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Sensitivity of cellular oxidative damage to biosynthetic rate and metabolic rate

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Department:

SENSITIVITY OF CELLULAR OXIDATIVE DAMAGE TO BIOSYNTHETIC RATE AND METABOLIC RATE

by

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ABSTRACT

The relationship between energy expenditure and longevity is a long standing question in aging studies. The empirical results have contradictory effects on the existing theories. A theoretical model and an experimental test of it were presented, revealing the detailed tradeoffs between metabolic rate, biosynthetic rate and cellular damage level. The dissipative mechanisms of oxidative metabolism cause various forms of cellular damages. To counteract the accumulation of damage, organisms have evolved highly efficient maintenance mechanisms. If there is no other energy demand possess, then most of the cellular damage would be repaired, regardless of how metabolic rate varies. However, the repairing mechanisms cost energy. When the energy that could be allocated to repairing is otherwise channeled to biosynthesis during growth, the damage is inevitably accumulated, despite the high repairing efficiency and the variation in biosynthetic rate, and will have a significant impact on the cellular damage level. The model predicts that cellular damage is more sensitive to biosynthetic rate than metabolic rate. To test the prediction, a broad variation in the metabolic and biosynthetic rate was induced in 5^{th} instar hornworms, and assayed the corresponding lipid peroxidation as an indication of cellular damage. The results showed that the metabolic rate had a negligible effect on lipid peroxidation, and the biosynthetic rate had increased the peroxidation. Our study answers a long-standing question regarding the oxidative stress theory of aging: that the treatments that vary the metabolic rate but fail to change the biosynthetic rate have no effects on cellular damage or lifespan, whereas the treatments that change the biosynthetic rate but keep the metabolic rate unchanged will vary animal life span.

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1. GENERAL INTRODUCTION

The relationship between metabolic rate (MR) and longevity is a long standing theme in the biology of aging. The contrasting ideas about how the energy metabolism is associated with longevity has made this relationship more controversial to understand (Speakman, Selman, McLaren, & Harper, 2002). The existing theories have supporting and contradicting empirical evidence.

The oldest theory is the rate of living theory (RLT), which explains that the rate of energy expenditure or the MR is negatively correlated with longevity (Pearl, 1928). The two primary determinants of MR are individual body mass and body temperature (Pearl, 1928; Rubner, 1908). Empirical data supports RLT, even after the confounding effect of body mass is removed (Speakman et al., 2002). Within individual taxonomic groups, the ones with a higher mass specific MR have a shorter lifespan (McCoy & Gillooly, 2008). Under experimental conditions, lowering the MR by decreasing body temperature has been shown to extend the lifespan in ectotherms (Klass, 1977; Partridge, Piper, & Mair, 2005; Van Voorhies & Ward, 1999) and endotherms (Conti et al., 2006).

However, there are several problems with the RLT. The negative correlation between MR and longevity is not present across taxon. One example is birds live much longer than mammals although they have a higher MR compared to mammals with the same body mass (A. Hulbert, R. Pamplona, R. Buffenstein, & W. Buttemer, 2007). Diet restriction (DR) and genetic interventions for longevity do not alter the mass specific metabolic rate (C. Hou, 2013; Westbrook, Bonkowski, Strader, & Bartke, 2009). Experimental manipulations that increase metabolic rate do not shorten lifespan in mice

(Vaanholt, Daan, Schubert, & Visser, 2009) or in voles (Selman, McLaren, Collins, Duthie, & Speakman, 2008b). These results suggest that the relationship between energy expenditure and longevity is non-monotonic.

Another widely described theory for aging is the oxidative stress theory (OST). This explains that progressive declines in physiological functions are the result of the accumulation of oxidative damage caused by reactive oxygen species (ROS) (M. E. Harper, L. Bevilacqua, K. Hagopian, R. Weindruch, & J. Ramsey, 2004; Rajindar S Sohal, Robin J Mockett, & William C Orr, 2002). OST is supported by the extended life span in mice and flies with an overexpression of antioxidants (Muller, Lustgarten, Jang, Richardson, & Van Remmen, 2007). The level of oxidative damage to DNA, lipids, and protein increases with age in various tissues in animal models (Bokov, Chaudhuri, & Richardson, 2004). However, several challenges address OST. Genetic alteration in the antioxidant enzymes failed to affect longevity (Pérez et al., 2009; Van Raamsdonk & Hekimi, 2012) and endogenous ROS defense was blocked by antioxidants in diet (Ristow et al., 2009).

DR is a common interventions to increase the active and healthy life-span of many species (Mair & Dillin, 2008; E. J. Masoro, 2005). One of the earliest explained mechanisms of DR induced longevity is that under poor nutrient conditions, the organism reallocates resources from reproduction to health maintenance (Holliday, 1989). DR retards the accumulation of oxidative damage in DNA (Sohal, Agarwal, Candas, Forster, & Lal, 1994), peroxidation of lipids (Matsuo, Gomi, Kuramoto, & Sagai, 1993), and accumulation of oxidized proteins (Dubey, Forster, Lal, & Sohal, 1996). This suggests that the accumulation of oxidative damage is retarded by DR. DR associated longevity

can be explained from an energetic viewpoint. Suppressing growth by DR, channels the energy to somatic maintenance, which is an energy tradeoff between growth and longevity. Here, the somatic maintenance repairs oxidative damage in protein, lipids, and DNA (C. Hou, 2013). Overall, many discrepancies exist between theories and experimental results for energy expenditure and longevity.

We developed a theoretical model based on the first principle of energy tradeoffs. This model predicts that, when repair efficiency is high, the damage level caused by the metabolic rate is negligible compared to damage caused by the biosynthetic rate. The framework of the theory is illustrated in Figure 1.1.

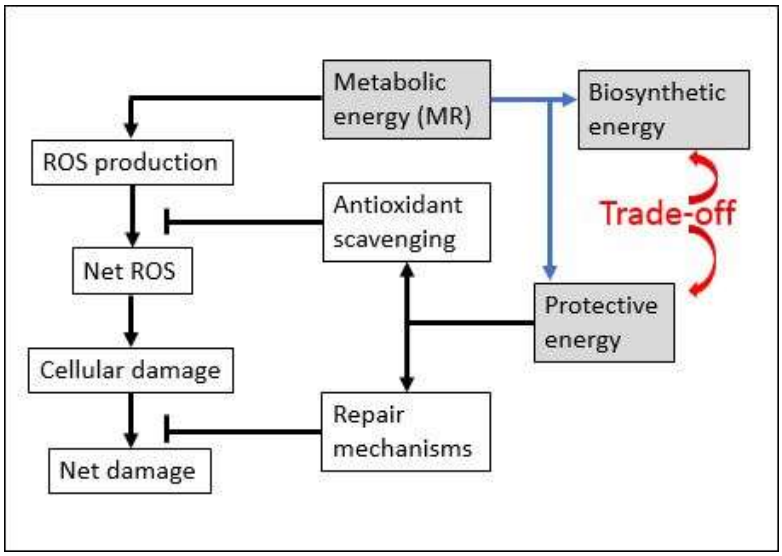


Figure 1.1. The framework of the theory. The metabolic energy (MR) is partitioned between biosynthesis and protective energy. MR is proportional to ROS production. The protective energy is allocated to antioxidant scavenging and repair mechanisms.

