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Energetics of metamorphosis in *Drosophila melanogaster*

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ABSTRACT

We measured the energetic cost of metamorphosis in the fruitfly, *Drosophila melanogaster*. Metabolic rates decreased rapidly in the first 24 h and remained low until shortly before eclosion, when the rates increased rapidly, thus creating a U-shaped metabolic curve. The primary fuel used during metamorphosis was lipid, which accounted for >80% of total metabolism. The total energy consumed during metamorphosis was lowest at 25 °C, compared to 18 and 29 °C, due to differences in metabolic rates and the length of pupal development. Temperature differentially affected metabolic rates during different stages of metamorphosis. Prepupal and late pupal stages exhibited typical increases in metabolic rate at high temperatures, whereas metabolic rates were independent of temperature during the first 2/3 of pupal development.

We tested two hypotheses for the underlying cause of the U-shaped metabolic curve. The first hypothesis was that pupae become oxygen restricted as a result of remodeling of the larval tracheal system. We tested this hypothesis by exposing pupae to hypoxic and hyperoxic atmospheres, and by measuring lactic acid production during normoxic development. No evidence for oxygen limitation was observed. We also tested the hypothesis that the U-shaped metabolic curve follows changes in metabolically active tissue, such that the early decrease in metabolic rates reflects the histolysis of larval tissues, and the later increase in metabolic rates is associated with organogenesis and terminal differentiation of adult tissues. We assayed the activity of a mitochondrial indicator enzyme, citrate synthase, and correlated it with tissue-specific developmental events during metamorphosis. Citrate synthase activity exhibited a U-shaped curve, suggesting that the pattern of metabolic activity is related to changes in the amount of potentially active aerobic tissue.

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1. Introduction

Holometabolous insects undergo a remarkable transition from the larva (a relatively simple, legless, wingless feeding individual) to the active, flying, reproductive adult. During metamorphosis most of the larval tissues are histolyzed and recycled to support the re-architecture of the animal into the adult form. Because pupae do not feed, all of the energy required to complete metamorphosis must be acquired during the larval stages (Boggs and Freeman, 2005; Boggs, 2009). These energy stores are brought forward by the cells of the larval fat body, which are retained in the pupa and are present in the immature adult (Hoshizaki, 2005; Aguila et al., 2007). Larval energy stores can have important adult functions. For example, autogenous mosquitos use larval resources to produce an egg clutch before their first blood meal (Telang and Wells, 2004). In Ephemeroptera and other insects with non-feeding adults (*e.g.* silkworm moths, *Bombyx mori*), larval stores must support the entire life and reproductive output of the adult. Even in those species which feed regularly as adults, larval-derived resources can provide a significant contribution to reproduction (Fischer et al., 2004; Boggs and Freeman, 2005; Min et al., 2006). Thus, metamorphosis represents a critical period in which energy stores established from larval feeding are allocated between fueling pupal development and supporting the needs of the adult for reproduction and survival.

Metamorphosis would seem to be an energy-intensive process, and thus metabolic rates should increase to support the complete restructuring of the organism. Surprisingly, this is not the case. Several studies have demonstrated that metabolic rates actually decrease after pupariation and remain low until shortly before eclosion to the adult stage (*i.e.*, there is a U-shaped metabolic curve). Orders examined include Diptera (Bodine and Orr, 1925;

Abbreviations: APF, after puparium formation; CS, citrate synthase; NEA, newly-eclosed adult; WPP, white prepupa.

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Wolsky, 1938), Lepidoptera (Crescitelli, 1935; Odell, 1998; Schmolz et al., 1999; Garedew et al., 2004; Hetz, 2007), Coleoptera (Odell, 1998; Kaiser et al., 2010) and Hymenoptera (Schmolz et al., 2005). Depending on the amount of accumulated larval energy stores, it is possible that animals entering metamorphosis are on the "metabolic edge." If energy stores are limited, the U-shaped curve might be necessary to complete metamorphosis. Alternatively, metamorphosis might be energetically cheap because of the U-shaped curve.

The underlying cause of the U-shaped metabolic curve is unclear. Several researchers have proposed that changes in metabolic rate reflect changing oxygen demand during destruction of the larval tissues and the assembly of the adult organs (Wolsky, 1938; Sacktor, 1951; Odell, 1998), although a comparison of metabolic rate changes with developmental events that transform the larva to the adult has not been carried out. An alternative hypothesis to explain the U-shaped metabolic curve is that the pupa becomes oxygen limited as the larval tracheae are remodeled to form the pupal and subsequently the adult tracheal systems. If pupal development is primarily fueled by lipid catabolism (Odell, 1998; Nestel et al., 2003), which requires oxygen, then remodeling of the tracheae might limit overall aerobic metabolism, and anaerobic metabolism may be necessary to complete metamorphosis.

In this study, we used the model insect, *Drosophila melanogaster* Meigen, to address several issues regarding the energetics of metamorphosis. Because metamorphosis relies solely upon energetic reserves accumulated during larval development, it is potentially an energetically vulnerable developmental period in insects and could have a direct impact on the success of the adult. We confirmed that *D. melanogaster* pupae exhibit a U-shaped metabolic curve during metamorphosis, and established an energetic budget for metamorphosis at different temperatures. We also tested predictions of the oxygen limitation and oxygen demand hypotheses to explain the U-shaped curve. We investigated the effects of varying oxygen levels on metabolic rate, as indicated by CO_2 production, and whether pupae produce lactic acid as an anaerobic end product. To provide an indication of metabolic demand, we measured pupal citrate synthase activity.

2. Material and methods

2.1. Drosophila rearing and pupal staging

D. melanogaster strain Oregon-R was obtained from A.J. Andres (University of Nevada, Las Vegas) and maintained at 25 °C in 500 ml bottles containing ~50 ml corn meal–sucrose–yeast medium (375 g cornmeal, 600 g sugar, 135 g agar, 250 g yeast in 8.8 l H_20). At the end of larval development, when the third-instar larvae began wandering in search of a pupation site, bottles were monitored for the presence of newly settled immobile larvae, *i.e.*, white prepupae (WPP). Animals were collected using a fine brush and transferred to 50 mm Petri dishes containing moist filter paper. The WPP were then transferred to incubators held at 18, 25 or 29 °C. To maintain a high humidity, Petri dishes were placed between stacking trays lined with damp paper. Thus, all animals were reared under the same conditions as eggs and larvae, and only the pupal rearing temperature differed.

