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Deciphering the Role of Mitochondrial Physiology and Thermal Acclimation in Shaping Whole Organismal Performance of an Invasive Forest Pest

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Abstract: Environmental factors such as temperature are substantial determinants of the spongy moth, *L. dispar*, distribution, reproduction, and growth. Accumulating energy reserves at the larval stage is particularly important to *L. dispar,* since the larvae metamorphose into a fully-grown, non-feeding adult. As non-feeding adults, the energy balance of the pupae must be adequate, to ensure enough energy reserves for adult dispersal, egg maturation and overall persistence of the species in the region. At this stage, environmental temperature also determines daily metabolic demands, and the overall cost of sustaining ecologically relevant activities. Various hypotheses describing a mismatched growth and metabolic rate at supraoptimal temperatures exist, with very few providing a mechanistic explanation of why growth decrease at supraoptimal temperature, while metabolic rate continues to rise on many ectotherms, including insect larval stages. Frequently, studies have shown positive and causative relationships between metabolism and growth suggesting that metabolism drives *L. dispar* and more broadly, ectotherms, performance. Such as relationship was integrated recently to mitochondrial energy transduction efficiency (METE), which is known to be thermosensitive. Current evidence suggest that mitochondrial METE may be pivotal in whole organismal thermal performance, and our study addresses such knowledge gap in the *L. dispar*. Post-diapause egg masses from a captive breeding population were manually disrupted and eggs were pooled. Subsamples of eggs were distributed in hatch-out containers at the corresponding acclimation temperatures. Acclimation setup consisted in two temperature treatments, 20°C and 30°C, each with a controlled with a photoperiod of 14L:10D. To assess the thermal sensitivity of mitochondria, we used a high-resolution respirometry system. Gut mitochondria were isolated and the performance of the electron transport system (ETS) was evaluated under multiple substrates. Proton conductance across the inner membrane (LEAK) did not differ between acclimation groups, with the exception of LEAK flux under complex I-activating substrates at 15°C. Mitochondrial oxygen flux linked to LEAK increased with assay temperature. Oxygen flux associated with LEAK also show an additive effect; the activation of additional electron carriers in the ETS increased the overall electron transfer capacity of the ETS, increasing the LEAK rates observed. Oxygen flux associated with oxidative phosphorylation (OXPHOS) displayed an increasing trend with assay temperature. Respiratory control ratios (RCR) were calculated from OXPHOS/LEAK significantly decreased in a bi-phasic fashion; lowest rate of change between 15-25°C, and a faster rate of change between 25-34°C. Results from this study show a reduction in METE with increasing temperatures, and aligns with the range thermal insensitivity described for the species in previous studies. Consequently, it is plausible that reductions in developmental performance (both time and rate) can be attributed to changes in METE. Both metabolism and development are energetically expensive, and as such influenced by the METE. Moreover, the lack of variation in RCR between acclimation groups in this study suggests that the bioenergetic machinery of *L. dispar* mitochondria adapts rapidly under short term acclimation (via regulatory processes controlling the ETS electron carrying capacity), and that adaptive changes in this fast-growing stage are beneficial to sustain energy-dependent processes at rapidly changing environmental temperatures.

Introduction

Economical and ecological importance of insect pests

In the upcoming decades, rising air temperatures, and the gradual warming of both forested an agricultural land pose unique challenges to the organisms living in these environments. From such organisms, nuisance species such as forest and crop pests are of particular interest, as the population dynamics of these species could have profound implications to the overall forest health of many ecosystems, and the food security of entire regions (Deutsch et al., 2018). For example, due to current and forecasted climate change, grain crop losses due to agricultural pests are expected to increase by 10-25% per each degree centigrade of surface (Deutsch et al., 2008, 2018). In a similar fashion, forest insect pests distributions are expected to change, and the forecasted increased in community metabolism and demographics could lead to devastating defoliation of forested areas, leading to changes in canopy cover, evapotranspiration of plants and the thermal regime of habitat occupied by many other species. As the energetic balance of insect pests are largely dependent on environmental temperature and availability of resources, a careful analysis of the individual bioenergetic machinery of each species is deemed necessary, to evaluate regional-scale losses, and accurately estimate the community metabolism in large-scale forecast models.

Insects play a crucial role in the U.S. economy, although the effect is often regional. In this regard, various studies recognize the ecosystem service of insects, including dung burial, pest control, pollination, and wildlife nutrition, as crucial in the agricultural sector (Fortuna et al., 2022; Jankielsohn, 2018; Losey & Vaughan, 2006). In

2006, the value of ecological services provided in the U.S. was estimated at $57 billion, translating to a similar amount in losses if insects were not available in nature. In such a case, insect value can be illustrated owing to human dependency, with billions of cross-pollinations facilitated by insects annually (Losey & Vaughan, 2006). In the absence of such services, millions in metric tons of food would be missing in the food economy. Additionally, Jankielsohn and colleagues (2018) state that despite herbivorous insects destroying an estimated 18% of global agricultural production, the benefits obtained from their existence outweighs their negative impact. In such a case, insects play critical functions in agroecosystems, including pollination, and maintaining ecological balance, which is useful in sustaining crop systems and growth. With an estimated 5.5 million species, insects contribute to the global biodiversity, and promote ecosystem cycling, predation, and parasitism, often manipulated to control the population of harmful pests, as well as in decomposition to enable nutrient recycling and waste elimination (Jankielsohn, 2018). However, in the U.S. and other regions, invasive species often disrupt ecological balance significantly. Studies also show that exotic species often replace native ones, modifying biological controls (Fortuna et al., 2022). Moreover, eradication and extinction is often impossible due to extensive colonization. When insects habituate to a new place, they can introduce diseases, disrupt the ecological balance, and initiate the extirpation of other species due to niche overlap, resource competition and diseases (Fortuna et al., 2022).

The L. dispar invasion: history and current status

Since its introduction from Europe to Massachusetts in 1869, the *L. dispar* has expanded its range over >900,000 km^2 (from Ontario to Virginia and North Carolina)

and across widely divergent climates and forest types (Thompson et al., 2017; Tobin et al., 2007, 2014). Part of its invasiveness undoubtedly stems from the caterpillars being extreme food generalists, with oaks (*Quercus* spp.) topping a long list of >300 food plant species (Liebhold, 1995). Since 1996, the USDA Forest Service Slow-the-Spread program (http://www.gmsts.org) has collected detailed data along the *L. dispar* invasion front providing extensive records of population spread rates: over the period 2000-2012. The dominant population dynamics revealed by these data include regional expansion of the invasion front in the northern portion of the range, but stasis or contraction in the middle and southern portions of the range (Thompson et al., 2017; Tobin et al., 2007). Understanding the population dynamics and ecological success of organisms like the invasive *L. dispar* is of fundamental importance for science and society (Allendorf & Lundquist, 2003; Mooney & Cleland, 2001; Parker et al., 2001; Sakai et al., 2001). Ultimately, ecological success of this and other species depends critically on achieving positive energy balance (Bunce et al., 1979; Dunham et al., 1989; Hall et al., 1992), such that organisms take in sufficient energy to pay the cost of maintenance, with enough accrued energy to fuel growth and reproduction.