The oxidative damage starts with overall energy expenditure, and it is proportional to the ROS production (Hou & Amunugama, 2015). The net oxidative damage is responsible for longevity. Two factors can control net oxidative damage; antioxidant scavenging and repair mechanisms. ROS scavenging is carried out by anti-oxidative enzymes, such as superoxide dismutase (SOD), peroxidredoxin (Prx), and glutathione peroxidase (GP), and by non-enzymatic antioxidants such as vitamins (R. S. Balaban, S. Nemoto, & T. Finkel, 2005). Only the net ROS can damage DNA, proteins, and lipids. These damaged molecules are repaired by different mechanisms such as removal of peroxidized acyl chains from phospholipids (A. Hulbert et al., 2007), DNA base excision repair or mismatch repair (Madhusudan & Middleton, 2005), and methionine sulfoxide repair (Tarrago & Gladyshev, 2012). The net damage can be altered by changing these two factors. Therefore, the correlation between net damage and metabolic energy is nonlinear. Further, the protective mechanisms of antioxidant scavenging and repair mechanism need energy, namely, protective energy. The total metabolic energy is partitioned between biosynthetic energy and protective energy. When growth is retarded by DR the extra energy is channeled to protective energy, resulting in longevity (Hou & Amunugama, 2015).

The goal of this thesis is to unravel the relationship between biosynthetic rate, metabolic rate, cellular oxidative damage and health maintenance. This thesis consists of two related projects to investigate the sensitivity of the oxidative damage to biosynthetic and metabolic rates. *Manduca sexta* larvae (horn worm) is used in the study because under laboratory conditions, the 1st instar larvae grows from 1mg to 13g at the end of 5th instar within 20 days, making it an ideal model. The body temperature can be changed by

altering the external environmental temperature. Thus, metabolic rate and growth rate can be disentangled in ectotherms.

The damage accumulated during the experiment period was estimated by measuring the lipid peroxidation level using plasma malondialdehyde (MDA). MDA is one of the main secondary products of poly unsaturated lipid peroxidation. Once it is formed, it can be metabolized by enzymes or react with other biomolecules such as, DNA, and proteins to form adducts. A portion of MDA is oxidized to CO_2 and H_2O and excreted in the urine. (Ayala, Muñoz, & Argüelles, 2014). It has been widely used as a biochemical marker of aging (Engelfriet, Jansen, Picavet, & Dollé, 2013), and the level of MDA increases with age (Massudi et al., 2012). One can explain this MDA accumulation with age as the protective mechanisms of antioxidant scavenging and the repair mechanisms becoming less efficient with age.

The first experiment was conducted to investigate how the energy tradeoffs between metabolism and growth affect health maintenance. During growth, a fraction of metabolic energy is allocated to biosynthesis, while the rest is used for health maintenance. Biosynthesis also induces damage accumulation. Although extensive literature exists on the collective effects of metabolism and biosynthesis on damage accumulation, studies on disentangled effects are rare. Based on our model, we predicted that if the repair efficiency were high, then the changes in damage level caused by the changes in metabolic rate would be negligible, and the damage level would be more sensitive to the changes in the biosynthetic rate. We tested the model on 5^{th} instar hornworms by manipulating the biosynthetic rate and metabolic rate by rearing them in different food treatments. The results strongly supported the predictions of the model.

In the second project, we experimentally manipulated the synthetic energy and metabolic energy. We used different food treatments, temperature conditions, and growth retardation (by rapamycin which targets the TOR signaling pathway). Compared to the observational study, the manipulative experiments offered a better understanding of, and more direct evidence for, the separate effects of biosynthesis and metabolism on cellular damage.

PAPER

I. CELLULAR OXIDATIVE DAMAGE IS MORE SENSITIVE TO VARIATION IN BIOSYNTHETIC RATE THAN IN METABOLIC RATE: A TEST OF THE THEORY ON HORNWORMS

ABSTRACT

A theoretical model was developed from an energetic viewpoint to unravel the entangled effects of metabolic and biosynthetic rates on oxidative cellular damage accumulation during animal's growth. The model was tested by manipulative experiments in hornworms. The theoretical consideration suggests that most of the cellular damages caused by the oxidative metabolism could be repaired by the efficient maintenance mechanisms, if the energy required by the repair is unlimited. However, during growth, a considerable amount of energy is allocated to biosynthesis, which entails tradeoffs with the requirements of repair. The model predicts that cellular damage is more sensitive to the biosynthetic rate than the metabolic rate. To test the prediction, we induced broad variations in metabolic and biosynthetic rates in hornworms and assayed the lipid peroxidation. We found that the increase in the peroxidation was mainly caused by the increase in biosynthetic rate, and the variations in metabolic rate had a negligible effect. The oxidative stress theory of aging suggests that high metabolism leads to high cellular damage and short lifespan. However, some empirical studies have shown that varying biosynthetic rate, rather than metabolic rate, changes the animal's lifespan. The conflicts between the empirical evidence and theory are reconciled by this study.

1. INTRODUCTION

The deleterious products of oxidative metabolism, such as reactive oxygen species, cause various forms of cellular damages, which in turn undermine the organism's health maintenance and longevity (R. Balaban, S. Nemoto, & T. Finkel, 2005; G. Barja, 2004). To counteract the accumulation of damage, organisms have evolved highly efficient repair mechanisms, such as oxidant scavenging and damage repair (Beckman & Ames, 1998; Monaghan, Metcalfe, & Torres, 2009). These repair mechanisms require energy and resources. If the resources and energy that could be allocated to repair are otherwise channeled to other biological processes, then damage will inevitably accumulate despite the high repair efficiency (Monaghan et al., 2009).

Biosynthesis during growth (one of the most intensively investigated biological processes that trades off with repair) is positively correlated with the oxidative damage level and proxies of it, such as declined performance and shortened lifespan at the whole organismal level (Chen Hou, 2013; Mangel & Munch, 2005; Mangel & Stamps, 2001; Miller, Harper, Galecki, & Burke, 2002) and the molecular and cellular levels (Bartke, 2005; Rollo, Carlson, & Sawada, 1996). Rapid growth leads to higher phospholipid peroxidation (Nussey, Pemberton, Pilkington, & Blount, 2009), protein carbonyl content (Forster, Sohal, & Sohal, 2000), decreased antioxidant defenses in red blood cells (Alonso-Alvarez, Bertrand, Faivre, & Sorci, 2007), elevated free radical processes (Rollo et al., 1996), declined locomotion ability (Mangel & Stamps, 2001), impaired immune function (De Block & Stoks, 2008), higher mortality rate, and shortened lifespan (Bartke, 2005; B.J. Merry, 1995; Miller et al., 2002). A special type of rapid growth: catch up

growth, referring to infants with low birth weight reaching to or exceeding the normal body weight later in life, increases the risk of adult-onset metabolic syndromes and short lifespan in humans and laboratory rodents (Metcalfe & Monaghan, 2001). In contrast, suppressed growth, usually induced by food restriction (E. Masoro, 2005; B. J. Merry, 2002; Weindruch & Walford, 1988) or genetic modification of growth hormones (Bartke, 2005; Brown-Borg, 2003), has been long known to keep animals in a relatively youthful and healthy state, and greatly extends the lifespan in a broad assortment of species, indicating the up-regulations of somatic damage repair in these animals.