We used the pupal staging system of Bainbridge and Bownes (1981) to stage animals during metamorphosis. This system divides post-larval development in *D. melanogaster* into 15 morphologically distinct stages, P1 through P15, based on traits visible by light microcopy in whole animals. Briefly, P1 is characterized by the white prepupa, and P15 ends with the eclosion of the adult. The developmental period spanning P1 to P15 is defined as metamorphosis and is often referred to as being equivalent to the pupal developmental period. More precisely, however, the developmental period from P1 to P4 makes up the prepupal stage and P5 to P15 represents the pupal stage. The transition from prepupa to pupa is marked by head eversion and the transformation to an animal with three distinct regions: head, thorax, and abdomen.

2.2. Metabolic rates

Pupal metabolic rates were measured at the respective pupal rearing temperature (18, 25 or 29 °C) using flow-through respirometry. Groups of 5–10 pupae or newly-eclosed flies (<1 h post-eclosion) were transferred to a 1 ml glass–aluminum respirometry chamber. In some cases, the chamber consisted of a 2 cm length of Tygon™ tubing. No differences were detected between measurements in the different chambers. Columns of silica gel and Ascarite™ were used to remove water vapor and carbon dioxide, respectively, from the air stream. Dry, CO_2-free air was pumped through the chambers at 50 ml min^{-1} to a Li-Cor Li-6262 infrared carbon dioxide sensor (Li-Cor, Lincoln, Nebraska, USA).

Data collection began within 10 min after pupae were placed in the chambers, and different groups of animals were used for each measurement. In preliminary experiments, we measured CO_2 release from individual animals and found that CO_2 was released in a regular manner for >45 min at all pupal stages, with no evidence for discontinuous gas exchange or other irregular breathing patterns (Chown et al., 2006; Quinlan and Gibbs, 2006). Metabolic rates from groups of flies were calculated from the release of CO_2 into the air stream over a period of 10 min. Data acquisition and analysis were performed using Datacan V software (Sable Systems, Las Vegas, Nevada, USA).

In addition to normoxic measurements, metabolic rates were measured at 25 °C under hypoxic (5% O_2) and hyperoxic (40% O_2) conditions. A gas mixer (Sable Systems) was used to generate hypoxic conditions by mixing air and N_2, and hyperoxia was achieved by mixing air and O_2.

2.3. Lipid content

Total lipid was determined gravimetrically. We froze individual WPP or newly-eclosed adult (NEA) flies at −70 °C and dried them overnight at 50 °C. Dried animals were weighed on a Cahn C-30 microbalance to a precision of 1 μg and extracted in 1 ml of ether overnight. The next day, ether-extracted flies were dried for one hour at 50 °C and re-weighed. Lipid mass was calculated as the difference between total dry mass and ether-extracted dry mass (Marron et al., 2003).

2.4. Carbohydrate and protein content

White prepupae and NEA flies were frozen at −70 °C. Sets of two animals each were homogenized in 120 μl lysis solution (1% NP-40, 0.5% deoxycholic acid, 0.1% Triton X-100, 100 mM NaCl, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.6). Homogenates were incubated at 70 °C for 5 min to denature hydrolases and spun in a microcentrifuge at 14,000 rpm for 2 min. The supernatant was removed and frozen at −70 °C until biochemical assays were performed. Supernatants were diluted as necessary to be in the linear range of the standard curves.

We measured carbohydrate levels by digestion of homogenates with *Rhizopus* amyloglucosidase (Cat. No. A7255, Sigma Chemical Co., St. Louis, Missouri, USA) at 25 °C overnight to release glucose subunits from glycogen and trehalose (Parrou and Francois, 1997). Glucose levels were then determined using a serum glucose kit (Cat. No. G7517, Pointe Scientific, Michigan, USA). Mussel glycogen (Cat. No. G1767, Sigma Chemical Co., St. Louis, Missouri, USA) was used as a standard. We used the bicinchoninic acid (BCA)

method to quantify protein levels (Smith et al., 1985). Homogenates were incubated with BCA reagent overnight at 25 °C, and absorbance was measured at 562 nm. Bovine serum albumin was used as a standard.

2.5. Lactic acid

To investigate the potential for anaerobic metabolism in *D. melanogaster* pupae, we exposed WPP and aged pupae to 0, 2 or 8 h of anoxia (100% N_2). Animals were placed in a 2 ml syringe, which was flushed with N_2 for 5 min and sealed. After the anoxic period, animals were immediately frozen in liquid N_2 and stored at −70 °C. We then quantified lactic acid accumulation using a blood lactate kit (Cat. No. L7596, Pointe Scientific, Michigan, USA). Individual pupae were homogenized in 100 μl cold 7% perchloric acid and centrifuged at 10,000 rpm at 4 °C for 10 min. The supernatants were neutralized by slow addition of 15 μl of 5 mM K_2CO_3 and mixed by flicking the tube. Solutions were cleared by centrifugation at 10,000 rpm in a microcentrifuge at 4 °C for 10 min. One hundred microliter of supernatant were incubated with 150 μl lactate oxidase (1000 U/l) for 5 min at 37 °C. One hundred microliter peroxidase (10,000 U/l) was added, and the supernatant was incubated for 10 min at 37 °C. Absorbance was measured at 550 nm. Lactate solutions included with the kit were used as standards.

2.6. Citrate synthase

As an indicator of aerobic capacity, we assayed activity of citrate synthase (E.C. 2.3.3.1), the entry enzyme for the tricarboxylic acid cycle. Sets of 5 pupae were homogenized in phosphate-buffered saline and centrifuged at 1000 rpm in a microcentrifuge for 5 min. An aliquot of homogenate was added to a reaction mixture containing: 100 mM Tris–HCl, pH 7.5; 0.2 mM ethylenediamine tetraacetic acid (EDTA); 0.1 mM dithio-*bis*-nitrobenzoic acid (DTNB); 0.2 mM acetyl-coenzyme A; and 0.5 mM oxaloacetic acid in a 25 °C thermostatted spectrophotometer cell. Activity was determined by measuring the change in absorbance at 412 nm.

2.7. Data analysis and statistics

We used Statistica version 7 to perform analyses of variance and post hoc tests. In general, data are reported as means (±standard error, SEM).

3. Results

3.1. Effects of temperature on metabolism and post-larval development of D. melanogaster

At 25 °C, metabolic rates of *D. melanogaster* during metamorphosis, as indicated by CO_2 release, decreased 67% in the first 24 h after puparium formation (APF; Fig. 1). They remained low for the next 48 h, then increased as pupae neared eclosion at ~96 h APF. Thus, we confirmed that the metabolic rate during metamorphosis in *D. melanogaster* exhibits a U-shaped curve (Wolsky, 1938). One potential explanation for the U-shaped curve is that energy stores are limited; therefore metabolic rates must decrease or the pupa would simply run out of fuel.