Cellular physiology influencing the organismal performance of L. dispar

Environmental factors such as temperature are substantial determinants of *L.dispar* distribution, reproduction, and growth (Allen et al., 1993; Knapp et al., 1986; Thompson et al., 2017), including metabolic rate (Casagrande et al., 1987). Metabolism is an essential process in the growth and activity of *L. dispar*, as it provides the energy that supports the different developmental and physiological processes, such as molting of the larva and the development of the egg to an adult moth (May et al., 2018).

The growth of *L. dispar* encompasses four stages that behave and appear differently. The entire developmental life cycle starts with eggs laid by an adult female. One female lays between 200 and 1000 eggs, overwintering at this stage until spring. As spring temperatures increase larvae hatches progressively and feed intensively on surrounding foliage (Elkinton & Liebhold, 1990; Knapp et al., 1986; Knapp & Casey, 1986). The feeding will last between five and six weeks. The growth of *L. dispar* larva is characterized by molting approximately every week. After the larva has attained total growth, it locates a suitable environment to pupate (Knapp & Casey, 1986). The transition from the larval stage to the pupal stage is defined by the shedding and hardening of the cuticle. After pupation, the larvae metamorphose into a fully-grown, non-feeding adult (Casagrande et al., 1987). As non-feeding adults, the energy balance of the pupae must be adequate, to ensure enough energy reserves for adult dispersal, egg maturation and overall persistence of the species in the region. At this stage, environmental temperature also determines daily metabolic demands, and the overall cost of sustaining ecologically relevant activities (Dunham et al., 1989).

Frequently, studies have shown positive and causative relationships between metabolism and growth, suggesting that metabolism drives *L. dispar* and more broadly, ectotherms, performance (Martinez et.al., 2017; Faske et al, 2019). In other words, a high metabolic rate is associated with a high growth rate. However, studies also demonstrated that while the relation between growth and metabolism is positive and causative within a narrow thermal range, supraoptimal temperatures result in a mismatch between the two life traits. With increased metabolism, the reproduction and growth of the moth are constrained at temperature beyond optimum. In a similar

fashion, Southern winter temperatures and controlled experiments at supraoptimal temperature have shown a reduction in pupal mass and fewer eggs at these supraoptimal temperatures (Faske et al, 2019). Thus, the linkages between metabolism and growth on *L. dispar*, as well as many other ectotherms in general is not straightforward, and depends on the physiological state and environmental factors that challenge each individual.

Temperature and mismatched growth-metabolism curves: what is the cause?

The association between growth and metabolism is understood as being positive and causative. However, in experiments by Kingsolver and Woods on *Manduca sexta,* supraoptimal temperatures leads to increase metabolic rate, while the growth rate decreases (Kingsolver & Woods, 1997). Such as relationship was integrated to mitochondrial energy transduction efficiency (METE); metabolism increases beyond 33℃, reaching 45℃, while METE and whole organismal decrease over supraoptimal temperatures (Martinez et al., 2017). Although METE may explain the unexpected relationship, such a relationship remains poorly understood. This view is also supported by studies in *Drosophila*, which showed that organismal physiology is determined by metabolic pathways, such as those occurring in the mitochondria, through ontogeny (Matoo et al., 2019). In such a case, the oxidative capacity in every mitochondrion had a significant influence on mass and metabolic rate in *Drosophila spp.* Although genetic variations often influence adaptability capacity depending on the larval or organismic instars, mitochondrial activity remains integral in such processes (Matoo et al., 2019). Additional studies propose an adaptability model, where mitochondrial function responds to physiological stressors to sustain growth, when such environments are

altered (Greenlee et al., 2014; Greenlee & Harrison, 2005). Current evidence suggest that mitochondrial METE is pivotal in whole organismal thermal performance, and to this end my study addresses such knowledge gap in the *L. dispar*.

Environmental temperature has strong, pervasive effects on the physiology of insects, which in turn affect populations persistence, abundance and distribution (Estay et al., 2014). For *L. dispar* larvae and adults, individuals must adapt to variations in temperature to maintain optimal biological processes (Allen et al., 1993). Behavioral thermoregulation increases the organisms' ability to achieve a positive energy balance, which rely in sustaining the coupling efficiency between oxygen consumption and ATP production in the mitochondrion (Martinez et al., 2017). For generations to persist, the *L. dispar* must consume adequate energy to sustain life. The energy must be sufficient to support reproductive and somatic growth, thus any alterations in the bioenergetic machinery will likely affect egg viability, larval development and reproductive output of adults (Elkinton & Liebhold, 1990; Liebhold, 1995; Logan et al., 1991).

Mitochondrial energy transduction efficiency (METE) as causative of whole organism thermal performance.

Energy balance at both cellular and tissue level determines the overall energy budget of animals. In ectotherms, environmental temperature is known to influence the energy transduction efficiency in the mitochondrion, imposing challenges to those organisms operating at supraoptimal temperatures. For example, in the crop pest *Manduca sexta*, the optimal temperature that is associated with the highest mitochondrial energy production efficiency is approximately 34°C (Chamberlin, 2004; Martinez et al., 2017). At supraoptimal temperatures between 34-42°C, respiration

coupled to ATP production plateau, while uncoupled respiration continues to increase, thus reducing the coupling efficiency of oxygen consumption to ATP production. Interestingly, reductions in mitochondrial coupling efficiency also parallels whole-organismal growth efficiency at the same temperatures (Kingsolver & Woods, 1997; Martinez et al., 2017), suggesting a direct link across levels of organization.

Mitochondria is a key organelle in eukaryotic cells, which account for approximately 90% of the entire energy production under aerobic conditions (Salin et al., 2018). Energy is produced through the continuous utilization of oxygen by the mitochondria. The activity of the electron transport System (ETS) generates a proton motive force, which can be coupled to the production of ATP via ATPase, or can be dissipated through uncoupling proteins or via intrinsic leakage of the inner membrane (Nicholls & Ferguson, 2002). ATP synthesis in mitochondria relies on specific substrates and processes. Within mitochondria, there is an inner membrane that contains proteins that participate in proton transport. Mitochondrial respiration is based on protein complexes I, III, and IV to transport protons to the intermembrane membrane .(Nicholls & Ferguson, 2002) .Electron transport rely on NADH and $FADH_2$ availability, as these are reducing agents of complexes I and II which feds electron into the Electron Transport System (ETS). As the electrons are transported along the mitochondrial membrane, the protein complexes I, III and IV translocate protons to the intermembrane region. The proton movement establishes a gradient in proton concentration and ATP synthase utilizes the concentration gradient to synthesize ATP, in the presence of ADP and inorganic phosphate(Brand, 2005). In both proton cycling processes, coupled or uncoupled to ATP production, molecular oxygen is used as the terminal electron

acceptor, thus the flux of oxygen can be used as an indicator of energy transduction efficiency (Gnaiger, 2011, 2020).