Attempting to interpret the positive correlation between biosynthesis and cellular damage, many researchers have argued that an increased biosynthetic rate causes a raised metabolic rate, which, as a primary source of free radicals, leads to increased cellular damage (Monaghan et al., 2009; Nussey et al., 2009). However, although biosynthesis is fueled by metabolism, the relationship between them is not simply proportional. When one of them increases, the other may increase (Ricklefs, 2003; West, Brown, & Enquist, 2001), decrease (M. Hayes et al., 2015; Steyermark, 2002), or keep roughly the same (Álvarez & Nicieza, 2005; McCarter & Palmer, 1992). The complex relationships between them make their effects on cellular damage difficult to isolate. Rates of metabolism and biosynthesis may have different degrees of impacts on cellular damage, i.e., the same degree of variations in these rates may lead to different relative changes in damage. However, in most studies, the observed changes in cellular damage reflect the combined influences of changes in both metabolic and biosynthetic rates. When these two rates vary independently, or even in the opposite direction, the separate effects of each on cellular damage are obscured.

The goal of this paper is to unravel the effects of biosynthetic and metabolic rate on cellular damage accumulation. We first developed a simple theoretical model based on the first principle of energy tradeoffs and real physiological parameters. The model predicts that, if the repair efficiency is high, then the changes in damage level caused by the changes in metabolic rate will be negligible, and the damage level will be more sensitive to the changes in biosynthetic rate. The model was then tested by experiments on 5^{th} instar tobacco hornworms (the last instar of *Manduca sexta* larvae). We measured lipid peroxidation as an index of cellular damage accumulation in larvae with different rates of growth and metabolism.

2. THE THEORETICAL MODEL

Recently, we developed a theoretical model grounded on empirical data to understand how animals alter their energy budgets for damage repair, biosynthesis, and energy storage in the face of environmental changes and to understand how the alteration in the energy budget affects cellular damage accumulation (Chen Hou, 2013; Hou, 2014; Hou, Bolt, & Bergman, 2011; Hou et al., 2008). Some of the quantitative predictions on the relationship between growth suppression and lifespan extension are strongly supported by data collected from wild animals across a broad range of species and more than 200 empirical studies on small laboratory rodents (Chen Hou, 2013; Hou et al., 2011).

In this paper, we extend the model and make predictions on the relationship between cellular damage, metabolic rate, and biosynthetic rate. The detailed assumptions and derivation of the equations of the model are available in the supplementary material and references (Chen Hou, 2013; Hou, 2014). Here, we only introduce the main results. The model estimated the accumulation of cellular damage caused by the oxidative metabolism. Based on the first principles of energy conservation and biochemistry, the model estimates net damage as the difference between damage production and the damage repair effort. The former is associated with metabolic energy expenditure, and the latter depends on energy that is available for repair, which entails tradeoffs with the energy required for biosynthesis during growth. Thus, high energy expenditure leads to high damage, and high growth rate, which drains energy from repair and also leads to high damage. The main equation of the model reads:

$$D(t) = (1-\varepsilon) \times ME + \varepsilon E_{\mathrm{m}} \Delta m \big|_0^t = (1-\varepsilon) \times ME + \varepsilon \times SE \quad (1)$$

where *ME* is the metabolic energy spent during growth from age 0 to age *t* (in joules); *Δm* is the increase of body mass during growth, and E_{m} is the energy required to synthesize one unit of biomass, so $SE = E_{\mathrm{m}} \Delta m$ is the synthetic energy spent during growth (also in joules). The parameter ε is the effective repair efficiency. The repair efficiency is expressed as $\varepsilon = \eta/(f\delta)$, where δ is a constant, indicating the amount of damage produced by one unit of metabolic energy expenditure; η is another constant, indicating the amount of damage repaired by one unit of energy spent on repairing, and *f* indicates the activity (exercise) level of the animals. The detailed derivation of Eq. 1 and estimation of ε are available in the supplementary material.

Equation 1 decomposes the net damage accumulation in two terms, $D_B = (1-\varepsilon) \times ME$ and $D_{\mathrm{syn}} = \varepsilon \times SE$, estimating the effects of metabolism and biosynthesis on damage accumulation separately. Both terms are proportional to energy factors (*ME* and *SE*) with coefficients $1-\varepsilon$ and ε respectively. The sensitivities of damage to the changes in metabolic and biosynthetic rate depend on the coefficients of these two terms, $1-\varepsilon$ and ε. Based on the first principle of biochemistry and the fitting of empirical data, the repair efficiency (ε) has been estimated to be in the neighborhood of 0.99 ((Chen Hou, 2013; Hou et al., 2011) and the supplementary material). For such a high efficiency, the metabolic term in Eq. 1, $(1-\varepsilon) \times ME$, is close to zero, regardless of how metabolic energy (*ME*) changes. The major contribution to the net damage, therefore, comes from the biosynthetic term, $\varepsilon \times SE$. This means that the damage accumulation is more sensitive to the biosynthetic term (*SE*) than to the metabolic term (*ME*). This theoretical result can

also be understood as evidence that oxidative metabolism causes cellular damage. Without growth (biosynthesis), the highly efficient repair mechanism repairs most of the damage so that the damage accumulates at a low rate without growth, i.e., $(1-\varepsilon)\times ME$. However, during growth, biosynthesis costs a considerable amount of metabolic energy that could be spent on repair. Therefore, the contribution of biosynthesis to net damage is positive, i.e., $\varepsilon\times SE$, and damage accumulates quickly in the presence of growth.

To test this theoretical prediction, we induced broad variations in metabolic energy (*ME*) and synthetic energy (*SE*) among individual 5th instar hornworms by varying food supply levels in two separate experiments, namely 4-day food treatment and 6-day treatment. The details of the treatments are described in the method section and the supplementary materials.

