a period of 15 h in the presence of oxygen and under anoxia. Within 15 h in normal atmosphere the non-diapausing pupae incorporated labeled glucose into trehalose, glycogen, and glycerol. Over the same time period under anoxia more label was incorporated into glycerol and major accumulation of alanine occurred, but trehalose was catabolized. The diapausing pupae show a pattern similar to the non-diapausing pupae with most label incorporated into trehalose and alanine in the presence of oxygen and into alanine under anoxia

(Fig. 5). The non-diapausing pupae showed the presence of glycogen (101 ppm) in addition to the compounds found in the D pupae. The spectra of hemolymph confirmed the presence of alanine and glycerol and their accumulation in response to anoxia in both ND and D pupae (Fig. 5 inserts).

Atmosphere saturated with oxygen at 95% concentration caused a depletion of carbohydrate storage compounds, trehalose and glycogen, in D and ND pupae, whereas glycerol concentration remained unchanged (data not shown).

Influence of cold acclimation on steady-state metabolites in non-diapausing pupae

Cold acclimation did induce the synthesis of metabolites different from those detected in the warm-acclimated ND pupae. Major peaks were attributed to glycerol, suggesting an elevated concentration (data not shown). Warm acclimation of the previously cold-acclimated pupae did not result in glycerol degradation within 24 h.

Aerobic and anaerobic metabolism of glucose by non-diapausing and diapausing pupae

Labeled glucose was metabolized similarly by ND and D pupae. Both developing and diapausing pupae incorporated the 1-^{13}C label intially into trehalose and then into glycogen in the presence of oxygen (Fig. 6). However, in the D pupae the conversion of glucose to trehalose and glycogen was much slower; ND pupae converted nearly all the label within 8 h, whereas D pupae incorporated only 50% of label into storage compounds within this period. Based on signal intensity (i.e., peak height), relatively more label was incorporated into glycerol by the ND pupae than D pupae, which shunted more label into alanine under atmospheric PO_2 (Fig. 6B).

During anoxia both the D and ND pupae incorporated the labeled glucose within 15 h primarily into

alanine (Fig. 6A). More glycerol was labeled under anoxia in the ND pupae than in the D pupae (Fig. 6 A, B). Upon removal from anoxia, after 24 h, glycerol degradation and synthesis of lactate was evident in both the D and ND pupae (data not shown). Label was not incorporated into anaerobic metabolites or storage compounds under an oxygen-saturated atmosphere (data not shown). The absence of labeled intermediates or endproducts indicates that the glucose was metabolised for energy when the pupae exposed to oxygen atmosphere were depleted of storage compounds.

Discussion

This study demonstrates that development in non-diapausing flesh fly pupae is arrested by anoxia during a critical developmental phase, shortly after pupariation. Although these developmentally-arrested, viable ND pupae are morphologially indistinguishable from D pupae, they fail to resume development in response to 20-hydroxyecdysone and hexane, treatments that readily initiate development in D pupae (Zdarek and Denlinger 1975). This suggests that the arrested state observed in ND pupae fundamentally differs from diapause.

Though development was arrested by anoxia in non-diapausing pupae, diapausing pupae initiated development in response to oxygen deprivation. The time at which anoxia was administered was critical. This suggests that 4–6 days post-pupariation the pupae are receptive (sensitive) to developmental changes. Previous studies with *S. crassipalpis* have demonstrated that diapause-programmed pupae at this stage are also highly sensitive to other stimuli, such as high temperature and ecdysteroids, which avert diapause (Denlinger et al. 1988).

Oxygen-saturated atmospheres had no apparent effect on the developmental program in either the D or ND pupae. This result differs from the previous findings by Sonobe et al. (1979), where increased O_2 supply to the eggs of *Bombyx mori* prevented diapause. Moreover, metabolites detected in the eggs resembled the ND eggs, suggesting a switch in metabolic pattern induced by increased O_2 concentration. The metabolites of *S. crassipalpis* after the oxygen treatment were different from those found in D or ND pupae before and after anoxia. The O_2-saturated atmosphere influenced the energy storage compounds; trehalose and glycogen were present at very low concentrations following the O_2 exposure. Injected glucose was used for energy and dissipated as CO_2 in both D and ND pupae. Contrary to these results, pupae under anoxia synthesised trehalose, glycogen, glycerol, and alanine from the injected glucose. The depletion of storage carbohydrates during anaerobic metabolism is well documented (Storey and Storey 1989). However, when provided with a labeled precursor (i.e., a carbon source), carbohydrates and anaerobic by-products were synthesized by the pupae.

Metabolism was similar in the diapausing and non-diapausing pupae. The steady-state concentrations of compounds present in live pupae and their hemolymph were virtually identical provided that the animals were raised under the same environmental conditions. Similarly, the metabolism of labeled glucose injected into pupae produced the same intermediates and endproducts in D and ND pupae; initially trehalose was labeled and the final fate of the glucose label was glycogen. Despite the lack of qualitative differences between metabolites of D and ND pupae, the rates of turnover were much lower in the D pupae. This is consistent with the depressed oxidative metabolism and low rate of protein synthesis observed in D pupae (Joplin and Denlinger 1989).

Suppressed oxidative metabolism resulted in the production of anaerobic metabolites, glycerol, and alanine, in both the D and ND pupae. Apparently, these compounds can accumulate during the diapause state of internally-curtailed oxidative phosphorylation or during anoxia when oxygen availability is externally limited. The consequent channeling of carbon through pathways of substrate phosphorylation occurs in both the D and ND pupae. Metabolites produced anaerobically have been confirmed to function as antifreezes/cryoprotectants. For instance, the increased levels of glycerol in *S. crassipalpis* correlate with enhanced cold tolerance (Chen et al. 1987). Other anaerobic by-products detected in this study probably also contribute to the cold-hardening process in the flies. Trehalose (Crowe and Crowe 1982) has been shown to protect membrane proteins from dehydration induced by low temperatures. Alanine was also found at increased concentrations in the flesh fly pupae and may serve as an energy source during recovery from anoxia (cf. Hochachka and Somero 1985), in addition to having a cryoprotective function (Loomis et al. 1989).

Even though exposure of D and ND pupae to anoxia led to accumulation of metabolites presumed to confer cold hardiness, the ability of pupae to survive cold shock remained unchanged. Previous findings of high levels of glycerol in the last larval instar indicate a similar lack of correlation with a change in cold tolerance (Lee et al. 1987b). Undoubtedly, other metabolites and /or protein changes are also contributing to cold tolerance in flesh flies. For instance, unique proteins, possibly related to heat shock proteins (Joplin and Denlinger 1990), appear to be synthesized in response to anoxia. Further investigation is needed on the protective mechanisms of these compounds and their advantages to insects not subjected to selective pressures enhancing cold tolerance.

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