Since most physiological processes occurring in ectotherms are energy dependent, Mitochondrial ATP production efficiency had a significant impact on animal performance, including reproduction, development, and growth of ectotherms (Chung & Schulte, 2020). Furthermore, the efficiency of ectotherm's mitochondrial ATP production is temperature dependent. As a result, cellular energy production, in ectotherms rely on the individual's ability to operate at or close to its thermal optimum, particularly during stages of rapid development, such as the larval stages of holometabolus insects. Despite many observations of this phenomenon on various insect genera (e.g.*Manduca*, *Bombix*, *Limantria*) , the mechanistic links explaining the thermal dependence remains poorly studied.

As proposed in a previous study, METE parallels developmental rate in holometabolus larvae (Martinez et al, 2017). The present study centers on testing this hypothesis by expanding it to other Lepidoptera species, and determined the applicability of the model to other ectotherms. In addition, the present study tests the role of acclimation temperature shaping the larvae's ability to modify its METE. Particularly, the study aims to address the following questions:

Research questions:

Q - Are reductions in mitochondrial ATP production efficiency an underlying mechanism of reductions in whole-organism performance in relation to high temperature?

H – It is hypothesized that a main factor contributing to reductions in growth performance at supra-optimal temperatures is a reduction in the mitochondrial energy transduction efficiency.

Q - Does METE altered through short-term acclimation?

H - The overall metabolic demands of larvae of holometabolus insects are profoundly dependent on temperature. It is therefore hypothesized that profound changes occur in the mitochondrion through acclimation, to satisfy the changes in energy demands.

Materials and Methods

Sourcing of L. DISPAR egg masses - Post-diapause egg masses from a captive breeding population were kindly provided by the USDA APHIS Otis Laboratory, under interstate transport permission USDA P526P-21-00138 (permitee: Eloy Martinez). A shipment containing approx. 20 egg masses arrive at the Biological Sciences Building, and promptly transported to the USDA APHIS inspected containment facility, located in the first floor, room 1040. Egg masses were stored at 4°C until used.

Larval rearing and temperature acclimation setup - Individual egg masses were manually disrupted and eggs were pooled. Subsamples of eggs were distributed in four hatch-out containers at the corresponding acclimation temperatures. Every 24hrs, each container was inspected for hatching first instars, which were relocated to grow-out containers with fresh diet as they hatched. Diet was prepared according to the USDA-APHIS Otis Laboratory Diet, each liter consisting of 120.0g of wheat germ (Honeyville grain, Inc.), 25.0g of casein (Frontier), 8.0 g for Wesson Salt Mix (Animal Specialties and Provisions), 2.0g sorbic acid (Frontier), 1.0g of methyl paraben (Frontier), 10.0g of USDA vitamin premix (Bio-serv), 15g of *Gracillaria* agar (MoorAgar) and 0.0428g ferric citrate (Sigma-Aldrich).

Acclimation setup consisted in two temperature treatments, 20°C and 30°C, each with an electronically controlled with a photoperiod of 14L:10D. Daily inspection of larvae and diet took place through the developmental period of larvae from 1st to 5th instar (total time varied depending on hatching date and acclimation temperature), which consisting on replacing dried diet, removing diseased or sick individuals, and removing any condensate from each container.

Individuals who reached 5th instar were relocated to empty enclosures overnight, to void their gut before mitochondrial isolation trials.

Tissue extraction and mitochondrial isolation - The isolation process followed after the eggs reached the 5th instar. The process used to excise tissue from each larva has been described previously (Martinez et al 2017), and minor modifications were added to ensure cleaner samples and a better homogenization process. Briefly, each larva was dissected ventrally, and a total of 20 digestive tracts were pooled for a single mitochondrial isolation. After each tube excision, the peritrophic membrane of each tube was removed, and the clean digestive tube pooled in 1mL of ice-cold Isolation Medium (IM; 250mM sucrose, 2mM ethyleneglycol-bis (β-aminoethyl ether) N, N'-tetraacetic acid [EGTA], morpholinopropane sulfonic acid [MOPS], 0.5% fatty-acid-free bovine serum albumin [BSA], pH = 7.4, 20C, 272 mmol kg^{-1}). After mincing with ice-cold scissors, the sample was homogenized in 5mL of IM, using a 7mL Potter Elehjem homogenizer coupled with a variable speed drill. Five passes at low speed (~400rpm) was enough to homogenize the tissue. Homogenate was centrifuged at 700g for 10min at 4C to remove cellular debris and undisrupted tissue. The supernatant was collected and centrifuged at 9,600g for 15min at 4°C to sediment the mitochondrial fraction. Pellets were washed with IM, resuspended and recovered by centrifugation at 9,600g for 15min at 4°C two consecutive times. The final pellet was resuspended in 250uL of IM and stored on ice for 1h before trials.

To assess the thermal sensitivity of mitochondria, high-resolution respirometry systems were employed. The unit comprises two 2.0-mL thermo-controlled respirometric chambers and Clark-type polarographic oxygen electrodes (Oroboros Instruments; O2k Oxygraph). Polarographic O_2 electrodes were calibrated for each

assay temperature (20 and 30 ◦C) across a range of O_2 tensions, in the presence of 2.0 mL of respiration medium (RM; respiration medium (RM) prepared according to Keeley (1973), modified by Martinez et. al. (2017) consisting of 250 mM sucrose, 0.3% w/v BSA, 15 mM KCl, 5.0 mM $MgCl_2$, 0.1 mM EGTA, 25mM K_2PO_4, and50mM MOPS (pH=7.4, 20°C), to account for temperature effects and background O_2 consumption by the probes. In addition, zero calibration of the probes at each assay temperature was achieved by injecting 20 µL of a freshly prepared 40 mg ml^{-1} sodium dithionite solution into each chamber. The background flux was recorded before mitochondrial injection and subtracted from each run. Oxygen concentration was maintained between 350 and 200 nmol mL^{-1} by gently opening each respirometric chamber to gradually fill the gas phase above the respiration medium if needed. Respiration rates were normalized to mitochondrial protein content.

For each run, 25uL of purified mitochondria (154-369 mg of mitochondrial protein) was injected into the respirometer chamber containing 2.0 mL of RM. Electron transport via complexes I, II, and glycerol-3-phosphate dehydrogenase (GPDH) to the ETS was assayed concurrently in each run. The respiration not associated with ATP production and broadly defined in this study as oxygen consumption associated with proton conductance, proton slip, and cation cycling at saturating substrate concentrations (LEAK) was initiated by adding 2 mM malate (M), 10 mM glutamate (G), 5 mM pyruvate (P), and 10 mM proline, which supplies electrons to complex I via production of NADH by mitochondrial dehydrogenases. Convergent electron entry to the ubiquinone pool via $FADH_2$ was initiated by the addition of 10 mM succinate, and electron entry via GPDH was initiated by adding 10mM sn-glycerol-3-phosphate. To engage OXPHOS, 10 mM ADP was added to the

chamber. The proportion of the total respiration coupled to OXPHOS (termed the RCR) was calculated by diving OXPHOS rates by the LEAK rates. Mitochondrial protein was quantified using a Coomassie Plus Reagent Assay (Thermo Scientific, Rock-ford, IL).

Statistical Analyses

Significant differences in OXPHOS, LEAK and calculated RCR as a function of assay temperature and acclimation temperature were determined using a two-way ANOVA, with Holm-Sidak multiple comparison method (Sigmaplot, v12.0).