To test this simple idea, we attempted to change the energetic cost of metamorphosis by changing the temperature. Larvae were grown at 25 °C, collected as white prepupae (WPP, *i.e.*, 0 h APF), and shifted to either 18 or 29 °C or retained at 25 °C. Thus, each animal underwent larval development under the same conditions and began metamorphosis with approximately the same energy content. Metabolic rates at 18 or 29 °C were measured and also followed a U-shaped pattern (Fig. 1). Surprisingly, even though metabolic rates were inversely correlated with length of pupal development, total CO_2 output was higher at 18 and 29 °C (222 μl CO_2 and 129 μl CO_2, respectively) than at 25 °C (96 μl CO_2), as calculated from the area under the curve for each temperature. Thus, pupae have additional energy reserves that can be allocated towards metamorphosis under varying environmental conditions. We note that there was a trend towards lower viability at the extreme temperatures (18 or 29 °C), but over 80% of larvae eclosed to adulthood (data not shown). Thus, it is unlikely that differential mortality can explain the differences in metabolic rate (*i.e.*, we were not assaying injured or dead animals).

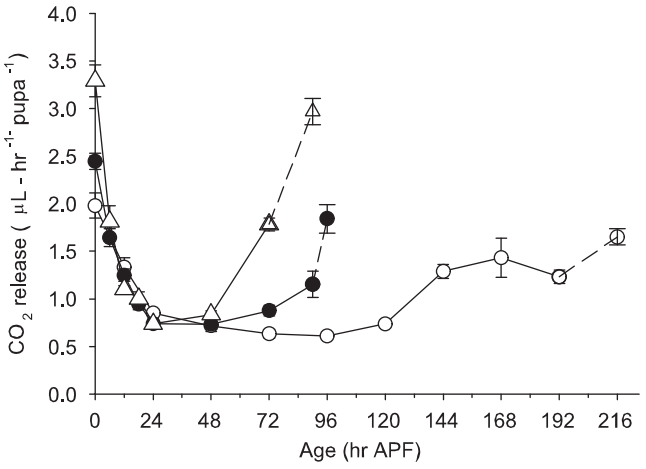

Fig. 1. Metabolic rates of groups of *D. melanogaster* pupae at 18–29 °C. Filled circles, 18 °C; open circles, 25 °C; filled triangles, 29 °C. Points after dashed lines are for newly-eclosed adults. Data are means (±SEM; n = 4–18).

We examined the effects of temperature on metabolic rates at four pupal stages: P1, P5, P9–P10, and P14–P15). Only at the beginning (P1) and the end of metamorphosis (P14–P15) did metabolic rates increase with temperature (ANOVA; $F_{2,15} = 29.4$, $P < 0.0001$ for WPP; $F_{2,26} = 108$, $P < 10^{-6}$ for P14–P15 pupae). The Q_{10} values for WPP and for P14–P15 pupae (72, 90 and 192 h APF at 18, 25 and 29 °C, respectively) were ~1.7. In contrast, at 24 h, corresponding to stage P5 for most pupae at all temperatures, metabolic rates were unaffected by temperature (ANOVA; $F_{2,39} = 3.04$, $P = 0.06$). Inspection of the data revealed that mean CO_2 production was actually highest at 18 °C, perhaps because the metabolic decline was slower at the lower temperature. Almost all pupae reached stages P9 or P10 after 48 h APF at 29 °C, 72 h at 25 °C, and 120 h at 18 °C. Metabolic rates were unaffected by temperature in these pupae ($F_{2,25} = 2.95$, $P = 0.095$). Thus, higher temperatures increased metabolic rate in prepupal and late pupal stages, but had no effect during intermediate developmental stages.

3.2. Progression through metamorphosis is not differentially affected by temperature

Not surprisingly, the overall length of metamorphosis was negatively correlated with temperature (Fig. 1). It is not clear, however, whether only certain developmental stages were affected by temperature or if all 15 stages of metamorphosis were uniformly affected. These two possibilities are outlined in Fig. 2, using a simple, two-stage developmental program. In the upper panel of Fig. 2, the length of only one stage (P2) is sensitive to the temperature change, so that differences in overall development time can be ascribed to just that stage. In the lower panel, the lengths of both stages are affected proportionally by temperature. The difference in the cost of metamorphosis at different temperatures will

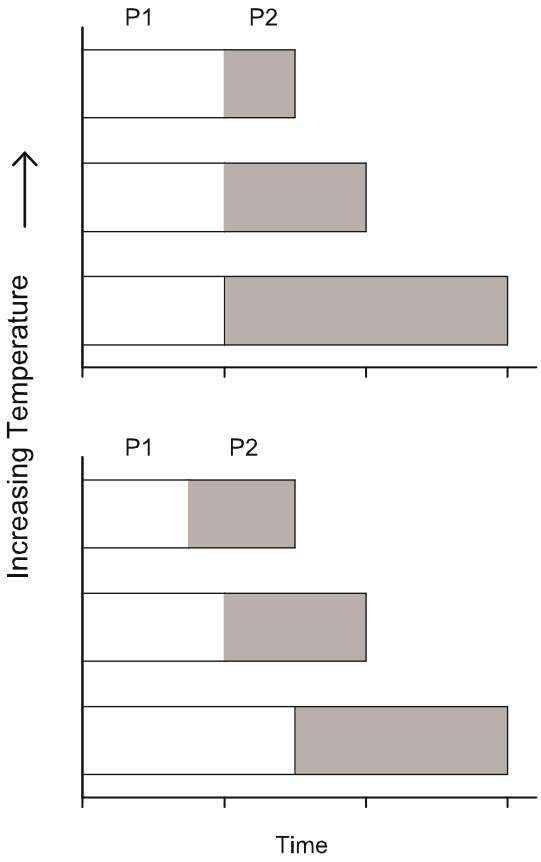

Fig. 2. Hypothetical effects of temperature on pupal development, using a simplified two-stage model. Within each panel, higher developmental temperatures are on top. Upper panel: the length of the first pupal stage is unaffected by temperature, and changes in total developmental time result from differential effects of temperature on the second stage only. Lower panel: both pupal stages are proportionately affected by temperature, so that the absolute lengths of both stages change, but the relative lengths do not.