We then assayed the corresponding lipid peroxidation levels in individual hornworms using plasma malondialdehyde (MDA) as a surrogate. Lipids are one of the major targets of oxidative stress. Lipid peroxidation generates many secondary decomposition products of poly unsaturated fatty acids. Known as a universal biomarker of lipid peroxidation, MDA is one of the secondary decomposition products, and is widely used to assess oxidative damage levels (Hall, Blount, Forbes, & Royle, 2010; Monaghan et al., 2009; Nussey et al., 2009). We assumed that the level of MDA is proportional to the total cellular damage (variable, *D*, in Eq. 1) with a factor of *g*, as $MDA = g\times D$. Therefore, Eq. 1 becomes:

$$MDA = g\times(1-\varepsilon)\times ME + g\times\varepsilon\times SE \qquad (2)$$

We need to emphasize that damage accumulates over the entire period of growth, so a considerable fraction of the MDA assayed in this study was accumulated during the

first four instars of the larval lives, whereas the manipulations of growth and metabolic rate only started when the larvae entered the 5th instar. Thus, to test how variations in these rates influenced the damage accumulation, we removed the effects of un-manipulated *ME* and *SE* in the first four instars from the assayed MDA level. Previous studies, as well as this study, showed that both *ME* and *SE* (the metabolic and synthetic energy) spent during a period of growth, are linearly proportional to the body mass at the end of this period (see Figure.1A, 1B, and (West et al., 2001)). Therefore, the effects of *ME* and *SE* can be removed during the first four instars by removing the effect of the body mass at the end of the 4th instar from the assayed MDA. We linearly regressed the MDA level on this body mass. The residual of MDA after the removal of this mass was then considered to be the damage caused by *SE* and *ME* during the 5th instar period—the experimental period. The MDA level during the 5th instar, *SE*, and *ME* were all linearly correlated to the final body mass at the end of the experimental period, *M* (Figure 1). This means that the final body mass has a confounding effect when performing a linear regression of the MDA level during the 5th instar on *ME* and *SE*. We investigated the confounding effect of final mass on these variables in two ways. First, we controlled for the final mass by including it in a multiple linear regression analysis with *SE* and *ME* to predict the *MDA* level at the 5th instar (details are described in the methods section). We also ran an alternative model by removing the confounding effect of final mass by performing separate linear regressions of *MDA*, *ME*, and *SE* on the final body mass and calculating the mass residuals in each of the three analyses. We then regressed the mass residual of MDA on the mass residuals of *ME* and *SE*, as:

$$\hat{MDA}_{\text{residual}} = \hat{\beta}_0 + \hat{\beta}_1 \times ME_{\text{residual}} + \hat{\beta}_2 \times SE_{\text{residual}} \quad (3)$$

where $\hat{\beta}_0$, $\hat{\beta}_1$, and $\hat{\beta}_2$ are estimated regression coefficients. Eq. 3 allows us to write the model in the form of Eq. 2 for comparison. We present results from both approaches, but used the model employed for regression Eq. 3 as the focus of our predictions.

Comparing the theoretical Eq. 2 and the regression Eq. 3, we made three predictions. First, the fitted regression coefficient of the metabolic term, $\hat{\beta}_1$, was smaller than that of the biosynthetic term, $\hat{\beta}_2$; second, the partial correlation between MDA level and the metabolic term (*ME*) was insignificant after accounting for *SE* (P-value > 0.05), whereas that between MDA and the synthetic term (*SE*) was significant after accounting for *ME* (P < 0.05); and third, and most importantly, the ratio of the coefficients, $\hat{\beta}_1$ and $\hat{\beta}_2$, gives $\hat{\beta}_1 / \hat{\beta}_2 = (1 - \varepsilon) / \varepsilon$. We predicted that the repairing efficiency (ε) estimated from this equation was in the neighborhood of 0.99, which is the value that was previously estimated from the biochemistry principles (Chen Hou, 2013; Hou et al., 2011) (details of estimating ε are given in the supplementary material).

3. METHODS

3.1. ANIMAL REAR AND FOOD SUPPLY LEVELS

We induced variations in metabolic energy (*ME*) and biosynthetic energy (*SE*) by varying food supply levels in 4-day food treatment and 6-day treatment, each with approximately 80 hornworms. The details of animal rear are available in the supplementary material. In short, on the first day, the 5th instar larvae were randomly separated and treated with four levels of food supply. The four cohorts were free-feeding (AL), short-term food restriction-A (SFR-A), short-term food restriction-B (SFR-B), and long-term food restriction (LFR). The length of free feeding and food restriction and the level of food restriction for each cohort are described in Table 1. All larvae were sacrificed on the sixth day and fourth day in the 6-day and 4-day experiments for MDA measurements. Sample size for 6-day experiment and 4-day experiment is 61 and 72 respectively.

3.2. SYNTHETIC ENERGY SPENT DURING THE EXPERIMENTAL PERIOD

The body mass of each larva in every cohort was measured approximately at the same time every day from the first day of the 5^{th} instar to the nearest 0.1 mg using a digital microbalance (Perkin-Elmer AD6). The energy spent on biosynthesis during the experiment, *SE*, in joules, was calculated as the increment of body mass from the 1^{st} day to the last day of the experiment, Δm, multiplied by the energy required to synthesis one unit of biomass, E_m , i.e., $SE = \Delta m \times E_m$. The value of E_m in the 5^{th} instar hornworm was taken to be 168 Joules/gram, which was estimated previously by Sears et al (Sears,

Kerkhoff, Messerman, & Itagaki, 2012) (1197 Joules/gram of dry mass, and dry/wet mass ratio was 14% throughout the 5th instar). Our independent assays used the method described in Peterson et al. (Peterson, Walton, & Bennett, 1999) to give a range of this parameter from 143 to 212 Joules/gram (details in the supplementary materials). We used the value estimated by Sears et al., 168 Joules/gram, and the upper and lower limits of the range estimated by us (143 and 212 Joules/gram) to perform the data analysis.

Table 1. Description of 6-day and 4-day food treatments.

Cohort	6-day Experiment		4-day Experiment	
	Day 1-3	Day 4 and 5	Day 1 and 2	Day 3
AL	AL	AL	AL	AL
SFR-A	AL	FR	AL	FR
SFR-B	FR	AL	FR	AL
LFR	FR	FR	FR	FR

AL: *ad libitum*; FR: Food restriction. For larvae under food restriction, the amount of food was given based on the individual's body mass, as $F = 0.27 \times m + 0.44$, where F and m are the wet mass of food and body in grams. This restriction level was designed based on our previous result (M. Hayes et al., 2015), so that the food uptake rate of food restricted cohorts was about 50% of that of the AL cohort.

3.3. METABOLIC ENERGY SPENT DURING THE EXPERIMENTAL PERIOD

The details of respirometry are available in the supplementary material and reference (M. Hayes et al., 2015). In short, we measured the exchange rate of O_2 and CO_2 of each larva for a 7-10 minutes time interval every day using Sable System International

(Las Vegas, U.S.A.) CA-10 CO_2 and FC-10 O_2 analyzers at 25 ºC. We then converted them to metabolic rate (in Watts). We assumed that the metabolic rate of each caterpillar would increase linearly between two successive measurements (approximately 24 hours apart) due to the body mass increasing during the day. Based on this assumption, we calculated the metabolic energy consumed in a particular day as 24 hours multiplied by the mean value of the rates measured at the beginning and the end of the 24-hr period in Joules. The metabolic energy (*ME*) was defined as the sum of larval metabolic energy expended each day during the experiment in Joules.

Due to the random activity rhythm of hornworms, metabolic rate was not constant, even after the effect of increasing body mass was removed. Thus, the random activity produced an inherent unknown measurement error. We incorporated the amount of variability in the measurement errors into the statistical analysis. Measurement errors were estimated for each individual. The gas analyzers took samples every second, so the ~10 minutes of respirometry generated a time-series curve. We calculated the standard deviation of each curve, which is the estimated measurement error of metabolic rate of one individual caused by the random activity. We then calculated the percentage of the standard deviation as SD/mean×100%, and assumed that this percentage would represent the random activity during the day. The standard deviation in the estimated measurement errors across individuals was then used as the estimated measurement error standard deviation in the ensuing statistical analysis. This approach to estimating measurement error variation is described in another study (Bland & Altman, 1996).