Results

Proton conductance across the inner membrane (LEAK) is shown in Figure 1, with each subpanel representing LEAK under various ETS substrates. The use of multiple substrates resulted in an additive effect, where complex II substrates contributed the most to maximal LEAK observed. Reaction norms calculated as Q_{10} between data points shows Q_{10} ranging from 1.6-15.8, where the highest values were observed for 30°C acclimated samples under complex I substrates, between assay temperatures of 25 and 28°C (Figure 1A). The lowest Q_{10} observed was on 30°C acclimated samples under complex I and PDH substrates, assayed at 30°C (Figure 1B). Significant differences in LEAK between acclimation groups at a given assay temperature were found only under complex I substrates ($p = 0.039$; Two way ANOVA; Table 1and Figure 1A). Significant differences were also observed within acclimation groups, across assay temperatures, for all substrates. Overall, mitochondrial oxygen flux linked to LEAK increased with assay temperature. Interestingly, oxygen flux associated with LEAK also showed an additive effect; the activation of additional electron carriers in the ETS increased the overall electron transfer capacity of the ETS, increasing the LEAK rates observed (Figure 1 A-D). Detailed p and F values are shown in Table 1.

Oxygen flux associated with oxidative phosphorylation (OXPHOS) displayed an increasing trend with assay temperature (Figure 2). Reaction norms calculated as Q_{10} between data points was lowest between temperatures 25 and 28°C for both 20 and 30 acclimation groups, with a Q_{10} of 0.92 and 0.72, respectively (Figure 2). Acclimation group OXPHOS did not differ significantly at any assay temperature. Interestingly,

between 28 and 30°C, both acclimation groups showed a decrease in oxygen flux associated with OXPHOS. Within the aforementioned temperature range, reaction norms for OXPHOS across assay temperature departed from the expected Q_{10} of ~2. Significant differences within acclimation groups were found, with the exception of OXPHOS rates found between 25-30°C. Detailed p and F values are shown in Table 1.

Respiratory control ratios (RCR) are calculated from OXPHOS/LEAK are summarized in Figure 3. Acclimation groups exhibited a similar thermal performance curve for RCR, where 15°C assay temperature showed RCR values of 7.0 for both acclimation groups, indicating better coupling of oxygen flux with ATP production at low temperatures. As assay temperatures increased to 25°C, the coupling efficiency significantly decreased, and progressively resulted in a bi-phasic, progressive decrement of RCR. The initial slope show a modest decrease between 15-25°C, then warmer assay temperatures resulted in a faster rate of change between 25-34°C (Figure 3).

Integrating the findings of the present study to relevant life history traits of the *L. dispar*, yields interesting results, shown in Figure 4. Particularly, when comparing metabolic demands and the respiratory control ratio, there is strong alignment of the observed fast RCR decrement from this study and the onset of peak metabolic activity (Martinez, unpublished data), reductions in the developmental time (Data from Casagrande et al, 1987: Figure 4A) and a reduction in developmental rate (Logan et. al., 1991: Figure 4B) recorded for *L. dispar* 4th instar larvae.

Discussion

Various hypotheses describing a mismatched growth and metabolic rate at supraoptimal temperatures exist, with very few providing a mechanistic explanation of why growth decrease at supraoptimal temperature, while metabolic rate continues to rise. To this end, Martinez and colleagues (2017) associate the discrepancy to reduced mitochondrial efficiency in larvae from the tobacco hornworm, *Manduca sexta*, although the etiological association between the two variables remain unclear. In *L. dispar* development, Casagrande et al. (1987) propose a constant-temperature model, with current evidence placing supraoptimal temperatures beyond 30°C, described as experimentally uncommon. In this regard, as temperature increases above the supraoptimal, *L. dispar* development rates decrease proportionately, with various explanations offered, including protein unfolding. Diurnal analyses indicate sigmoid curves in a proportional correlation between percentage development and days, up to a maximum, where the trait asymptotes (Casagrande et al., 1987). At first-instar development over time, Casagrande et al. (1987) identify a proportional association in low and high-temperature regimes in the absence of extremes. Between 7°C-33°C, the cumulative first-instar development shows a normal curve, with growth peaking at 100% in 16 days before tapering (Casagrande et al., 1987). Another study conducted stage-specific experiments of the *L. dispar*'s growth as determined by temperature, yielding a non-parametric distribution, suggesting population specific differences in developmental rates (Logan et al., 1991). At the first instar, optimal temperatures were found at 30℃, with the fourth instar indicating reduced developmental time as temperature departs from the optimal 28°C (Casagrande et al., 1987). Although temperature plays a vital role in the development rate, it is clear that ontogenic shifts in thermal performance also

apply. Despite Martinez et al. (2017) study associating reduced mitochondrial efficiency above supraoptimal temperatures with reduced growth, the mismatch between growth and metabolism remains complex and unexplored for the *L. dispar* and ectotherms at large.

Linkages between sub cellular energy-generating process and upper, whole organism performance have been a focus of research in the past decades, particularly because of the recognition of how mitochondrial ATP production can be influenced by both organismal and environmental factors. From those externa factors, the role of environmental temperature on bioenergetic pathways is within the most widely studied effectors (Chung & Schulte, 2020; Guderley & St-Pierre, 2002). Multiple holometabolus insect study systems have been employed to describe the reaction norm between temperature and their physiological processes, all to show an exquisite suite of variations and remarkable traits that are, more often than not, species specific (Angilletta et al., 2004; Kingsolver & Woods, 1997; Knapp et al., 1986; Knapp & Casey, 1986; Sears et al., 2012; Thompson et al., 2017). Regardless, the underlying mechanisms of cellular energy transduction and their thermal performance seem to reveal interesting similarities among species.

This study aimed to address the knowledge gap between the individual variations observed in previous studies, and the underlying mechanism responsible for energy production in the cell. Based on our results, and integrating those to existing data from previous studies, we found evidence to support the role of METE in whole-organismal traits. Metabolic and developmental stasis correlates with significant reductions in RCR at supraoptimal temperatures for 4^{th} instar *L. dispar,* which suggest a causative relation

between METE and upper level, energy dependent processes. This also has been demonstrated in *Manduca sexta* (Martinez et. al., 2017), adding to the current body of literature attempting to describe the subcellular mechanisms responsible of whole organism fitness in ectotherms.

