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Biphasic metabolic rate trajectory of pupal diapause termination and post-diapause development in a tephritid fly

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ABSTRACT

Metabolic depression is a highly conserved feature of insect diapause, and an increase in metabolism is a reliable indicator of diapause termination and the initiation of post-diapause development. The trajectory of metabolic rate following diapause termination can guide the identification of important physiological and developmental landmarks during this developmental transition, yet quantitative descriptions of these trajectories are relatively rare. Here we track changes in metabolic rate from diapause through diapause termination and pharate adult development in *Rhagoletis pomonella* (Diptera: Tephritidae), a univoltine tephritid fly that diapauses in the pupal stage. Using respirometric monitoring we show that diapause termination and subsequent pharate adult development is characterized by a biphasic increase in metabolic rate. Respiration rate initially increases logistically, reaching a plateau that is followed by a final, exponential increase terminating in adult eclosion. We develop a non-linear model describing this pattern with easily interpretable landmarks, and we map visible landmarks of morphogenesis onto the trajectory. The bulk of visible morphogenesis in pharate adult development occurs relatively late in the trajectory during the final, exponential phase, that matches the U-shaped trajectory of non-diapause individuals. These results mirror a qualitative description of the same trajectories of diapause and non-diapause flesh flies (*Sarcophaga*). Overall, our results suggest that (1) the course of diapause breakage and post-diapause development may be evolutionarily conserved in higher flies with pupal diapause, and (2) the biphasic trajectory is distinct from a more continuous trajectory observed during direct development.

1. Introduction

Diapause, a seasonally programmed developmental arrest, is a life history stage that allows insects to mitigate stressful periods and synchronize their life cycles to exploit favorable periods. The role of diapause in life cycle synchronization is particularly apparent in insect herbivores, many of which are adapted to feed on a specific stage of a particular plant, such as newly flushed leaves or fruits (Feder and Filchak, 1999; Danks, 2007). Many specialized herbivores have a single generation each year and insects must terminate diapause at the appropriate time to exploit a narrow window of peak resource quality before the next generation enters an obligate diapause lasting until the following year. Therefore, characterizing the physiological mechanisms underlying diapause development and termination is a critical step towards understanding seasonal life history adaptation.

Many studies have explored the environmental cues that promote diapause termination (Tauber et al., 1986; Danks, 1987). However, less is known about the physiological mechanisms that regulate diapause termination. Diapause is not just a cessation of normal development. It is a dynamic alternate developmental pathway that is characterized by several physiologically distinct states including diapause induction, maintenance, and termination (Kostál, 2006). The transition from diapause maintenance to termination is typically associated with changes in the insect's endocrine milieu (Denlinger, 2002; Denlinger et al., 2005). However, the physiological and molecular mechanisms that translate ecologically relevant environmental cues into endocrine signaling cascades at diapause termination are poorly understood.

Although most insect species enter and remain in diapause in only one life stage, a diversity of strategies exist among species, with different species diapausing in every stage from embryonic blastoderm formation to adults that have already begun reproducing. While some components of the diapause program are conserved across species having different diapause strategies (e.g., increased stress tolerance), the mechanisms that terminate diapause may vary radically among strategies. Many economically

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important crop pests in the Lepidoptera and Diptera diapause as pupae. Thus, understanding the physiological mechanisms underlying pupal diapause termination, and therefore life history timing, is an issue of fundamental importance for pest management.

Metabolic depression is an almost universal characteristic of diapausing insects (Hahn and Denlinger, 2007). While the degree of metabolic depression during diapause may vary to some degree among species and diapausing life stages, and according to environmental circumstances, hundreds of studies have shown that diapausing individuals have lower metabolic rates than non-diapausing individuals of the same stage. Moreover, metabolic rates are typically lower during the diapause stage than in those preceding and following diapause. Metabolic rate is therefore a useful marker for identifying diapause termination, facilitating studies of the physiological and molecular events regulating diapause termination.

Although many studies have measured metabolic rates in diapausing insects, very few have quantified metabolism in unmanipulated individuals allowed to terminate diapause on their own. In most studies diapause is broken in groups of individuals in a coordinated manner, either by dramatically manipulating the environment or by application of pharmacological agents. One common treatment involves rapidly shifting diapausing individuals from cooler (20 °C) to warmer temperatures (25 °C) to immediately terminate diapause with little or no latency to respond (e.g., Fujiwara and Denlinger, 2007). Another common method involves treating individuals with pharmacological agents, such as hormones or their analogues to induce diapause termination (e.g., Bodnaryk, 1985). These intensive manipulations make it difficult to discern which aspects of the observed physiological and molecular responses are caused by diapause termination and which are caused by the manipulations themselves.