3.4 MDA ASSAY

The MDA-HPLC method described by (Lin, Huang, Zhou, & Ma, 2006) was optimized and validated for hemolymph samples. The details are available in the supplementary material. In short, we used HPLC with an Alltima C18 column to assay the total plasma MDA (both free and protein-bound). The assay depended on the formation of adducts between MDA and thiobarbituric acid (TBA) under heat. The fluorescence detector wavelength was set as 515 nm (excitation) and 553 nm (emission). The sample ran 7 min and the retention time of MDA-TBA was around 2.5 min.

The MDA concentration (in nM/ mL) is a body mass-specific quantity, whereas our model (Eqs. 1-3) makes predictions on the total damage, metabolic energy, and biosynthetic energy in the whole body. Thus, we multiplied the MDA concentration by the larval body mass on the last day of the experiment and used this value to test our theoretical model. To keep the dimensions the same in the equation, we could have also used the per ml values of MDA, and divided *ME* and *SE* by body mass, so that all the variables were mass-specific. However, this would introduce the variable of body mass into the regression equation twice (to *ME* and *SE*) and give less accurate results compared to the method that only introduces body mass once (to MDA). Moreover, the mass-specific (per mass) quantity can still be strongly correlated to body mass. There are many such examples in physiology (Hou et al., 2008; Kooijman, 2010). In our study, MDA per ml, mass-specific *ME*, and mass-specific *SE*, were all correlated to body mass ($R = 0.3$, $P<0.02$ for MDA per ml, $R = 0.6$, $P < 10^{-6}$ for mass-specific *SE*, and $R = 0.24$, $P < 0.037$ for mass-specific *ME*). Thus, even if we took the mass-specific quantities to perform the

multiple linear regression, we would still need to remove the confounding effect of body mass by estimating the mass-residuals of the variables.

3.5. DATA ANALYSIS AND STATISTICS

In each experiment, we tested if food treatments induced a significant difference in the MDA level between each cohort. We performed ANCOVA with MDA as the dependent variable, *ME* and *SE* as the covariates, and food treatment as the fixed factor, using SPSS 21. ANCOVA yielded $p > 0.1$ between each pair of these four treatments (see results), indicating that the food treatments did not induce any difference in the MDA level. Thus, in each experiment we pooled data from four cohorts and regressed the MDA level on *ME* and *SE*.

However, this regression caused two problems. First, these three variables were linearly proportional to the final body mass on the last day of the experiment. Thus, the confounding effect of body mass may have given false correlations between the dependent and independent variables. Second, because of the confounding effects of body mass, *ME* and *SE* are correlated to each other. Thus, there may have been multicollinearity between the independent variables, which often leads to unreliable and unstable estimates of the regression coefficients in multiple regression. When two independent variables are highly correlated, the one measured less accurately would usually fall out as being non-significant.

To address these issues, we first fit a multiple linear regression model (Model A) with *ME*, *SE*, and final body mass (*M*) as predictors of *MDA*w (the whole body MDA during the 5^{th} instar). This model controls for the confounding effect of final body mass,

but introduces severe multicollinearity due to the high correlation between final body mass and the other two predictors. Since the parameter estimates in the presence of multicollinearity often have a high standard error and can change drastically when different variables are in the model, we removed the final mass from the model (Model B) to observe the stability of the parameter estimates. In our final regression model (Model C), we removed the confounding effect of final mass on the variables by calculating the mass residuals of each variable, and then regressing the mass residual of MDA on the mass residuals of *ME* and *SE*, as shown in Eq. 3.

Model A: $\hat{MDA}_{\mathrm{W}} = \hat{\beta}_0 + \hat{\beta}_1 \times ME + \hat{\beta}_2 \times SE + \hat{\beta}_3 \times M$

Model B: $\hat{MDA}_{\mathrm{W}} = \hat{\beta}_0 + \hat{\beta}_1 \times ME + \hat{\beta}_2 \times SE$

Model C (Eq. 3): $\hat{MDA}_{\mathrm{residual}} = \hat{\beta}_0 + \hat{\beta}_1 \times ME_{\mathrm{residual}} + \hat{\beta}_2 \times SE_{\mathrm{residual}}$

To make sure that the independent variables (the mass residuals of *ME* and *SE*) did not have multicollinearity in the multiple regression, we calculated the variance inflation factors (VIF) and condition index of the multiple regression. It has been commonly recommended that if the value of VIF is below 10, and the condition index is below 30, multicollinearity is not significant (Hair, Anderson, Tatham, & Black, 1995). We compared the estimates for $\hat{\beta}_1$, $\hat{\beta}_2$ and ε in all three models. These values should be most alike between models A and C since both are ways to adjust for the confounding effect of the final mass.

We then included the measurement errors in the final model (Model C). A linear regression model ($MDA_i = \beta_0 + \beta_1 ME_i + \beta_2 SE_i + error_i$) was initially fit using *ME* and *SE* as explanatory variables to predict MDA. In the standard linear regression model, the

explanatory variables are assumed to be measured without error, but this assumption is known to be untrue since *ME* cannot be measured perfectly, as we described above. Thus, a linear regression model that accounts for the measurement error in *ME* was fit using a latent variable approach (Fuller, 2009) in PROC CALIS, SAS v.9.4. In this model, $ME_i^* = ME_i + u_i$ where ME_i^* represents the observed *ME* value, ME_i represents the true (latent) value, and u_i represents the measurement error for individual *i*. It is assumed that the measurement error is independent from the true value. The size of the measurement error standard deviation (σ_u) is required to estimate the regression coefficients. We estimated the standard deviation of metabolic rate as the percentage of the mean value of each sample curve. The procedure was done to obtain a distribution of the measurement errors (percentage values). We then estimated the standard deviation of the distribution, which is considered the size of the measurement error standard deviation (σ_u) and is used to estimate the regression coefficients (Fuller, 2009).

4. RESULTS

In the 6-day experiment, food treatments induced broad ranges of variation in metabolic energy (*ME* from *ca.* 4850 Joules to 16540 Joules), synthetic energy (*SE* from 370 Joules to 1480 Joules), and MDA level from 3510 nmol×g/ml to 35610 nmol×g/ml (Figure 1). All these variables were linearly proportional to the final body mass on the 6th day. The 4-day experiment had similar results (Figure 1).