The present study aimed to characterize the METE as a function of temperature in *L. dispar*, and to link this important efficiency index to whole-organism processes for the species. In Figure 4, linkages between METE and whole-organism performance are shown, where thermoneutral metabolic rate and developmental time stasis correlates with significant reductions in RCR. In a similar study carried by Martinez et al (2017) on *Manduca sexta*, METE paralleled a growth curve replotted from a different study (Kingsolver & Woods, 1997), for the same species and the same larval stage. By linking our findings to other available studies on *L. dispar*, an integrative analysis can be made, particularly when it concerns to test the hypothesis pointing to the role of METE in shaping whole organismal performance (Figure 5). For example, Lindroth and colleagues (Lindroth et al., 1997) found that high temperature treatments did not affect survival of individuals, but did decrease the long-term developmental rate of *L. dispar*. In another study of the *L. dispar*, Casey and Knapp described thermal ecology of the species, and found metabolic rates to be thermally insensitive between 25°C and 35°C, in addition to displaying thermoconforming behavior in the wild (Casey & Knapp, 1987). This thermoneutral zone was also found for the population surveyed in our study, as shown in the Figure 4 (Agosta and Martinez, unpublished data). Conversely, they found thermoregulatory behavior on a tent caterpillar species evaluated in the same study, which suggest a high level of physiological variation among co-occurring species. Other studies carried in *Manduca sexta* larvae suggest that growth also follows the same

pattern of insensitivity within the same temperature range (Reynolds et al., 1985). While there are obvious advantages to thermally independent physiological processes, the causes of these observations are unclear. Further work on this area would be highly insightful, as apparent patterns of thermal insensitivity and often a result of a fast-adapting biochemical machinery.

It is clear that a reduction in METE aligns with the range of temperature where thermal insensitivity is described (see Figure 4). Consequently, it is plausible that reductions in developmental performance (both time and rate) can be attributed to changes in METE with temperature. Both metabolism and development are energetically expensive, and as such influenced by the efficiency of METE (Figure 5). Moreover, the lack of variation in RCR between acclimation groups in this study suggests that the bioenergetics machinery of *L. dispar* mitochondria adapts rapidly under short-term acclimation (e.g. via regulatory processes controlling the ETS electron carrying capacity), and that adaptive changes in this fast-growing stage are beneficial to sustain energy-dependent processes at rapidly changing environmental temperatures.

Previous studies in *L.dispar* from the Northeast coast of the US suggest no benefit of thermoregulation on metabolic activity (Casey & Knapp, 1987). Diurnal temperature fluctuations fall within a thermoneutral region, which does not result in a net increment of metabolic activity. Although the present study is the first instance of describing compensatory mechanisms on insects resulting in constant LEAK and OXPHOS with acclimation, mitochondrial coupling did show a decrement in efficiency over the thermoneutral range of assay temperatures. Thermally insensitive metabolic demands operating under a supraoptimal METE results in reduced efficiency of ATP

production, and may contribute to the reduction in development rate observed by Logan and colleagues (Logan et al., 1991). Therefore, this study adds evidence to a mechanistic explanation describing the role of mitochondria function in shaping the thermal sensitivity of energy dependent processes of ectotherms.

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Figure Legends:

Figure 1: Oxygen flux associated with proton conductance (LEAK) of *L. dispar* gut mitochondria. Additive LEAK rates observed through the sequential addition of complex I, PDH, GPDH and II activating substrates (subpanels A-D). Significant differences are shown (Two-way ANOVA, Holm-Sidak pairwise comparisons; mean ±SEM; n = 4). Black numbers represent significant difference in the mean within the 30°C acclimation group, while lowercase letters represent significant difference in the mean within the 20°C acclimation group, among assay temperature. Colored numbers represent the Q_{10} values between data points. Asterisks (*) denotes a significant difference between acclimation groups at a given assay temperature.

Figure 2: ADP-induced oxygen flux (OXPHOS) as a function of acclimation temperature and assay temperature for *L. dispar* gut mitochondria. OXPHOS rates observed in the presence of complex I, PDH, GPDH and II activating substrates. Significant differences are shown (Two-way ANOVA, Holm-Sidak pairwise comparisons; mean ±SEM; n = 4). Numbers represent significant difference in the mean within the 30°C acclimation group, while lowercase letters represent significant difference in the mean within the 20°C acclimation group, among assay temperature. Colored numbers represent the Q_{10} values between data points.

Figure 3: Thermal performance curve of respiratory control ratios (RCR: OXPHOS/LEAK) of 4th instar *L. dispar* gut mitochondria after 20 and 30°C acclimation. Significant differences are shown (Two-way ANOVA, Holm-Sidak pairwise comparisons; mean ±SEM; n = 4). Numbers represent significant difference in the mean within the 30°C acclimation group, while lowercase letters represent significant difference in the mean within the 20°C acclimation group, among assay temperature.

Figure 4: Integration of mitochondrial thermal performance (RCR) obtained in this study, with whole organismal thermal performance traits. A) RCR plotted with metabolic rate of 4th instar *L. dispar* (Metabolism; Martinez, unpublished data) and cumulative developmental time (Casagrande et.al, 1987) as a function of temperature. B) RCR and metabolic rate graphed along a developmental rate model published by Logan et.al. (1991).

Figure 5: Conceptualized Mitochondrial Energy Transduction Hypothesis (METEH) initially proposed by Martinez et al (2017), and modified to include the findings of this study.