Several laboratories have quantified the pattern of increasing metabolism during diapause termination in unmanipulated moth larvae by continuously monitoring oxygen consumption (e.g., Wipking et al., 1995; Kostal et al., 1998; Singtripop et al., 2007). However, quantitative descriptions of the transition from pupal diapause to diapause termination and through pharate adult development in unmanipulated individuals are rare and limited to large moth species. Because metabolic rates in diapausing insects are low, many studies report values for groups of individuals measured simultaneously rather than single individuals. Furthermore, most studies do not achieve a temporal resolution sufficient for monitoring specific physiological and molecular events occurring during diapause termination. For example, Teixeira and Polavarapu (2005) quantified CO_2 production as a metric of metabolism in pupae of the blueberry maggot, *Rhagoletis mendax*, and showed that metabolism increased between pupal diapause and pharate adult development. However, these authors' estimates of metabolism were performed on groups of 10 pupae, limiting individual-level resolution. Denlinger et al. (1972) monitored the termination of pupal diapause in unmanipulated individuals of several species of flesh flies in the genus *Sarcophaga* and qualitatively (verbally) described the pattern of metabolic rate change. Their data suggest two distinct periods of rapid metabolic rate increase, the first associated with diapause break and the second associated with the rapid morphogenesis that occurs late during pharate adult development. Slama and Denlinger (1992) quantify the initial logistic phase of increasing oxygen consumption associated with diapause termination in the flesh fly *Sarcophaga crassipalpis*, but did not distinguish respiration at diapause termination from the remainder of adult development. Thus, quantitative characterization and confirmation of metabolic patterns from pupal diapause termination through adult development in flesh flies and other insects are generally lacking in the literature.

Here, we present results of several experiments tracking daily CO_2 production as a metric of metabolic rate in individual diapausing and non-diapausing pupae of the apple maggot fly *Rhagoletis pomonella* (Diptera: Tephritidae, Walsh). We develop a non-linear mathematical model that describes changes in metabolism observed during the transition from pupal diapause to diapause termination and through pharate adult morphogenesis until eclosion. We also map visible developmental landmarks of pupal diapause and adult morphogenesis onto the distinct phases of metabolism identified by the model. Finally, we compare the metabolic rate trajectories of flies undergoing diapause versus non-diapause developmental programs to test predictions that diapausing individuals should initially be metabolically depressed compared to non-diapause individuals, but should follow a metabolic trajectory similar to non-diapausing individuals after diapause termination. To test this hypothesis, we ask whether a simplified, nested version of the non-linear model describing metabolic rates for diapause termination and post-diapause development fits the data for non-diapause individuals developing directly from pupae to pharate adults.

2. Materials and methods

2.1. Study system

R. pomonella is a univoltine, frugivorous specialist. The fly historically infested hawthorn fruits (*Crataegus* sp.) native to the USA and Mexico. However, in the last 150 years, a novel, genetically distinct host race has evolved the ability to attack introduced, domestic apple (*Malus domesticus*). The hawthorn to apple host shift was accompanied by a corresponding shift in the timing of adult fly emergence to track the 2–4 weeks earlier fruiting time of apples compared to hawthorns. The seasonal timing of adult emergence in *R. pomonella* is regulated by the timing of pupal diapause (Feder and Filchak, 1999). Larvae exit host fruits in late summer, burrow into the soil, and pupate. Most pupae will enter and remain in diapause until the following summer when they will terminate diapause and emerge as adults to infest the next season's fruit crop. A few individuals in each host race, however, will avert diapause and undergo direct non-diapause development if exposed to extended periods of elevated temperature. The resulting "non-diapausing" adults will emerge when no fruit is available and die without reproducing (Boller and Prokopy, 1976). Therefore, diapause is functionally an obligate part of the cycle.

2.2. Animals and rearing

In the fall of 2007 we collected hawthorn fruits infested with *R. pomonella* pupae at a field site near Fennville, MI, USA. We brought the fruits back to the laboratory where they were kept in an environmentally controlled room at 24 °C and 14L:10D light cycle. Fruits were placed in wire-mesh baskets suspended over plastic collecting trays so that wandering larvae exited the fruits and pupated in the trays. Pupae were collected daily and placed in moist vermiculite at 21 °C for 10 days. After 10 days, pupa were removed from vermiculite and transferred to clean petri dishes that were placed at ~85% relative humidity and 4 °C to simulate overwintering, as done in previous studies (Feder et al., 1997). Pupae for the two diapause termination experiments performed in the study (experiments I and II) were drawn from this overwintering pool. Pupae collected in the fall of 2008 from hawthorn fruits at another site near Grant, Michigan (~80 km north of Fennville) were used in a third experiment comparing metabolic rates between diapause initiation and non-diapause flies progressing through pupal and pharate adult development. The indivi-

duals used for experiment III were reared under conditions identical to those described above until pupariation. However, instead of subjecting pupae to cold winter temperatures 10 days after pupariation, we kept individuals continuously at 24 °C and tracked their metabolic rate for 33 days.