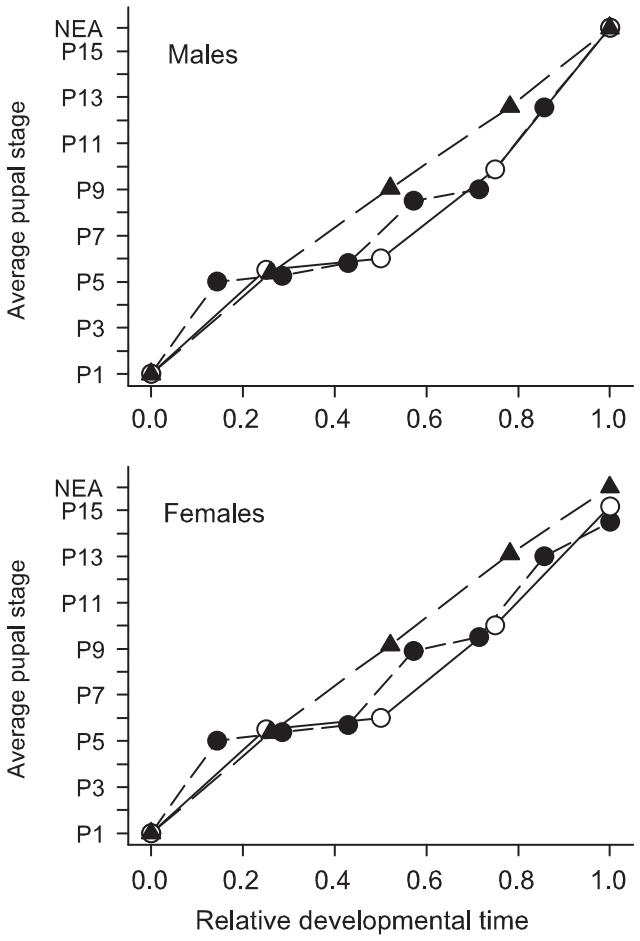

Fig. 3. Effects of temperature on relative developmental rates. Sex was determined in third-instar larvae. The *X*-axis shows relative development, scaled to 216 h at 18 °C, 96 h at 25 °C, and 90 h at 29 °C. The *Y*-axis shows mean developmental stage at 24-h intervals during metamorphosis. $n = 10$ pupae staged per time point. Symbols as in Fig. 1.

depend on whether the stages most affected by temperature are associated with higher rates of metabolism, such as at the beginning or end of metamorphosis.

To determine which of the models depicted in Fig. 2 better fit the pupal response to a temperature shift, white prepupae (P1) were shifted to 18 or 29 °C or retained at 25 °C. Individuals were examined at 24 h intervals APF and staged according to the criteria of Bainbridge and Bownes (1981). The lengths of pupal development including eclosion, at 18, 25 and 29 °C were then scaled to 100% of developmental time (216, 96 and 90 h, respectively). Fig. 3 depicts mean pupal stage as a function of percent development time. Because the stages differ in length, formal statistical analysis is not appropriate, but we observed that both prepupal (P1–P4) and pupal (P5–P15) development progressed at the same relative rate across temperatures. These data are consistent with the proportional effect model in the lower panel of Fig. 2. In Fig. 4, metabolic rates are plotted as a function of relative pupal development and scaled to the initial metabolic rates of the WPP. The relative timing of metabolic changes was similar across temperatures, suggesting that metabolic rate depends on the developmental stage rather than how long pupae have undergone metamorphosis.

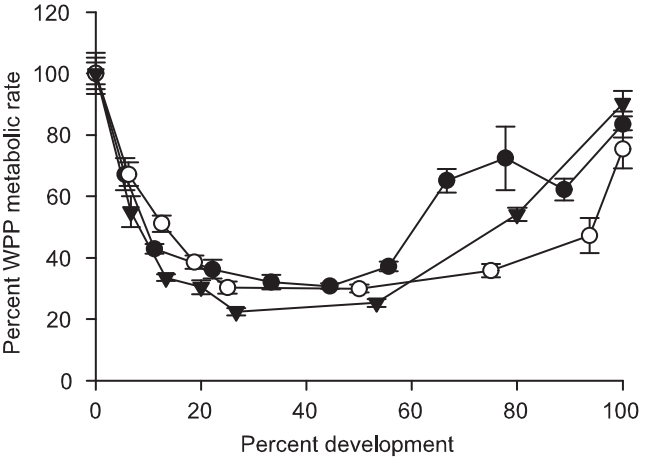

Fig. 4. Metabolic and developmental profile of *D. melanogaster* pupae incubated at 18–29 °C. On the *X*-axis, development time is expressed relative to the normal length of pupation. Symbols as in Fig. 1.

3.3. Energetic substrates consumed during metamorphosis

Our measurements of CO_2 release suggest that metamorphosis is energetically cheaper at 25 °C than at 18 or 29 °C (see Section 3.2). As an independent estimate of the cost of development, we measured the energy content of WPP reared at 25 °C and newly-eclosed adults (NEA) that had completed metamorphosis at 18, 25 or 29 °C. Because all animals were reared at 25 °C until the WPP

stage, they entered metamorphosis with similar energy stores. Thus, differences in energy content of NEA flies should reflect differences in energy consumed by the pupae.

Fig. 5 shows the amounts of three potential energy sources (lipids, carbohydrates and proteins) in WPP and in NEA flies reared throughout metamorphosis at the three temperatures. As expected, white prepupae contained significantly higher levels of lipids, carbohydrates, and protein compared to NEA flies raised at 18–29 °C (ANOVA for lipids, $F_{3,59} = 40.6$, $P < 10^{-6}$; for carbohydrates, $F_{3,28} = 3.10$, $P = 0.043$; for protein, $F_{3,28} = 5.20$, $P < 0.006$). Animals which developed at 25 °C retained significantly more lipid (100 μg) as newly-eclosed adults than those which underwent metamorphosis at 18 °C (75 μg) or 29 °C (79 μg) (Tukey post-hoc test, $P < 0.02$ for both comparisons). Post-hoc tests revealed no significant differences in protein or carbohydrate levels among NEA flies reared at different temperatures, indicating no differences in consumption of these compounds.

We used the data in Fig. 5 to calculate theoretical CO_2 production based on the disappearance of lipid, carbohydrate, and protein during metamorphosis. The data in Fig. 1 were used to calculate measured CO_2 production (obtained by integrating under the curve for each temperature; Table 1). At 25 °C and 29 °C, calculated and measured values of CO_2 production were in excellent agreement, but measured CO_2 release was substantially higher at 18 °C (Table 1). Using data in Fig. 5, we also calculated total heat produced during metamorphosis (Table 1; for protein, we assumed uric acid production; Schmidt-Nielsen, 1994). Despite the opposing responses of developmental time and metabolic rate, the energetic cost of metamorphosis at both the lowered and elevated temperature was greater than at the normal laboratory rearing temperature of 25 °C.