Figure 1

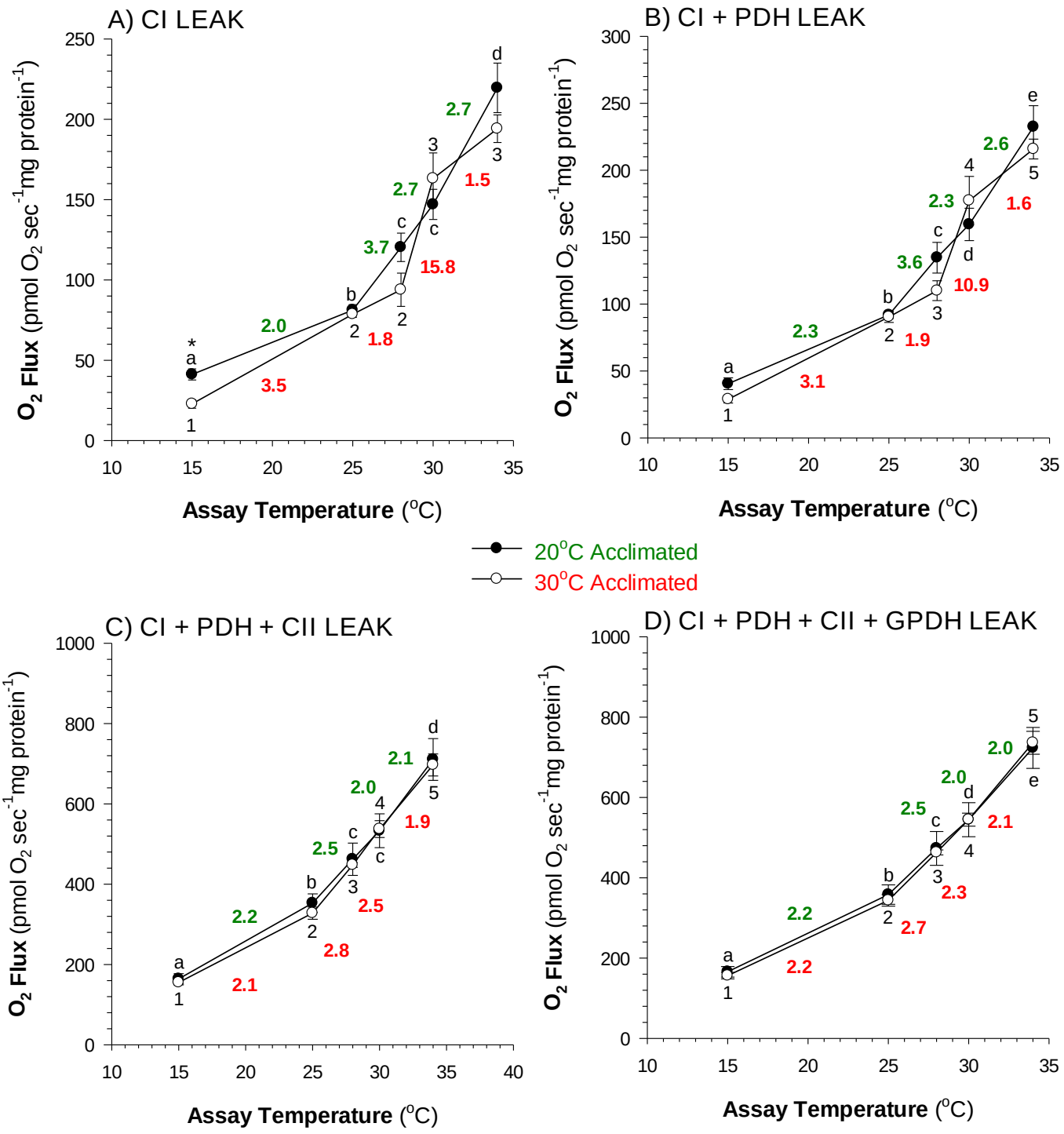

A) CI LEAK
B) CI + PDH LEAK
C) CI + PDH + CII LEAK
D) CI + PDH + CII + GPDH LEAK
O2 Flux (pmol O2 sec-1mg protein-1)
Assay Temperature (°C)
20°C Acclimated
30°C Acclimated

Figure 2

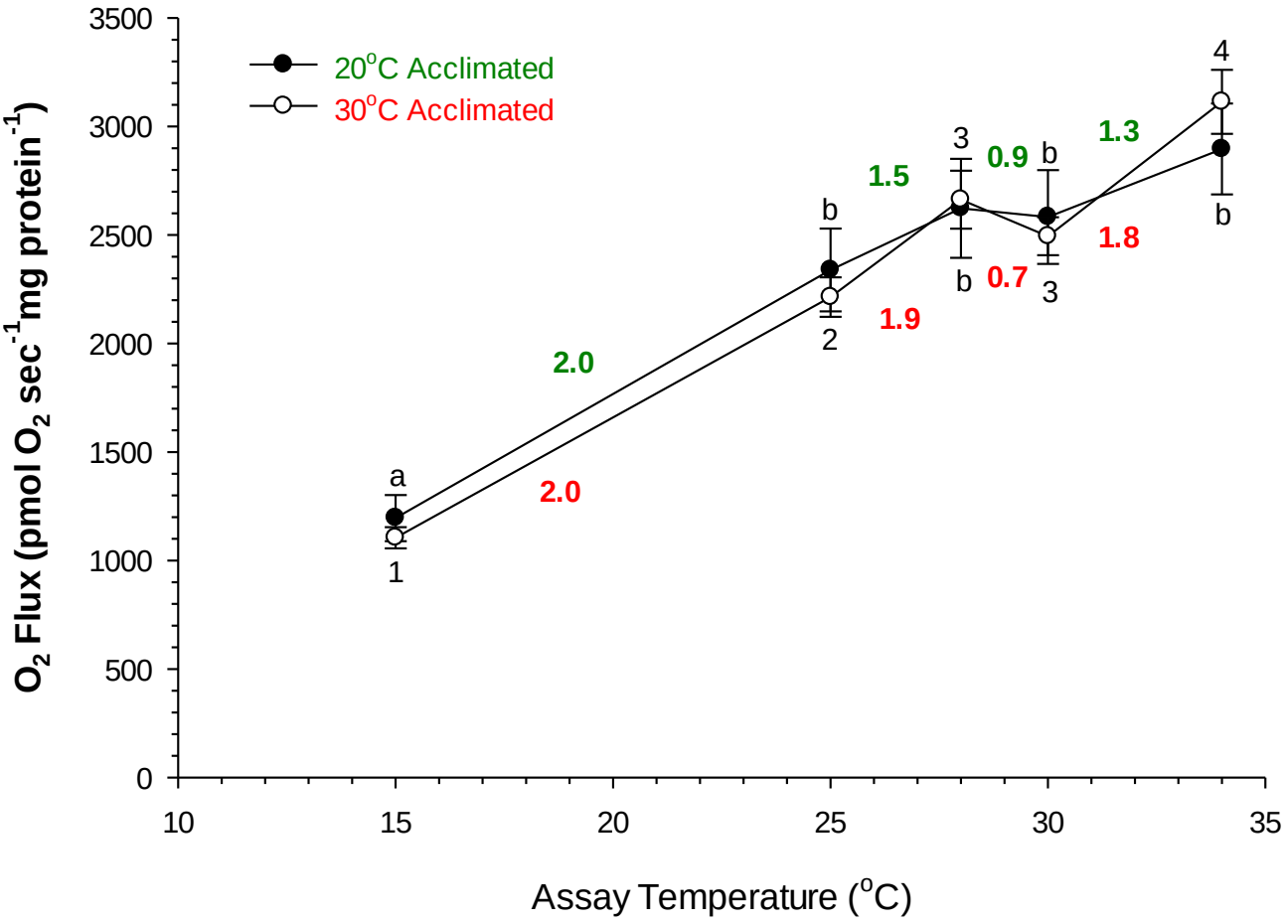

20°C Acclimated
30°C Acclimated
O2 Flux (pmol O2 sec-1mg protein-1)
Assay Temperature (°C)
3500
3000
2500
2000
1500
1000
500
0
10
15
20
25
30
35
a
b
b
b
b
1
2
3
3
4
2.0
2.0
1.5
1.9
0.9
0.7
1.3
1.8