2.3. Experimental setup

2.3.1. Experiment I

The goal of experiment I was to determine the general pattern of fly metabolism during diapause termination and development following winter. After 30 ± 1 weeks at 4 °C, we weighed 45 randomly selected pupae to the nearest 0.001 mg and transferred them to a 24 °C incubator at ~85% humidity. After 10 days at 24 °C we placed the pupae individually into 5 ml syringes, each fitted with a three-way luer valve and a 26-gauge needle to act as a constant-volume respirometry chamber. We purged the syringes with air that was first scrubbed of CO_2 using a drierite™–ascarite™–drierite™ column and then re-humidified by bubbling through acidified water (pH ~4.0). Syringes were sealed with the plunger drawn back to produce a chamber of 1 ml internal volume. We also purged three empty syringes to serve as controls. Every 24 h the full volume of each syringe was injected into a flow-through respirometry system consisting of a Li-Cor 7000 infrared CO_2 analyzer (Lincoln, NE, USA) with a resolution of 0.1 parts per million (ppm) CO_2 interfaced to Sable Systems International Expedata data logging software (Las Vegas, NV, USA). *R. pomonella* pupae were small enough to fit into one arm of the luer valve, allowing the full volume of the syringe to be injected. Flow rate was fixed at 150 ml/min using a Sierra Instruments mass flow controller (Monterey, CA, USA). CO_2-free air, produced as above, served as the baseline for measurements, and the system was routinely calibrated with CO_2-free air and a certified standard mixture of 500 ppm CO_2 in nitrogen. Following injection, each syringe was immediately re-purged with CO_2-free, re-humidified air. Most of the 45 pupa were periodically sampled for a parallel experiment, but a subset of 7 individuals were injected and purged every 24 h until they eclosed.

2.3.2. Experiment II

The goal of experiment II was to associate patterns of metabolic rate change for *R. pomonella* with morphogenic landmarks demarcating progressive developmental stages from diapause termination to adult eclosion. After 34 ± 1 weeks at 4 °C, we transferred 30 randomly selected pupa to 24 °C. We removed the operculum (the portion of the puparium covering the head) after 5 days at 24 °C to allow visual monitoring of diapause termination and pharate adult development. Pilot studies suggested that removing the operculum does not adversely affect development if humidity is sufficiently high (Ragland, unpublished data). We found that *R. pomonella* pupal and pharate adult development followed the same morphological progression previously described for other higher flies such as *Sarcophaga* and *Drosophila* (Denlinger and Zdarek, 1994; Ashburner, 2005). Fraenkel and Hsiao (1968) have described in detail the visible changes occurring in the head region during the transition from pupal diapause through adult morphogenesis in the flesh fly *Sarcophaga argyrostoma*. They note the appearance of the imaginal antennae under the fat body cells of the head as the first visible landmark of adult morphogenesis, followed by antennal migration and elongation, progressive accumulation of colored pigments in the eyes, and finally accumulation of darkened bristles on the head. All four of these landmarks occur after pupal–adult apolysis. For *R. pomonella*, we were unable to identify the imaginal antennae prior to elongation. However, beginning with elongated imaginal antennae, we were able to identify the same three remaining developmental landmarks in *R. pomonella* that were previously identified in *S. argyrostoma*. We then mapped these three landmarks to our respirometric data to determine how the parameters of our metabolic model correspond to morphogenesis from pupal diapause to pharate adult.

To measure metabolic rates in experiment II, uncapped diapausing pupae were secured in modeling clay and affixed to the plunger of a 5 ml syringe affixed with a luer valve and needle as described above. Syringes were purged as described above except that the plunger was set to a volume of 3 ml. Only 2 ml of the 3 ml volume was injected into the respirometer to leave adequate clearance for the pupa. For this experiment, we purged the syringes only every Sunday, Tuesday, and Thursday, injecting 2 ml of their volume into the respirometer 24 h later every Monday, Wednesday, and Friday until fly eclosion.