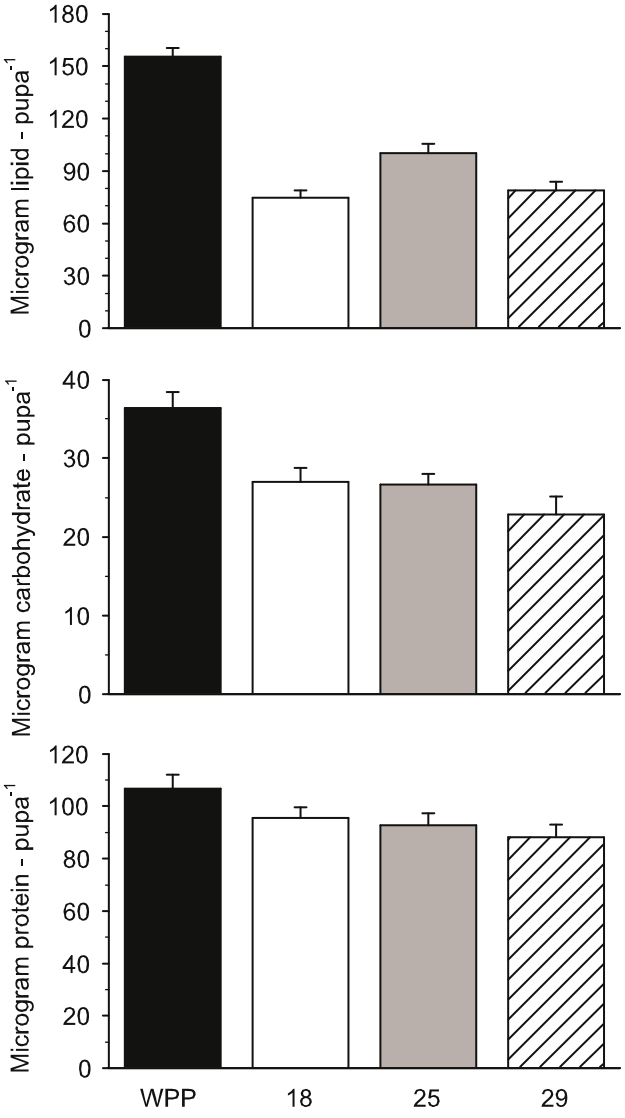

Fig. 5. Energy content of *D. melanogaster* white prepupae and newly eclosed adults. Animals were reared as larvae at 25 °C and either maintained at 25 °C or shifted to 18 or 29 °C. Black bars, WPP; open bars, NEA reared at 18 °C; gray bars, NEA reared at 25 °C; hatched bars, NEA reared at 29 °C. Data are means (±SEM); $n = 10$–18 individual animals for lipid assays; $n = 8$ pairs of animals for glycogen; $n = 8$ pairs of animals for protein assays.

3.4. Pupal metabolism is not oxygen limited

The experiments described above indicate that temperature does not affect the overall shape of the metabolic curve during metamorphosis. However, the underlying cause of the U-shaped curve is not clear. One possibility is that pupae become oxygen limited. During pupal development, the tracheal system is remodeled to replace the larval tracheae with the adult system. Lack of adequate oxygen uptake could cause a drop in apparent metabolic rate, as indicated by CO_2 production. If this is correct, pupae exposed to higher oxygen levels would at least partially overcome this limitation and have higher metabolic rates. Conversely, exposure to hypoxic conditions should depress metabolic rates even further. To test whether the drop in CO_2 release is due to oxygen limitation, we reared pupae under normoxic conditions at 25 °C. At 24 h intervals, we measured the metabolic rates of pupae placed under normoxic (21% oxygen), hypoxic (5% oxygen) or hyperoxic (40% oxygen) conditions (Fig. 6). An analysis of variance indicated a significant effect of age ($F_{3,60} = 230$; $P < 10^{-6}$) associated with the drop in metabolic rate during the first 24 h APF. Oxygen level significantly affected CO_2 production ($F_{2,60} = 5.29$; $P < 0.01$), and there was a significant age-by-oxygen level interaction ($F_{6,60} = 21.5$; $P < 10^{-6}$). Tukey post-hoc comparisons indicated that the significant effect of oxygen level was caused by low CO_2 production by WPP at 5% oxygen. White prepupae released significantly less CO_2 at 5% oxygen than at 21% or 40% O_2 (Tukey post-hoc tests; $P < 0.002$), whereas CO_2 release was unaffected by oxygen level at later stages (Tukey post-hoc tests; $P > 0.25$ for all comparisons). To summarize, these data suggest that low metabolic rates during metamorphosis are not due to oxygen limitation.

To further test whether oxygen is limiting during metamorphosis, we determined whether pupae produced ATP anaerobically by measuring the anaerobic end product, lactic acid. We measured

Table 1
Energy budgets for metamorphosis at 18–29 °C. Lipid, carbohydrate, and protein consumption were calculated as the difference between WPP and NEA flies (Fig. 6). Total pupal CO_2 production was measured using flow-through respirometry (Fig. 1), theoretical CO_2 release was calculated from energetic substrate consumption (Fig. 6), and heat production was calculated from consumption of energetic substrates.

Pupal temperature	18 °C	25 °C	29 °C
Measured CO_2 production (μl)[a]	222	97	129
Lipid consumed (μg)[b]	80	55	77
Carbohydrate consumed (μg)[b]	9.4	9.8	13.6
Protein consumed (μg)[b]	11	14	19
Calculated CO_2 production (μl)[c]	129	96	134
Calculated heat production during metamorphosis (J)[c]	3.5	2.6	3.6
% J from lipid	90	84	84

[a] Calculated from data in Fig. 1 by integrating under the metabolic curve.
[b] Calculated from data in Fig. 5.
[c] Calculated from data in Fig. 5 using standard conversion factors (Schmidt-Nielsen, 1994).

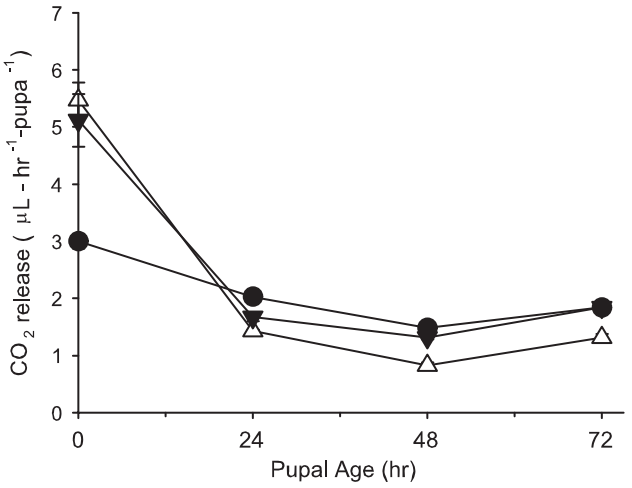

Fig. 6. Effects of environmental oxygen level on metabolic rates of *D. melanogaster* pupae at 25 °C. Filled circles, 5% O_2; open triangles, 21% O_2; filled triangles, 40% O_2. Data are means (±SEM); $n = 6$.