Figure 3

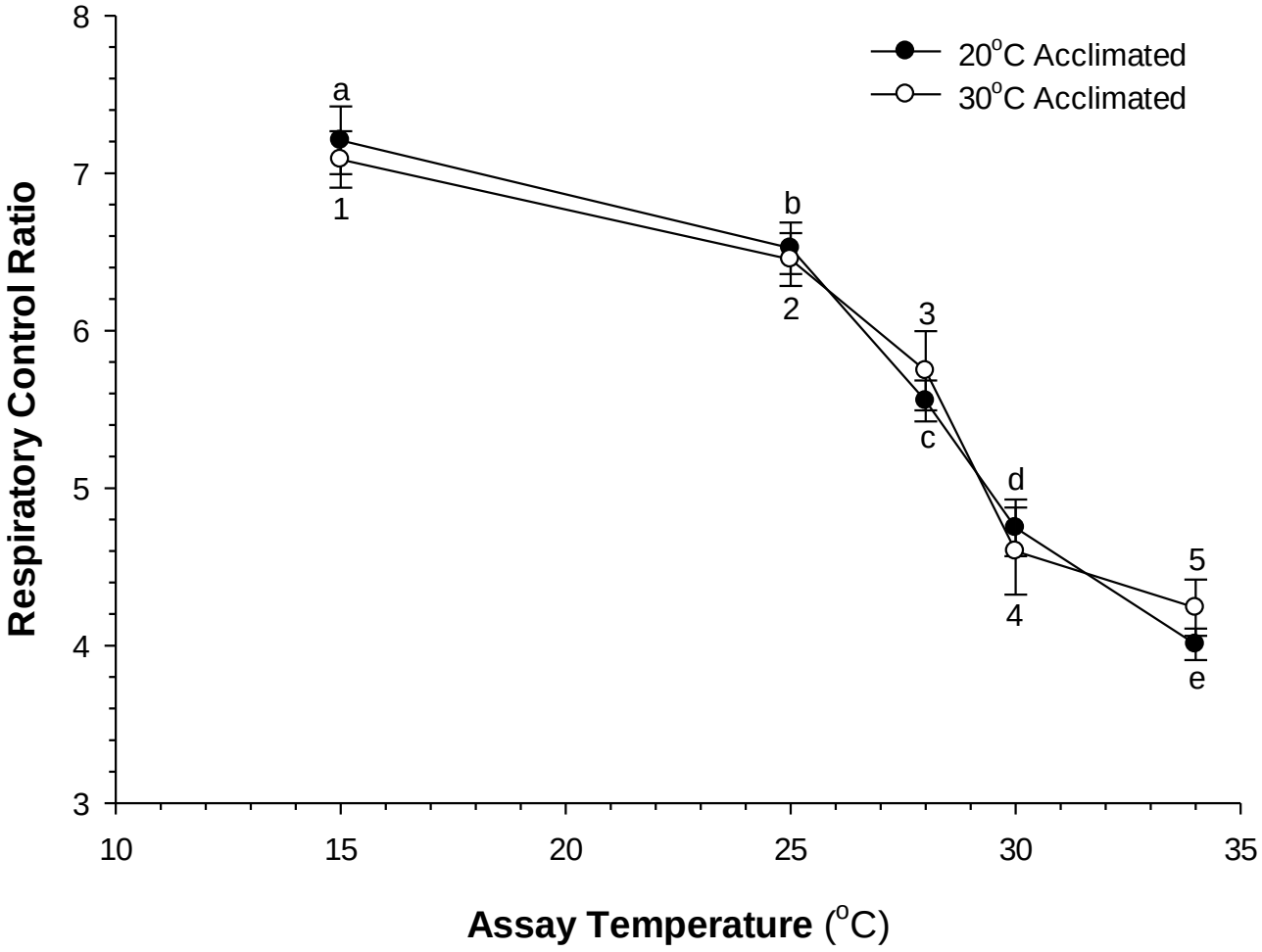
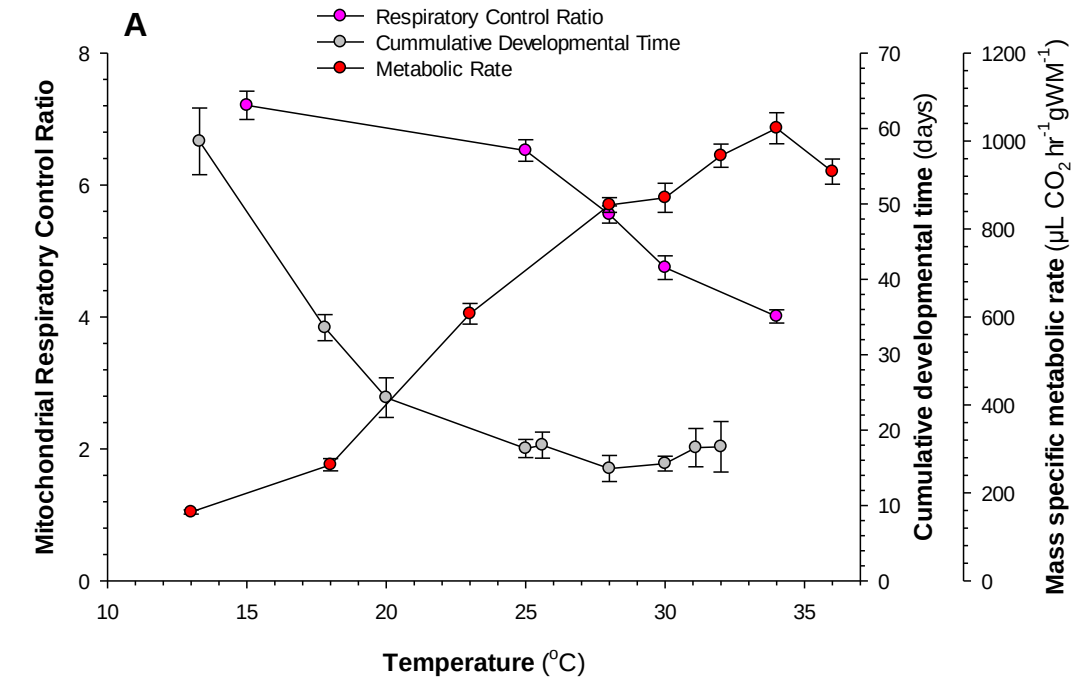

20°C Acclimated
30°C Acclimated
Respiratory Control Ratio
Assay Temperature (°C)
8
7
6
5
4
3
10
15
20
25
30
35
a
b
c
d
e
1
2
3
4
5

Figure 4


A
Respiratory Control Ratio
Cummulative Developmental Time
Metabolic Rate
Mitochondrial Respiratory Control Ratio
Cumulative developmental time (days)
Mass specific metabolic rate (µL CO2 hr-1gWM-1)
Temperature (°C)