2.3.3. Experiment III

The goal of experiment III was to determine the trajectory of metabolic rate during direct, non-diapause development. On the day of pupariation, we transferred a sample of 310 pupae to a 24 °C incubator at ~85% humidity. We tracked metabolic rate daily in a subset of 10 individuals from the day after pupariation to 5 days post-pupariation to establish the general pattern of initial metabolic rate decline following pupariation (Denlinger, 1981). We tracked metabolic rate in the remaining 300 individuals every third day from 5 days post-pupariation to 33 days post-pupariation on days 5, 8, 12, 15, 19, 22, 26, 29, and 33. Respirometric measures were performed as described above described for experiment I. Individuals that did not eclose in 33 days were moved into a simulated winter treatment for a separate experiment. After accounting for mortality, we were left with data for 7 and 270 individuals for the 1–5 and 5–33 day intervals, respectively. Initial inspection of the 5–33 day data yielded two clear groups: trajectories where metabolic rate declined asymptotically and monotonically (diapause development), and trajectories with the characteristic U-shape of non-diapause pupal development (Denlinger, 1981). About 12% of all experimental individuals displayed non-diapause development. We included a randomly selected subset of 34 individuals in an analysis modeling the metabolic rate trajectory of direct development. We also present data from a randomly selected subset of 23 diapause-destined individuals to calculate minimum pre-winter metabolic rates during diapause.

2.4. Data analysis

Expedata data logging software records instantaneous measures of CO_2 versus time. We used the manual bolus integration method of Lighton (2008) to calculate the rate of CO_2 production for an individual pupa as

$$R = \frac{\int_{t_i}^{t_f} CF \cdot dt}{e} \quad (1)$$

where R is the calculated respiration rate in μl CO_2/h, C is the instantaneous measure of CO_2 concentration in μl/l, F is the flow rate in l/s, e is the elapsed time (hours) from purge to injection, and $t_i - t_f$ is the time interval (seconds) over which the concentration data are integrated. Data from experiment II were multiplied by a factor of 3/2 to account for total volume of the syringe (3 ml) versus injected volume (2 ml).

Visual inspection of the data from experiments I and II strongly suggested an initial, logistic increase in respiration rate followed by a second, exponential increase terminating in eclosion. We constructed a five parameter logistic plus exponential function describing this trajectory:

$$R = \frac{\alpha}{1 + \beta e^{-t}} + ce^{at} + b \quad (2)$$

where R is the respiration rate, t the time in days out of winter treatment, c is a fitted scaling parameter, and α, β, a, and b are fitted parameters that determine respiration rate at the transition between the logistic and exponential increase, the timing of the initial logistic increase, the timing of the transition between logistic and exponential increase, and the diapausing baseline respiration rate, respectively. This model could have been simplified by fixing some parameter values, but we chose a relatively flexible model instead to account for individual to individual variation in CO_2 production. Models were fit separately for each individual in experiments I and II using a least squares estimation procedure in Proc NLIN, SAS ver. 9.1 (SAS Institute 2004, Cary, NC). We analyzed wet mass-equivalent metabolic rate (units of μl CO_2/(h mg)) for experiment I to account for the effects of body size on inter-individual variation. We did not correct for body mass in experiment II because the objective of this experiment was to characterize only the time course of development, and we only included similarly sized individuals in the 7–8 mg range in this experiment. We report associated F statistics and coefficients of determination either for each model (experiment I) or for the mean and variation across models (experiment II). We also calculated the positions of both inflection points for each fitted curve as convenient landmarks. The first inflection point marks a time point just after diapause termination, while the second marks the start of the second, exponential increase in respiration rate.

Visual inspection of the combined data from days 1 to 5 and days 5 to 33 from experiment III suggested an initial, exponential decline in metabolic rate from days 1 to 5 followed by an exponential increase from days 8 to 33 in the non-diapause group. We constructed a three-parameter model for the exponential increase during direct development. We expected that metabolic rates during direct development would be concordant with the metabolic trajectory we observed after diapause termination. Therefore, we used a simplified nested version of Eq. (2) to determine whether the increasing portion of the direct development curve tracked a metabolic trajectory similar to the exponential phase modeled for post-diapause development:

$$R = ce^{at} + b \quad (3)$$

where c is a fitted scaling parameter, a describes the timing of the exponential increase, and b represents the baseline respiration rate at the bottom of the U-shaped curve before the exponential increase. We fit this model to wet mass-equivalent metabolic rate values for each individual using the same procedures described above. In addition, we estimated the average minimum metabolic rate across both non-diapausing and diapausing groups from a rolling average of two data points from each individual (a rolling average minimizes outlier influence).

3. Results

3.1. Experiment I

Our non-linear model provided a very good fit to the daily respiration rate data with an average r^2 of 0.97 significant at $p < 0.001$ (Table S1, Fig. 1a). After removal from the 4 °C winter treatment, pupae retained relatively low metabolic rates for 10–15 days, suggesting that no pupa had broken diapause as an immediate consequence of the transition from cold to warm conditions. All pupae eventually increased their respiration rates in an initially sigmoidal pattern (Fig. 1). This initial increase was followed by a relatively stable plateau in respiration rate that lasted about 10 days. After the plateau, a second, exponential increase in respiration rate was observed that terminated in adult emergence. The first inflection point (see IP1 in Fig. 1a) marked the midpoint of the initial sigmoidal increase, while the second inflection point (IP2 in Fig. 1a) marked the transition from logistic to exponential increase in respiration rate at the mid point of the plateau.