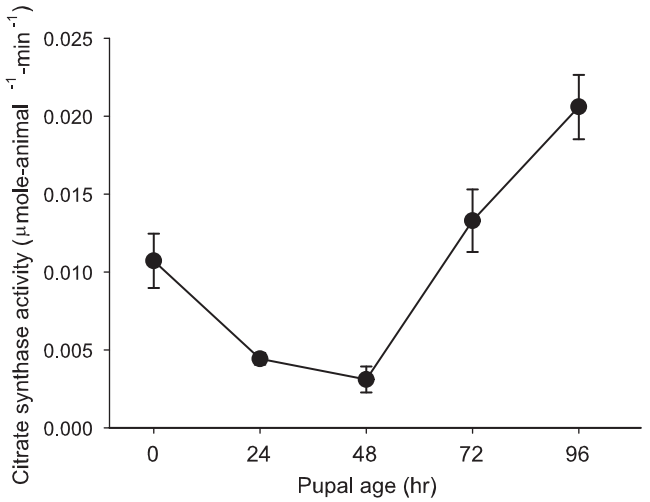

Fig. 8. Citrate synthase activity of *D. melanogaster* pupae. Data are means (±SEM); $n = 5$.

lactate levels in WPP and pupae reared under normoxic conditions at 24-h intervals APF (Fig. 7). Lactic acid was undetectable until 72 h APF, when modest amounts of lactate were measured. To establish whether pupae are capable of significant anaerobic metabolism, we exposed WPP and aged pupae to anoxia for 2 or 8 h at 24-h intervals APF and measured lactate production. Pupae were capable of anaerobic metabolism, as indicated by significant production of lactate when they were exposed to anoxia (Fig. 7). An ANOVA revealed highly significant effects of age, the length of the anoxic period, and their interaction (F-ratio = 49–273, $P < 10^{-6}$ for all effects). White prepupae produced more lactate than older pupae (Tukey post-hoc test; $P < 0.0002$ for all comparisons), consistent with their higher metabolic rates (Fig. 1).

Although pupae are capable of anaerobic metabolism (Fig. 7), it is not clear whether anaerobic ATP production would be sufficient to support normal pupal development. To address this issue, we exposed WPP and 24, 48 and 72 h-old pupae to an 8 h period of anoxia. After treatment, the pupae were allowed to complete development. Pupae were staged at 24-h intervals APF based on the criteria of Bainbridge and Bownes (1981). Animals were closely monitored immediately before eclosion to determine the time of eclosion. Progress through metamorphosis after anoxia treatment was normal, but eclosion was delayed by 10–13 h (data not shown). These data suggest that anaerobic metabolism cannot adequately support energetic needs for metamorphosis, and that anoxic stress inhibits pupal development. Overall, our data suggest that oxygen availability is not a significant factor in the depression of metabolic rates during metamorphosis under aerobic conditions.

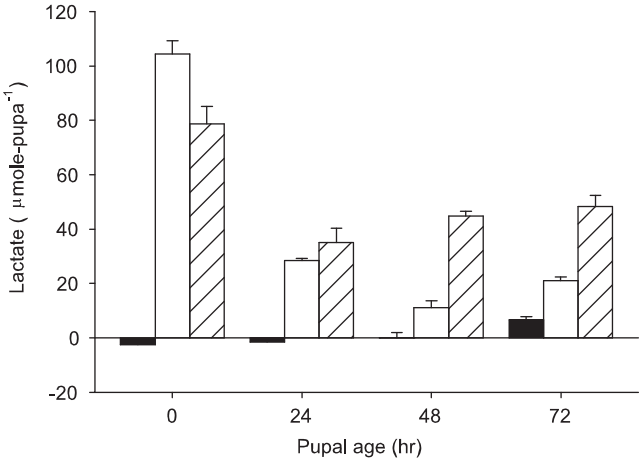

Fig. 7. Lactic acid production by *D. melanogaster* pupae after 0, 2 and 8 h of anoxia at 25 °C. Filled bars, 0 h; open bars 2 h; hatched bars, 8 h of anoxia. Data are means (±SEM); $n = 8$.

3.5. Citrate synthase activity

Several authors have suggested that the shape of the metabolic curve reflects the loss of larval tissues at the beginning of metamorphosis and the increase in the mass of adult tissue at the end of metamorphosis (Wolsky, 1938; Sacktor, 1951; Odell, 1998). An underlying assumption is that the increase in adult tissue corresponds to an increase in metabolically active tissue. We used citrate synthase (CS), an indicator of mitochondrial density, to monitor aerobic capacity. We measured CS activity in pupae at 24-h intervals APF in animals reared at 25 °C. CS activity followed a U-shaped curve; it dropped rapidly in the first 24 h APF, remained low through 48 h APF, then increased (ANOVA; $F_{4,23} = 19.1$, $P < 10^{-5}$; Fig. 8). The rise in CS activity was faster than the rise in metabolic rate at 72 h APF. Pupae at 72 h APF had the same CS activity as WPP (Tukey post-hoc test; $P = 0.75$), despite their depressed metabolic rates relative to the WPP (Fig. 1), and NEA flies had significantly higher CS activities than WPP and pupae (Tukey tests, $P < 0.04$ for all pair-wise comparisons).

4. Discussion

4.1. Cost of metamorphosis – energetics of the U-shaped curve

Metamorphosis in holometabolous insects is characterized by a U-shaped metabolic curve (Bodine and Orr, 1925; Crescitelli, 1935; Wolsky, 1938; Odell, 1998; Schmolz et al., 1999, 2005; Garedew et al., 2004; Hetz, 2007; Kaiser et al., 2010). In this study, we investigated the energetic budget of metamorphosis and examined the underlying developmental and biochemical changes that might contribute to the shape of the metabolic curve. The shape of the curve suggests that metamorphosis might be an energetically inexpensive process overall, but this is not the case. Pupae consumed 35% and 27% of their initial lipid and carbohydrate reserves, respectively, and produced 97 µl of CO_2 to support metamorphosis at 25 °C (Fig. 1, Table 1). Had pupae at 25 °C maintained the initial high metabolic rates of WPP, they would have produced ~230 µl of

CO_2 during metamorphosis (2.4 μl hr^{-1} for 96 h). Because the initial lipid and carbohydrate contents at the beginning of metamorphosis are only sufficient to produce 240 μl of CO_2 (calculations based on data in Fig. 5), nearly all the lipid and carbohydrate stores would have to be consumed to support this level of metabolism. Compounding the energetic cost is the energetic need of the immature adult. Newly-eclosed flies do not begin to feed until ~8 h post eclosion (Chiang, 1963). If high metabolic rates were maintained throughout pupal development, any remaining triglyceride and carbohydrate reserves would be depleted in order to support the young adult. Thus, the successful transition from the larva to the adult might require a reduction in metabolic demand based on the amount of lipid and carbohydrate stored during the larval stage. An alternative hypothesis is that pupae could develop faster if their metabolic rates remained high, which would increase fitness by shortening development to adulthood, but are constrained by the need to coordinate developmental changes in different tissues.