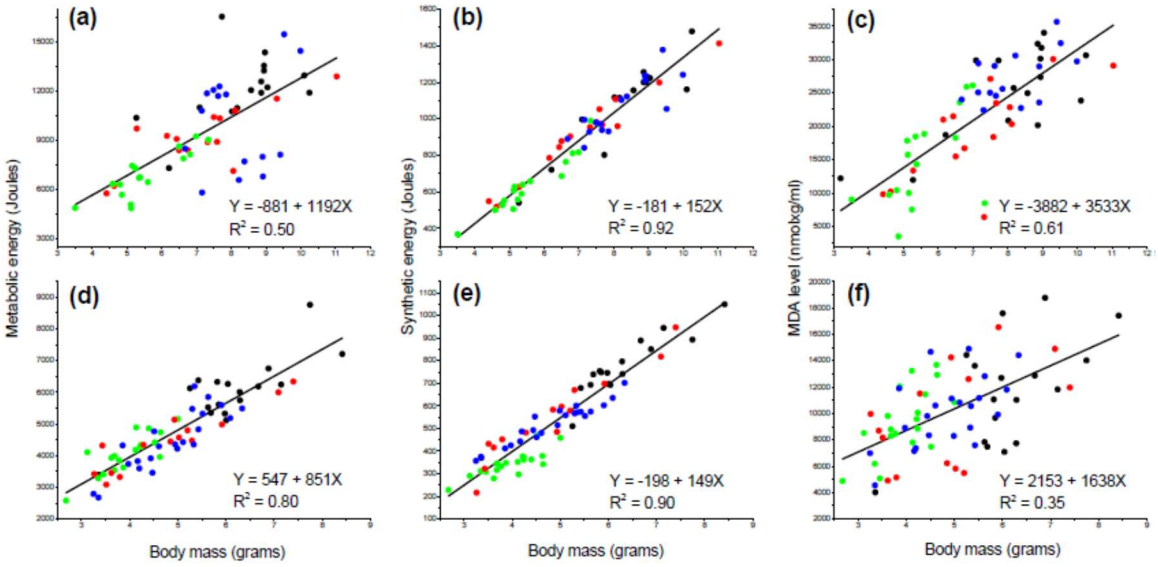


Figure 1. Variables are linearly proportional to the final body mass in the 6-day and 4-day experiments. Food treatments in the 6-day (top row) and 4-day (bottom row) experiments induced 4-fold, 4-fold, and 9-fold variations in metabolic energy (a) and (d), synthetic energy (b) and (e), and MDA level (c) and (f), respectively. Each dot represents an individual caterpillar belonging to different cohorts. Four cohorts are represented: black: AL, red: SFR-A, blue: SFR-B, and green: LFR.

The starting and ending body masses for 6-day and 4-day treatments are given in Table 2. The treatments did not induce any difference in MDA levels in either the 4-day or 6-day experiment. ANCOVA, using MDA as the dependent variable, *ME* and *SE* as covariates, and food treatments as fixed factors, showed that the assumption of homogeneous regression slopes was satisfied ($P > 0.05$), and there was no difference in the MDA level between each pair of these four treatments ($P > 0.1$).

Table 2. Starting and ending body masses in 6 and 4 day treatments.

Cohort	6-day Experiment			4-day Experiment		
	n	1st day body mass (g) Mean, SD	6th day body mass (g) Mean, SD	n	1st day body mass (g) Mean, SD	4th day body mass (g) Mean, SD
AL	15	1.822, 0.568	8.335, 1.657	17	1.670, 0.365	6.146, 1.100
SFR-A	15	1.665, 0.428	7.116, 1.721	14	1.559, 0.377	4.830, 1.309
SFR-B	15	1.872, 0.539	8.148, 0.999	22	1.738, 0.380	4.852, 0.843
LFR	16	1.765, 0.309	5.560, 1.024	19	1.923, 0.352	3.967, 0.580

Statistical values of Model A (Table 3) shows that *ME* and *M* are insignificantly correlated with *MDA* ($P > 0.05$) in the 6-day study, but *SE* is significant ($P<0.005$). The

4-day results shows insignificance for *ME*, *SE*, and *M* (P>0.05), but *SE* is at the edge of significance (P=0.062). The VIF values for *SE* and *M* are larger than 10, and condition indexes are larger than 30 for both the 6-day and 4-day experiments. Therefore, we concluded that multicollinearity was present. We then removed the final mass from the model and regressed *MDA*w with *ME* and *SE* (Model B, Table 3). In this model *ME* was insignificant in both 4-day and 6-day experiments ($p > 0.05$), whereas *SE* was significant in both ($p < 0.05$). Multicollinearity was absent (VIF < 10 and condition indexes < 20). Results of the residual model (Model C) showed that *ME* was insignificant ($P > 0.05$) in both the 4-day and 6-day experiments, whereas *SE* was significant ($p<0.001$) in the 6-day experiment and close to significance ($P = 0.06$) in the 4-day experiment. All the VIFs and condition indexes in Model C were smaller than 1.6, indicating no multicollinearity. All statistic model assumptions were met via checking residual plots.

The results of the regression coefficients (Table 3) strongly supported the predictions. First, across Models A, B, and C, the coefficient of *SE* was much larger than that of *ME*, i.e., $\hat{\beta}_2 > \hat{\beta}_1$ (more than 20-fold and 80-fold in the 4- and 6-day experiments, respectively), indicating that *SE* has the highest correlation with *MDA* after accounting for the other variables (i.e., *SE* is more influential than either *ME* or final mass). Second, the P-values of *ME* across all the models for both experiments were larger than 0.2, indicating its insignificant effect on the MDA level, whereas the P-values of *SE* were smaller than 0.002 across all the models in the 6-day experiments, and smaller than 0.009 in Model B and 0.06 in Models A and C in the 4-day experiments. Third, and most importantly, the ratio $\hat{\beta}_1 / \hat{\beta}_2 = (1-\varepsilon)/\varepsilon$ gave similar estimates for ε (0.989, 0.995 and

0.989 for Models A, B, and C, respectively, in the 6 day experiment; 0.962, 0.982 and 0.962 in the 4-day experiment) that were remarkably close to the predicted value of 0.99.

The lower and upper bounds of the parameter E_m, 143 and 212 Joules/gram, gave slightly different results in the final model (Model C). The statistical parameters, i.e., the R-, P-, and t-values, and the coefficient of *ME* ($\hat{\beta}_1$) stayed the same as in Table 3. The coefficient of *SE* and the repair efficiency became smaller for the upper bound, and larger for the lower bound: for the upper bound of E_m, $\hat{\beta}_2$ = 25.89 (6-day) and 7.89 (4-day), and the repair efficiency ε = 0.986 (6-day) and 0.952 (4-day); and for the lower bound of E_m, $\hat{\beta}_2$ = 38.22 (6-day) and 11.70 (4-day), and ε = 0.99 (6-day) and 0.967 (4-day).

Finally, we estimated the effects of measurement errors in *ME*. The standard deviation of the measurement error is 17% of the mean values. Using this percentage value, we obtained the size of the measurement error standard deviation (σ_u) of *ME* in the 6- and 4-day experiments which was 338.3 Joules and 87.7 Joules, respectively. The regression coefficient estimates after fitting Model C with measurement error in *ME* were very similar to the initial Model C in both experiments (Table 3). The repair efficiency, ε, estimated from the coefficients in Model C with the measurement error was 0.988 and 0.961 in the 6-day and 4-day experiments, respectively, which were slightly smaller than the ones estimated without the measurement error.

Table 3. Linear regression results of the MDA level on metabolic energy (*ME*) and biosynthetic energy (*SE*), using Models A, B, and C.