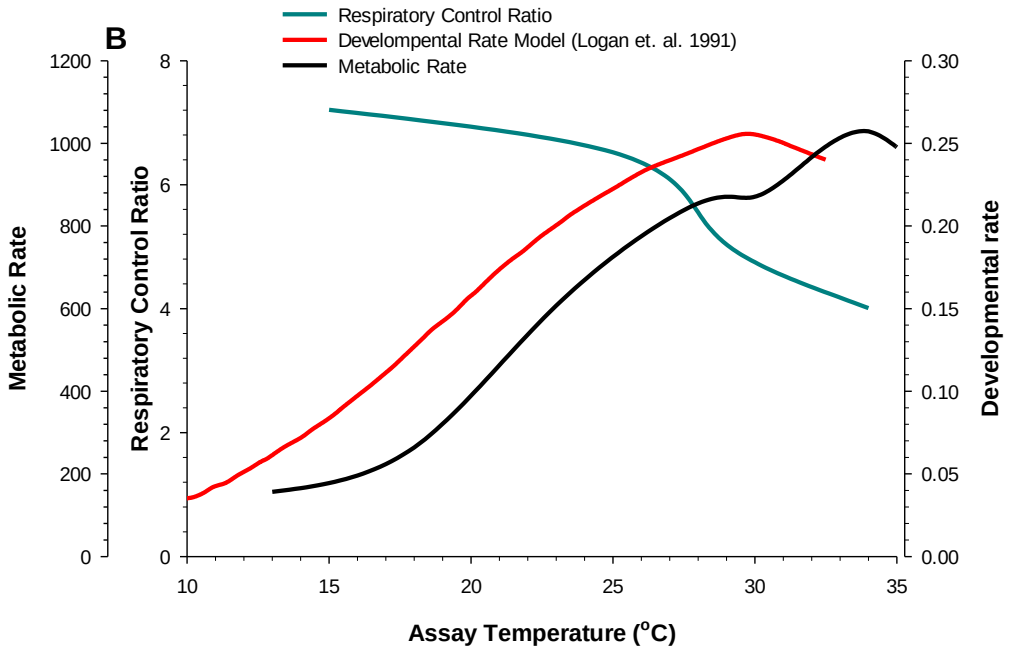
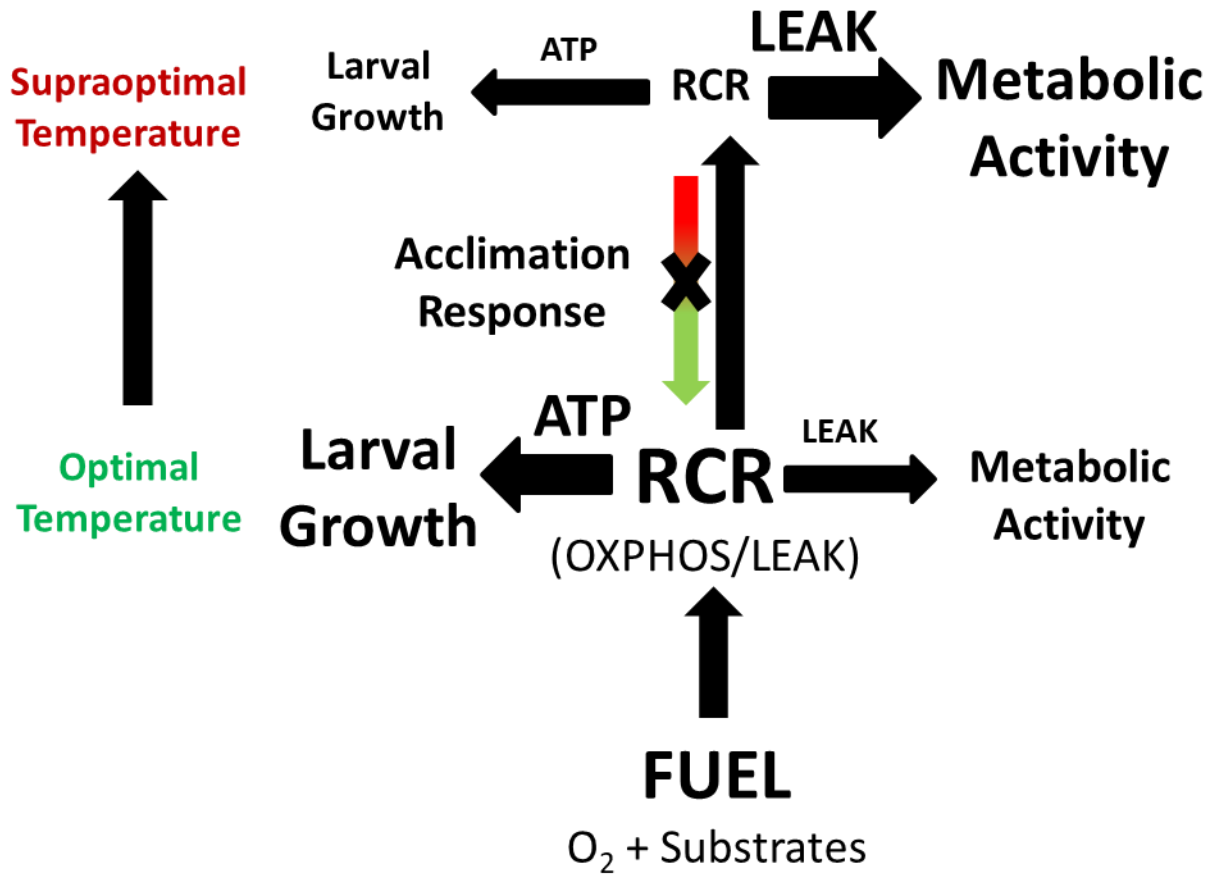

B
Respiratory Control Ratio
Develompental Rate Model (Logan et. al. 1991)
Metabolic Rate
Metabolic Rate
Respiratory Control Ratio
Developmental rate
Assay Temperature (°C)

Figure 5


Supraoptimal Temperature
Larval Growth
ATP
LEAK
RCR
Metabolic Activity
Acclimation Response
Optimal Temperature
ATP
LEAK
Larval Growth
RCR
Metabolic Activity
(OXPHOS/LEAK)
FUEL
O_2 + Substrates

Tables:

Table 1: Two-way ANOVA statistical analysis of ADP induced respiration (OXPHOS) and proton conductance (LEAK) under various substrates.

Complex I LEAK

Source of Variation	DF	SS	MS	F	P
ACCLIMATION TEMPERATURE	1	2186.537	2186.537	4.550	**0.039**
ASSAY TEMP	4	172798.009	43199.502	89.900	**<0.001**
ACCLIMATION T x ASSAY TEMP	4	4054.966	1013.741	2.110	0.098
Residual	40	19221.104	480.528		
Total	49	205692.428	4197.805		

Complex I + Proline dehydrogenase LEAK

Source of Variation	DF	SS	MS	F	P
ACCLIMATION TEMPERATURE	1	1239.790	1239.790	2.272	0.140
ASSAY TEMP	4	196853.939	49213.485	90.204	**<0.001**
ACCLIMATION T x ASSAY TEMP	4	3521.369	880.342	1.614	0.190
Residual	40	21823.233	545.581		
Total	49	233546.026	4766.245		

Complex I + Proline dehydrogenase + Complex II LEAK

Source of Variation	DF	SS	MS	F	P
ACCLIMATION TEMPERATURE	1	6806.855	6806.855	1.503	0.227
ASSAY TEMP	4	1521674.675	380418.669	84.012	**<0.001**
ACCLIMATION T x ASSAY TEMP	4	10406.034	2601.509	0.575	0.683
Residual	40	181125.695	4528.142		
Total	49	1790671.967	36544.326		

Complex I + Proline dehydrogenase + Complex II + Glycerophosphate dehydrogenase LEAK

Source of Variation	DF	SS	MS	F	P
ACCLIMATION TEMPERATURE	1	4328.017	4328.017	0.835	0.366
ASSAY TEMP	4	1620841.239	405210.310	78.207	**<0.001**
ACCLIMATION T x ASSAY TEMP	4	6741.025	1685.256	0.325	0.859
Residual	40	207249.295	5181.232		
Total	49	1936301.456	39516.356		

Complex I + Proline dehydrogenase + Complex II + Glycerophosphate dehydrogenase OXPHOS

Source of Variation	DF	SS	MS	F	P
ACCLIMATION TEMPERATURE	1	1004.795	1004.795	0.00969	0.922
ASSAY TEMP	4	19037241.864	4759310.466	45.910	**<0.001**
ACCLIMATION T x ASSAY TEMP	4	183785.879	45946.470	0.443	0.777
Residual	36	3732018.621	103667.184		
Total	45	24272531.857	539389.597		