There was substantial variation in the shape of the respirometric trajectories of diapause termination and adult morphogenesis among individual flies, including differences in the timing of the first inflection point (initial diapause break; $\bar{x} = 17$ days, S.E. = 0.97, CV = 0.15), in the elevation of the respiration plateau (respiration rate at the second inflection point; $\bar{x} = 8.90 \times 10^{-4}$ μl CO_2/(mg h), S.E. = 8.76×10^{-5}, CV = 0.26), in the baseline metabolism before the initial increase ($\bar{x} = 6.77 \times 10^{-5}$ μl CO_2/(mg h), S.E. = 1.22×10^{-5}, CV = 0.48) and in the timing of the transition to the final, exponential phase (second inflection point; $\bar{x} = 24$ days, S.E. = 0.88, CV = 0.10) (Fig. 1b). These values were, characterized in the model by parameters β, α, b, and a, respectively. Variation in respiration rate at eclosion was partially determined by differences in the time of day that adult flies emerged. Following eclosion, adult flies were mobile and respired at a substantial rate. Consequently, measures of CO_2 production on the day of adult eclosion reflected some combination of pre- and post-eclosion metabolism. Despite this variation, the overall pattern showed that the metabolic rate of diapausing individuals after overwintering was approximately 7.5% of that for individuals in the metabolic plateau stage

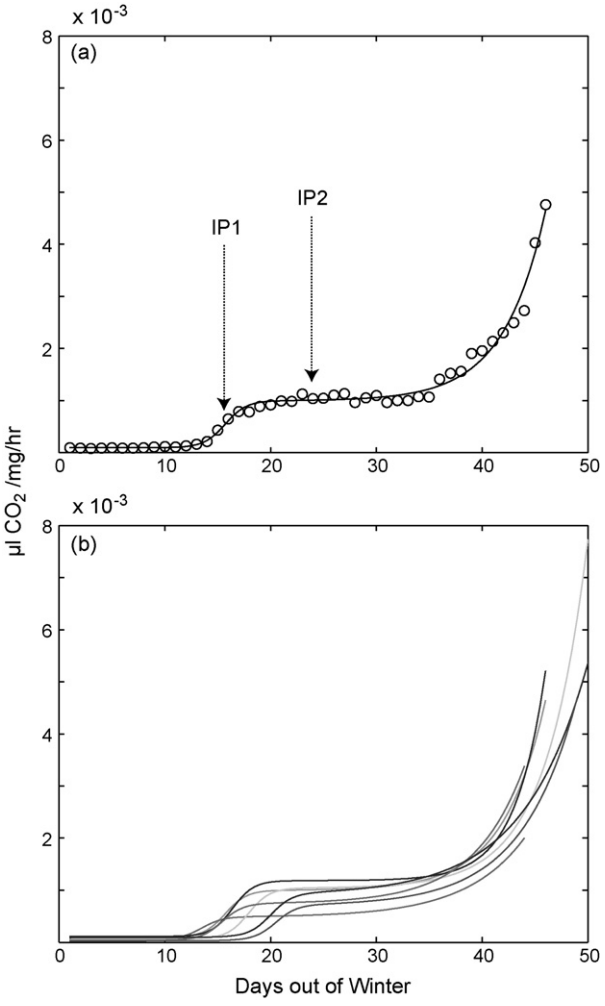

Fig. 1. (a) Fitted non-linear regression of metabolic rate on time (days) following winter treatment for a representative individual. Inflection points one (IP1) and two (IP2) are indicated by the arrows. (b) Estimated metabolic rate curves for all seven individuals measured in experiment I.

Table 1
Mean and standard error of the calculated r^2 and number of days out of winter treatment corresponding to the estimated inflection points and observed developmental markers from models fit to each individual in data set II. All model fits were significant at $p < 0.001$.

	r^2	Days to first inflection point	Days to second inflection point	Days to antennal elongation	Days to red-eye stage	Days to adult eclosion
Mean	0.95	12.8	20.3	28.0	33.2	45.4
S.E.	0.01	0.820	0.848	1.75	1.18	1.22

following the initial logistic increase in rate signifying diapause termination (8.90×10^{-4} μl CO_2/(mg h) vs. 6.77×10^{-5} μl CO_2/(mg h)).