One way to change the energetic cost of metamorphosis is to change temperature, which then should change metabolic rate. Countering this idea is the observation that temperature is positively correlated with metabolic rate and negatively correlated with the length of development, and these effects might cancel each other (Odell, 1998). We addressed this issue in *D. melanogaster* by shifting the pupal rearing temperature at the beginning of metamorphosis to either 18 or 29 °C. The U-shaped metabolic curve was retained at the two extreme temperatures, but the overall cost of metamorphosis was higher at both 18 and 29 °C compared to 25 °C, according to two independent measures. Thus, *D. melanogaster* pupae have energetic reserves to respond to increases in metabolic demands. This is in accordance with calculations by Odell (1998) indicating that *Manduca* and *Tenebrio* pupae enter metamorphosis with greater energy reserves than required to reach adulthood.

The length of metamorphosis was inversely correlated with temperature, and metabolic rates exhibited a positive correlation with temperature, but only at the beginning and end of metamorphosis. A surprising finding was that metabolic rates during the trough of the U-shaped curve were not affected by temperature, although increases or decreases in temperature appeared to shorten or lengthen, respectively, the development of all pupal stages (P1–P15) in a proportional manner (Fig. 3). Overall, our data suggest that metabolic depression is necessary for successful development at 29 and 18 °C. If metabolic rates were sustained at the WPP levels measured at 18 and 19 °C, then 288 μl of CO_2 would be produced at 29 °C and 432 μl at 18 °C (based on 3.2 μl CO_2 hr^{-1} and 2.0 μl CO_2 hr^{-1} at 29 °C and 18 °C, respectively). Under these conditions, metamorphosis at 29 °C would consume all lipid and carbohydrate stores and severely deplete protein levels. Metamorphosis at 18 °C would not even be energetically possible. As noted above, however, maintenance of a high metabolic rate could accelerate development, so that metamorphosis could be completed more rapidly. We discuss below two mechanistic hypotheses for the existence of the U-shaped curve.

Our measurements of total CO_2 production during metamorphosis and calculated CO_2 production based on macronutrient measurements are in excellent agreement at 25 and 29 °C (Table 1). At 18 °C, measured CO_2 production exceeded theoretical by ~70%. Part of this discrepancy might reflect the relatively high metabolic rates observed in pupae 6 and 7 days APF (Fig. 1). Respirometry and energetic measurements were conducted with multiple cohorts, and minor differences in larval or pupal rearing (larval density, medium quality, incubator temperature, etc.) might have affected our results. It is clear, however, that the energetic cost of metamorphosis is lower at 25 °C than at 18 or 29 °C. This may have important life history implications, as flies reared at 25 °C will have more larval-derived energy available for adult activities such as reproduction. Crescitelli (1935) likewise found that the metabolic cost of metamorphosis in the bee moth, *Galleria mellonella,* was lowest at intermediate temperatures.

These results are in contrast to the conclusions of Odell (1998), who reported that the energetic cost of metamorphosis was unaffected by temperature in *Manduca sexta* and *Tenebrio molitor*, because the effects of temperature on metabolic and developmental rates compensate for each other. Several factors might contribute to the discrepancies between these findings and our results. First, in *D. melanogaster* the metabolic rates characteristic of the trough of the U-shaped curve were unaffected by temperature (Fig. 1), whereas the species studied by Odell (1998) exhibited higher metabolic rates during all pupal stages as temperature increased. Second, whereas Odell (1998) reared animals at different temperatures as larvae and as pupae, we chose to make the larval environment constant. Body size is generally inversely related to rearing temperature in holometabolous insects (Atkinson, 1994), so it is likely that animals in the Odell's study differed in mass. Higher mass-specific metabolic rates at high temperatures could have been counteracted by smaller size of these animals. Animals in the current study were reared under the same conditions, so they would have pupariated at approximately the same size. Our experiments were designed to investigate the effects of manipulating one specific developmental stage, metamorphosis. Future work will address larval and pupal environments and the interaction between them.

4.2. Causes of the U-shaped metabolic curve – tracheal development and gas exchange

The U-shaped curve has been described in four orders of holometabolous insects (Bodine and Orr, 1925; Crescitelli, 1935; Wolsky, 1938; Odell, 1998; Schmolz et al., 1999, 2005; Garedew et al., 2004; Hetz, 2007; Kaiser et al., 2010). However, the physiological basis for it is unclear: either energetic demand changes dramatically during metamorphosis, or gas exchange capacity, especially oxygen delivery, is diminished so as to reduce metabolism. The latter hypothesis is attractive because of the tremendous structural changes that occur in the tracheal system. The anterior and posterior regions of the dorsal trunks collapse at the larval-pupal molt and new tracheae form in these areas during pupation, but filling of the tracheae with does not occur until just before eclosion (Manning and Krasnow, 1993). Thus, entrance into the true pupal stage coincides with the loss of approximately half of the major tracheae. Tracheal remodeling might also explain the observation that metabolic rates are unaffected by temperature in the trough of the U-shaped curve, as diffusion-based processes are much less affected by temperature than metabolism, with Q_{10} values of ~1.03 (Willmer et al., 2000). The thermal independence of metabolic rate during mid-metamorphosis is therefore consistent with metabolism being limited by diffusion.

Although consideration of both tracheal development and low Q_{10} are consistent with gas-exchange limitation, they are only suggestive. More direct evidence can be provided by manipulating ambient O_2 levels. When oxygen availability does not limit metabolism, metabolic rates are independent of atmospheric oxygen levels (Willmer et al., 2000). Only below a critical partial pressure of oxygen (P_{crit}) do metabolic rates begin to decline. P_{crit} values for insects are typically ~5 kPa (Harrison and Roberts, 2000). If O_2 availability limited metabolism, we would have expected metabolic rates to be particularly sensitive to atmospheric oxygen in the trough of the U-shaped curve. Wolsky (1938) found that pupal metabolism at all stages was lower in 10% oxygen than in normoxia, consistent with the oxygen limitation hypothesis. It should be noted, however, that carbon monoxide comprised the other 90%

of the gas mixture in that study. Inhibition of hemoglobin-O_2 binding (Hankeln et al., 2002) or cytochrome C oxidase activity (Alonso et al., 2003) by CO could have inhibited metabolism.