Model A: $\hat{MDA}_W = \hat{\beta}_0 + \hat{\beta}_1 \times ME + \hat{\beta}_2 \times SE + \hat{\beta}_3 \times M$						
	Experiment	Coefficients	*t*-values	P-values	Partial correlation, *r*	VIF
$\hat{\beta}_0$	6-day	-17821.505	-5.413	<0.001	–	–
	4-day	-3803.541	-2.400	.019		
$\hat{\beta}_1$ of *ME*	6-day	0.368	1.035	0.305	0.136	2.43
	4-day	0.394	0.646	0.520	0.078	5.591
$\hat{\beta}_2$ of *SE*	6-day	32.441	3.331	0.002	0.404	16.19
	4-day	9.949	1.899	0.062	0.224	11.22
$\hat{\beta}_3$ of *M*	6-day	-2141.796	-1.233	0.223	-0.161	20.71
	4-day	-687.775	-0.631	0.530	-0.076	19.77
Model A summary: R= 0.769 (6-day), and R = 0.517 (4-day). Condition index = 49.49 (6-day), and 43.37 (4-day).						
Model B: $\hat{MDA}_W = \hat{\beta}_0 + \hat{\beta}_1 \times ME + \hat{\beta}_2 \times SE$						
	Experiment	Coefficients	*t*-values	P-values	Partial correlation, *r*	VIF
$\hat{\beta}_0$	6-day	-20408.773	-8.006	<0.001	–	–
	4-day	-4390.689	-3.438	0.001		
$\hat{\beta}_1$ of *ME*	6-day	0.109	0.380	0.706	0.050	1.584
	4-day	0.126	0.289	0.774	0.035	2.868
$\hat{\beta}_2$ of *SE*	6-day	21.034	6.873	<0.001	0.670	1.584
	4-day	7.098	2.691	0.009	0.308	2.868
Model B summary: R= 0.762 (6-day), and R = 0.513 (4-day). Condition index = 9.78 (6-day), and 15.11 (4-day).						

Table 3. Linear regression results of the MDA level on metabolic energy (*ME*) and biosynthetic energy (*SE*), using Models A, B, and C (cont.)

Model C without measurement error: $\hat{MDA}_{residual} = \hat{\beta}_0 + \hat{\beta}_1 \times ME_{residual} + \hat{\beta}_2 \times SE_{residual}$						
	Experiment	Coefficients	*t*-values	P-values	Partial correlation, *r*	VIF
$\hat{\beta}_0$	6-day	1.22×10^{-11}	0.000	1.000	–	–
	4-day	2.5×10^{-8}	0.000	1.000		
$\hat{\beta}_1$ of $ME_{residual}$	6-day	0.372	1.060	0.294	0.138	1.20
	4-day	0.394	0.651	0.517	0.078	1.11
$\hat{\beta}_2$ of $SE_{residual}$	6-day	32.608	3.384	0.001	0.406	1.20
	4-day	9.949	1.913	0.060	0.224	1.11
Model C: R=0.408 (6-day), and R=.050 (4-day) Condition index= 1.54 (6-day), and = 1.38 (4-day)						
Model C with measurement error: $\hat{MDA}_{residual} = \hat{\beta}_0 + \hat{\beta}_1 \times ME_{residual} + \hat{\beta}_2 \times SE_{residual}$						
	Experiment	Coefficients	*t*-values	P-values	Partial correlation, *r*	VIF
$\hat{\beta}_0$	6-day	-1.64×10^{-11}	0.000	1.000	–	–
	4-day	-1.39×10^{-7}	0.000	1.000		
$\hat{\beta}_1$ of $ME_{residual}$	6-day	0.386	1.09	0.285	0.138	1.20
	4-day	0.407	0.66	0.512	0.078	1.11
$\hat{\beta}_2$ of $SE_{residual}$	6-day	32.762	3.44	0.001	0.406	1.20
	4-day	9.984	1.94	0.057	0.211	1.11
Model C: R=0.408 (6-day), and R=.050 (4-day) Condition index= 1.54 (6-day), and = 1.38 (4-day)						

5. DISCUSSION

The results of this study offer an answer to a long-standing question regarding the oxidative stress theory of aging. According to this theory (G. Barja, 2004; Beckman & Ames, 1998), a high metabolic rate causes a high production of reactive oxygen species, which leads to high cellular damage, and, thus, a short lifespan. However, two lines of evidence seem to conflict with this theory. The first of evidence comes from studies on food restriction, which extends the lifespan of a broad range of organisms and keeps them in a relatively healthy state (E. Masoro, 2005; Weindruch & Walford, 1988). While it largely suppresses growth, food restriction does not substantially decrease the metabolic rate of animals after body mass is corrected (McCarter & Palmer, 1992). This indicates that lowering the metabolic rate is not crucial in order for food restriction to extend lifespan. The conflicts between the empirical evidence and oxidative stress theory, which have been considered a long-standing puzzle (R. Balaban et al., 2005; Brys, Vanfleteren, & Braeckman, 2007; B. J. Merry, 2002; Selman, McLaren, Collins, Duthie, & Speakman, 2008a), can be explained by the theoretical model and empirical data presented in this paper. Our theory suggests that the highly efficient repair mechanisms scavenge and repair most of the free radicals produced by oxidative metabolism and the consequent cellular oxidative damage. If there are no other energy demanding processes, such as biosynthesis, then, no matter how high the metabolic rate is, the net damage is close to zero, because the repair efficiency is close to 0.99 ((Chen Hou, 2013; Hou et al., 2011) and this study). Thus, the variations in metabolic rate do not have significant effects on net damage level. However, the repair mechanism costs energy. When the energy, which

could be allocated to repair, is otherwise channeled to biosynthesis during growth, then damage inevitably accumulates quickly despite the high efficiency of repair. Our theory explains why food restriction extends lifespan without largely lowering metabolic rate—because it largely reduces growth. Indeed, reduction of growth plays a very important role in food restriction's effect on lifespan extension. Chen Hou (2013) analyzed the empirical data from more than 200 studies on food restriction in small rodents and found that lifespan extension by food restriction is linearly proportional to growth reduction.

The second line of evidence comes from the studies that experimentally elevated metabolic rate, but failed to change growth rate, and found no harmful effect on health or lifespan. For example, (Selman et al., 2008a) exposed voles to lifelong coldness, which elevated their metabolic rates by almost 100%, but had a minor effect on their growth rates. The control group and the group exposed to cold reached the same body mass at 20 months of age. The authors found "no treatment effect on cumulative mortality risk" and negligible effects on DNA oxidative damage, lipid peroxidation, and antioxidant protection. Similarly, moderate level of exercise were found to increase energy expenditure but had no effect on lifespan (J. O. Holloszy, 1997). In some cases exercise was even found to increase lifespan (John O. Holloszy, 1993; Navarro, Gomez, López-Cepero, & Boveris, 2004). In Eq. 1, $D = (1-\varepsilon) \times ME + \varepsilon \times SE$, the changes in damage induced by a large increase in metabolic energy (*ME*) can be offset by a slight decrease in *SE* (growth), because the coefficient of *ME* is much smaller than that of *SE*, i.e., $1-\varepsilon \ll \varepsilon$.