3.2. Experiment II

The non-linear model also significantly fitted the data from experiment II (Table 1 and Fig. 2). The appearance of fully elongated imaginal antennae occurred an average of 15 and 8 days after the first (IP1) and second (IP2) inflection points of the fitted model, respectively. The appearance of fully elongated imaginal antennae roughly corresponded to the midpoint of development from pupariation to adult eclosion. The remainder of the clearly visible morphogenic landmarks, when adult tissues were rapidly proliferating, occurred over a 28 day (on average) period between the second inflection point and adult eclosion.

3.3. Experiment III

Respiration rate curves for diapausing individuals monotonically declined to a steady baseline, while curves for non-diapausing individuals were strongly U-shaped with approximately 12% of

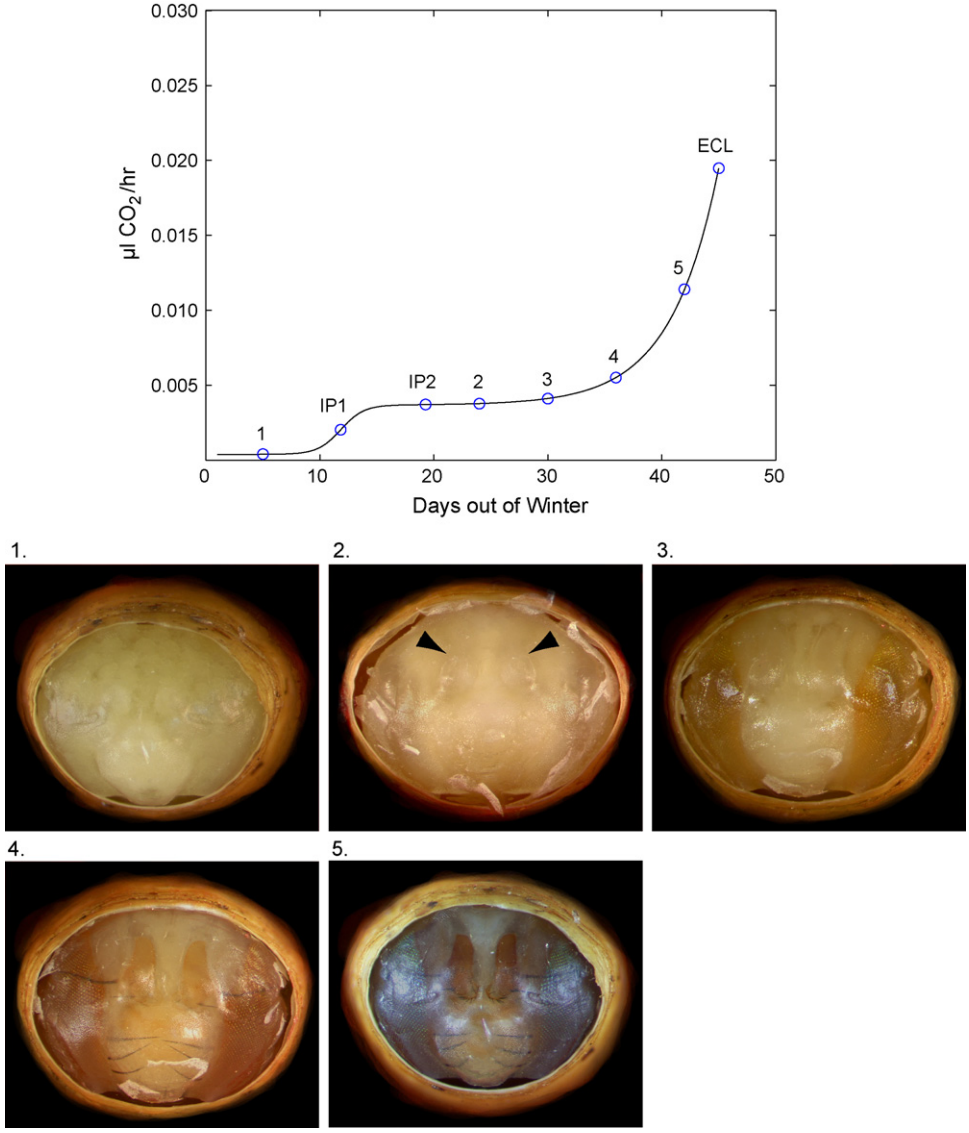

Fig. 2. Relationship between metabolic rate curve and developmental landmarks for a representative fly in experiment II. Shown on the curve are the positions for the two inflection points (IP1, IP2), the time of adult eclosion (ECL), and the five developmental stages based on visible morphology pictured in the lower panel: (1) diapausing pupa; (2) elongated antennal discs (black arrows); (3) red-eye; (4) black bristle; (5) late pharate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