We observed a significant effect of oxygen levels only in WPP under hypoxic conditions. It is possible that P_{crit} is higher in WPP than other stages, because of their higher metabolic rates. However, equal metabolic rates in 21% and 40% O_2 suggest that P_{crit} is below 21%, so that metabolism in normoxia is not O_2-limited. Alternatively, we note that because metabolic rates changed rapidly during early metamorphosis (Fig. 1), the oxygen-dependent differences detected in white prepupae could be the result of slight changes in the time between animal collection and metabolic measurement.

Although the developmental changes in the tracheal system suggest that oxygen could be a limiting factor in the metabolism of the pupae, our results from manipulating oxygen levels suggest that this is not the case. To further examine the potential for O_2 limitation, we investigated whether pupae supported some of their metabolic demands by anaerobic respiration. To our knowledge anaerobic respiration has not been examined in *D. melanogaster* pupae, but adult *D. melanogaster* produce lactic acid, acetic acid and alanine as anaerobic end products (Feala et al., 2007). We found that pupae reared in normoxic conditions produced little lactic acid, although they are capable of doing so when exposed to anoxia (Fig. 7). We did not assess other anaerobic end products, but taken together our experiments strongly suggest that metabolism of *D. melanogaster* pupae is not limited by oxygen availability.

4.3. Cause of the U-shaped metabolic curve – metabolically active tissue

The rapid decline in metabolic rates at the beginning of metamorphosis occurs during the prepupal period (0–12 h APF at 25 °C) and the beginning of pupal development (P5, 12–24 h APF). This period is associated with the histolysis of larval tissues and begins at puparium formation (0 h APF) with the selective histolysis of the larval muscle (Thummel, 2001). Larval muscle histolysis occurs in three waves and is completed by 8 h APF, with the exception of the mesothoracic longitudinal oblique muscles and intersegmental muscles that are used for head eversion (Fernandes et al., 1991). Autophagy of the midgut begins at apolysis (4 h APF) and is followed by loss of the salivary glands at the beginning of pupal development (13 h APF; Robertson, 1936; Thummel, 2001). The larval fat body does not undergo histolysis, although the cells of the fat body dissociate into individual cells during the prepupal-pupal transition (12 h APF; Nelliot et al., 2006). The developmental timing of the histolysis of the remaining larval tissues has not been defined, but is likely to occur at the beginning of pupal development. Histolysis of larval tissues could therefore contribute to the ~50% drop in metabolic rate in the first 4 h of metamorphosis.

In *D. melanogaster*, the construction of adult appendages and tissues, which Odell (1998) proposed to be associated with the late increase in metabolic rate, are initiated early in metamorphosis. During the initial period of metamorphosis, the leg and wing imaginal discs, which have undergone rapid proliferation throughout larval development (Nöthiger, 1972), undergo morphogenesis and give rise to the legs and wings which lie within the animal beneath the hypodermis (Robertson, 1936). Shortly after apolysis (6 h APF), but before pupation at 12 h APF, the legs and wings become visible. By the end of prepupal development (12 h APF) the adult cuticle has replaced the hypodermis by the proliferation of the histoblast cells (Robertson, 1936). At the beginning of pupal development (14 h APF) the dorsoventral flight muscles arise *de novo* (Fernandes et al., 1991). By 20 h APF, the remaining 3 larval bilateral mesothoracic longitudinal oblique muscles split to form 6 bilateral templates that will give rise to the dorsal longitudinal flight muscles of the adult (Fernandes et al., 1991). Thus, the initial 24 h of metamorphosis are characterized by tissue histolysis, cell proliferation, differentiation, and morphogenesis.

The period from 24 to 72 h APF represents the trough of the metabolic curve at 25 °C, during which time organogenesis of the major tissues is nearly completed, and flight muscle striation (54 h APF), new abdominal muscle (60 h APF) and leg muscle (60 h APF) are detected (Fernandes et al., 1991). Adult structures are therefore in place during the metabolic trough, although further changes do take place. The final period from 72 h APF to eclosion is characterized by fully differentiated flight muscle, the final darkening of the bristles and cuticle, and near completion of the alimentary tract (Robertson, 1936). Because of the complex patterns of cell proliferation and organogenesis, it is difficult to reconcile a simple model of histolysis and "new adult tissue" (Odell, 1998) to explain the U-shaped curve in *D. melanogaster*.

We investigated potential metabolic demand by measuring citrate synthase activity as an indicator of mitochondrial quantity. Citrate synthase exhibited a U-shaped pattern similar but not identical to that of metabolic rate. Although WPP and NEA flies had similar metabolic rates, NEA flies had twice the amount of CS activity. Furthermore, citrate synthase activity of pupae at 72 h APF was equivalent to that of WPP, even though 72 h APF pupae had much lower metabolic rates. Thus, although CS activity also exhibited a U-shaped curve, the rapid increase in mitochondrial density towards the end of metamorphosis preceded the increase in metabolic rates. We attribute these discrepancies to terminal differentiation of mitochondria-rich flight muscle in the latter half of pupal development. The maximal metabolic rate of adult *D. melanogaster* is ~35 μl CO_2 h^{-1} (Lehmann et al., 2000), whereas actively moving larvae produce only 7–8 μl CO_2 h^{-1} (Kaun et al., 2007). Higher citrate synthase activities in adults are consistent with differences in potential metabolic demand related to flight. We note that immature *D. melanogaster* require ~8 h to become flight ready, thus the newly-eclosed adults we examined would not have exhibited metabolic rates as high as flying adults. Citrate synthase activity provides a good indicator of potential metabolic activity, but because the types of tissue present change dramatically during metamorphosis, citrate synthase activity is less useful as an indicator of actual metabolic rate.

5. Conclusions

Metabolic rates decline sharply when holometabolous insects enter metamorphosis. Our data suggest that the U-shaped metabolic curve is essential for successful metamorphosis and highlight an important energetic constraint of the life cycle of holometabolous insects. The energetic reserves necessary for metamorphosis are drawn from energy stores that are acquired during larval feeding and provide the energy to fuel metamorphosis. They must also support the newly-eclosed adult, because *D. melanogaster* do not feed for the first 8 h of adult life (Chiang, 1963), or even the entire adult lifespan in species with non-feeding adults. The energetic cost of metamorphosis was lowest at 25 °C, the normal rearing temperature of *D. melanogaster*, suggesting an energetic optimum for development. The U-shaped metabolic curve was not caused by limited gas exchange associated with tracheal remodeling. Instead, our data are consistent with a decline in metabolism partially caused by histolysis of larval tissues and an increase in mitochondrial density, as muscle and other adult tissues are formed.

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