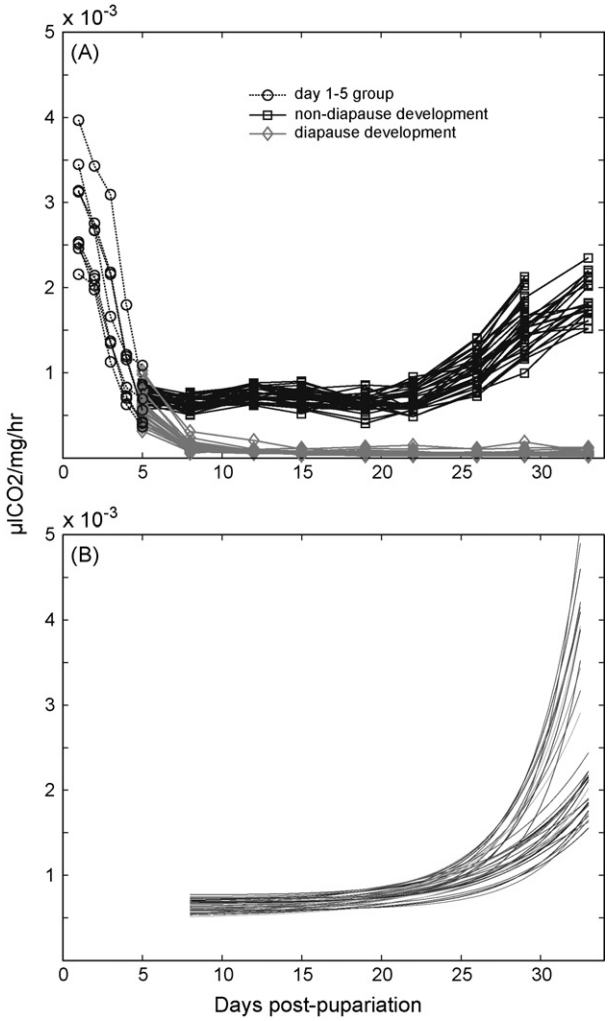

Fig. 3. (a) Metabolic rate following pupariation for non-diapause (—□—) and diapause (-◇-) development. Data for the first 5 days post-pupariation represent a separate group of eight individuals (··○··). All other data represent individuals measured from 5 to 33 days post-pupariation. (b) Fitted non-linear regression of metabolic rate on time (days) post-pupariation for non-diapause individuals only, estimated from data from days 8 to 33.

individuals not diapausing (Fig. 3a). Thirteen non-diapause individuals in experiment III eclosed between days 29 and 33. An additional 21 individuals of the 270 monitored in experiment III had respiratory rates indicating non-diapause development, but these flies were not followed until adult eclosion because they were placed in a simulated winter treatment for a different experiment. As a result, we cannot accurately estimate the average time to eclosion for all non-diapause individuals in the study population. However, the timing of the eclosion events we did observe in experiment III were similar to our estimates of eclosion time following diapause termination in experiments I and II of ~33 days (Fig. 2).

The exponential model provided a good fit to the metabolic data from days 8 to 33 post-pupariation for non-diapause individuals with an average r^2 of 0.94 significant at $p < 0.01$ (Table S1; Fig. 3b). The close fit suggests that after the initial metabolic decrease of the U-shaped curve, non-diapause individuals follow the same metabolic trajectory as the second, exponential phase of post-diapause development observed for overwintering flies. We obtained similar estimates of minimum metabolic rate for non-diapause individuals from the fitted intercept of the exponential model ($b = 6.25 \times 10^{-4}$ µl CO_2/(mg h), S.E. = 1.36×10^{-5}, CV = 0.13) and from a rolling average of the raw data through the bottom of the U-shaped curve ($\bar{x} = 6.23 \times 10^{-4}$ µl CO_2/(mg h), S.E. = 9.57×10^{-6}, CV = 0.09). As expected, the estimated stable metabolic rate for individuals in diapause from experiment III (based on a rolling average) is substantially lower ($\bar{x} = 4.78 \times 10^{-5}$ µl CO_2/(mg h), S.E. = 3.89×10^{-6}, CV = 0.40), representing just 7.6% of the metabolic rate of non-diapause individuals at the bottom of their U-shaped curve.

4. Discussion

Metabolic rate curves for *R. pomonella* and other non-diapausing fly pupa track a characteristic U-shaped trajectory (e.g., Denlinger et al., 1972). Metabolic rates decline rapidly following pupariation, bottom out during early pharate adult development, and then increase exponentially during the later phases of pharate adult morphogenesis until eclosion (Fig. 3a). In *R. pomonella*, an exponential function provided a good fit for the increasing portion of the curve. In contrast, individuals that entered diapause were metabolically depressed compared to developing pupae. While the diapause period itself is characterized by metabolic depression, the latter phase of the metabolic trajectory of post-diapause development in *R. pomonella* traces a curve very similar to the curve for the increasing phase of non-diapause pupal development. In fact, the non-diapause data fit the same function (Eq. (3)) as the portion of Eq. (2) modeling the exponential phase of increase in post-diapause metabolic rate.

Although they were similar, our metabolic rate estimates were slightly lower overall in experiment III than in experiment I for both individuals in diapause (4.78×10^{-5} µl CO_2/(mg h) vs. 6.77×10^{-5} µl CO_2/(mg/h)) and for individuals in the plateau prior to the exponential increase in metabolic rate nearing the end of pharate adult development (6.25×10^{-4} µl CO_2/(mg h) vs. 8.91×10^{-4} µl CO_2/(mg h)). A number of factors may have contributed to the small differences in metabolic rates among similar developmental phases between the two sets of experiments. Individuals from experiments I and III came from different populations of origin (Fennville vs. Grant, MI, respectively), and the respirometric measurements were taken at different times of the year (fall pre-winter vs. spring post-winter). Regardless, diapausing individuals were always clearly metabolically depressed compared to developing individuals. In experiment I the metabolic rates of individuals in diapause were on average 7.5% of the metabolic rates of those same individuals once they had reached the respiratory plateau after the initial increase in metabolism associated with diapause termination. Similarly, in experiment III the metabolic rates of diapausing individuals were on average 7.6% of the metabolic rates of non-diapausing individuals at the bottom of the U-shaped trajectory. Therefore, despite the small differences in absolute metabolic rates between the two experiments, the relative differences in metabolism between diapausing and developing individuals agree very well between experiments I and III. This evidence further supports a general concordance between metabolic trajectories of non-diapause and post-diapause pharate adult development.

Diapause termination and post-diapause development in *R. pomonella* appear to follow a similar metabolic trajectory to that verbally described for *Sarcophaga* species by Denlinger et al. (1972). During diapause, *Sarcophaga* pupae display a minimal respiration rate that is approximately 10% of the minimum of the U-shaped curve for direct development. The earliest visible landmarks for adult morphogenesis in *Sarcophaga*, migration of the imaginal antennae, can be observed several days after an increase in respiration rate up to the minimum of the U-shaped curve displayed by direct-developing pupae. For the next several days, respiration rate stabilizes briefly while the imaginal

antennae migrate to their final positions, then increases again exponentially as rapid adult morphogenesis occurs. This respirometric pattern mirrors our observation of a biphasic increase characterized by an initial, logistic increase followed later by a second, exponential increase. We observed elongated imaginal antennae, our first visible marker of morphogenesis, only after the final exponential increase in respiration rate had begun (i.e., after the second inflection point in the middle of the plateau). However, we were only able to identify imaginal antennae *after* they had fully migrated and elongated. We expect that pupal–adult apolysis and the earliest stages of morphogenesis occur earlier, probably before the second inflection point (mid-plateau) and potentially beginning at the first inflection point (just after the initial increase in metabolic rate). Certainly the bulk of visible morphogenesis occurs relatively late in the trajectory concomitant with the final, exponential increase in respiration rate. In general, our results match well with the qualitative description in *Sarcophaga*, suggesting that the metabolic pattern of diapause termination may be evolutionarily conserved in higher flies.

Our model provides a quantitative framework with well-defined metabolic landmarks to study the molecular and physiological events leading up to and during diapause termination and adult morphogenesis in *R. pomonella*. The biphasic increase in metabolic rate that we observed in *R. pomonella* suggests some element of discontinuity in the physiological transition from a diapausing pupa to a post-diapause pharate adult. Indeed, Schneiderman and Williams (1953) hypothesized that "The visible initiation of development is evidently the end-product of a brief period of endocrinological and biochemical preparation." after observing a similarly discontinuous increase in metabolic rate following diapause termination in *Cecropia* moth pupae. The initial increase in metabolism we observed is clearly an important physiological landmark in the process of diapause termination, and future work will involve documenting physiological changes prior to, during, and just after this transition. A major question that must be resolved is whether *R. pomonella* remains a pupa for an extended period after the initial increase in metabolism preparing for pupal–adult apolysis or if the initial increase marks pupal–adult apolysis itself. The former would suggest that there are some critical post-diapause steps that must be taken before development can ensue. Experiments are currently underway using a variety of histological, biochemical, and molecular approaches to distinguish between these two hypotheses and determine the physiological events that lead to the termination of diapause, a fundamentally important event in life history timing in *R. pomonella* and many other insects.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jinsphys.2008.12.013.

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