However, in some studies of cold exposure and mild exercise, growth seemed to be unchanged, while *ME* largely increased. In these cases, Eq. 1 predicts a net increase in damage, contradicting the empirical results. The key to understanding this contradiction

lies in the high efficiency. When animals are under stress, some repair and protection-related gene expression can be up-regulated, a phenomenon known as hormesis (E. Masoro, 2005; Rattan, 2004; Ristow & Zarse, 2010). It has been hypothesized that cold exposure and mild exercise can induce such a hormetic effect (Rattan, 2004). The effect may alter the structure of the macro-molecules and make them more resistant to oxidative insults. In the context of our model, this means that the coefficient (δ, which represents the amount of mass caused by one unit of metabolic energy), is reduced by the hormetic effects. Moreover, during exercise, the mitochondrial ROS production rate becomes lower when mitochondria transits from resting respiring state 4 to state 3 (the active phosphorylating respiration) (Gustavo Barja, 2007), and this transition also reduces δ. The mild stresses may also enhance the efficiency of repair or ROS scavenging, and increase the value of η. Recalling that the repair efficiency $\varepsilon = \eta / (\delta f)$, the reduced δ and increased η will increase ε.

The increase in ε induced by mild stress does not have to be large to offset the effect of the increased metabolic rate. Here we give an approximate estimate to show this point. Using the physiological data of a typical rat as an example ($B_{\text{rrest}}(\text{watts}) = 3.4 \times m^{3/4}$, M = 500 grams (Peters, 1983)), the total resting metabolic energy spent by a rat from birth to the age of 200 days is about $\int_0^{200\text{ d}} B_0 m(t)^{3/4}\, dt \approx 34000$ Kilojoules. The energy spent on bio-tissue synthesis from birth (~5 grams) to the age of 200 days is about 3000 Kilojoules (Moses et al., 2008). Taking the value of ε = 0.998 for rats previously estimated in (Chen Hou, 2013), the damage calculated by Eq. 1 is about $(1-\varepsilon) \times ME + \varepsilon \times SE \approx 3060$ KJ. Now, we assume that under mild stress, metabolic energy, *ME* increases 100%, from 34000 to 68000 KJ, while *SE* remains unchanged. It is

straightforward to see from Eq. 1 that an increase in ε from 0.998 to 0.999 is sufficient to offset the large increase in *ME*, and keep the damage level unchanged.

We need to emphasize that efficiency (ε) is high and robust as the result of natural selection (Rajindar S. Sohal, Robin J. Mockett, & William C. Orr, 2002). Not all the low dose stresses can induce hormetic effects or further increase them (E. Masoro, 2005). Growth rate, on the other hand, is much more plastic. A series of environmental factors, such as food supply, can change it and, therefore, change the damage in Eq.1. Thus, many interventions, such as food restriction, extend lifespan by changing growth rate and inducing energy reshuffling between biosynthesis and maintenance.

During growth, both metabolic and biosynthetic rate vary constantly. A variety of genetic, environmental, and physiological factors may cause independent or even opposite changes in these two rates. Since metabolic and biosynthetic rates may vary independently, their impacts on cellular damage may be different too. However, most studies on oxidative damage have only investigated the collective results of the concerted effects of these two rates. This study offers a departure point for better understandings of their relative effects on cellular damage. This study also provides a theoretical framework for estimating how genetic, environmental, and physiological factors influence children's health during growth.

6. SUPPLEMENTARY MATERIAL FOR THE PAPER

6.1. MODEL DEVELOPMENT

During growth, the total metabolic rate, B (in units of energy/time), is partitioned between the rate of resting metabolic energy, B_{rest}, and the rate of energy spent on activities (such as foraging) (Hou et al., 2008; West et al., 2001). The total metabolic rate is usually a constant multiple of the resting metabolic rate, i.e., $B = f \times B_{\text{rest}}$, where f is a dimensionless constant (Hou et al., 2008). The resting metabolic energy rate (B_{rest}) is further partitioned between the rate of energy required to maintain existing biomass, B_{maint}, and the rate of energy required to synthesize new biomass, B_{syn}, i.e., $B_{\text{rest}} = B_{\text{maint}} + B_{\text{syn}}$ (West et al., 2001). The maintenance term (B_{maint}) includes the energy spent on the repair mechanisms, such as oxidant scavenging and damage repair. The synthetic term (B_{syn}) can be expressed as $B_{\text{syn}} = E_{\text{m}} dm/dt$, where dm/dt is the growth rate (increase in body mass, m, per unit time, t), and E_{m} is the metabolic energy required to synthesize one unit of bio-tissue, such as the energy for assembling macromolecules from monomers. E_{m} is also called indirect cost of growth with the dimension of energy/mass (Hou et al., 2008; Peterson et al., 1999). Thus, biosynthetic rate, B_{syn}, is in units of energy/time. Here, as in other models of energy partition during growth (Kooijman, 2010; West et al., 2001), the biosynthetic energy only counts for the net growth (biomass gain). Energy for protein turnover, the net result of degradation and synthesis of damaged proteins, is included in the energy for maintenance, B_{maint}, because it does not contribute

to the net body mass gain (growth). Instead, it trades off with the energy for growing new biomass, as $B_{\text{rest}} = B_{\text{maint}} + B_{\text{syn}}$.

Based on this framework of energy allocation, we have made two assumptions for estimating the accumulation of oxidative damage. Assumption 1: Within a species, the rate of somatic damage production, *H*, caused by deleterious products of oxidative metabolism, such as reactive oxygen species (ROS), is proportional to the rate of oxygen consumption (metabolic rate, *B*). The assumption is based on the observations that metabolic and ROS generation rates are proportional to each other (see review in (Chen Hou, 2013)). Thus, we have the rate of damage accumulation (damaged mass/time), $H = \delta B$, where δ is a constant within a species, indicating the amount of damaged mass associated with one unit of metabolic energy. Here the damaged mass can be cell membrane, protein, DNA, or other macromolecules. The proportionality between ROS production and metabolic rate generally holds within one species. However, under certain conditions, ROS production can be disproportionally lower for a given metabolic rate (lower δ). Assumption 2: Repairing the damage requires metabolic energy. The rate of repair, *R* (repaired mass/time), is proportional to the energy available for maintenance (repairing damage), B_{maint}, with a coefficient η, i.e., $R = \eta B_{\text{maint}}$, where η is also a constant, indicating the amount of mass that can be repaired by one unit of available metabolic energy.

The net damage, $H - R$ accumulates. The accumulated damage can be integrated as a function of time, i.e., $F(t) = \int_0^t (\delta B - \eta B_{\text{maint}}) d\tau$. Using the relationship, $B = f \times B_{\text{rest}}$, we rewrite this equation as $D(t) = F(t) / (f\delta) = \int_0^t (B_{\text{rest}} - \varepsilon B_{\text{main}}) d\tau$, where $\varepsilon = \eta/(f\delta)$ is the effective repair efficiency, indicating the ratio of repaired mass and damaged mass for

one unit of energy, and $D(t)$ can be considered the recalibrated net cellular damage. To estimate damage, we substitute the equations $B_{\text{rest}} = B_{\text{maint}} + B_{\text{syn}}$ and $B_{\text{syn}} = E_{\text{m}} dm/dt$ in $D(t)$, and obtain:

$$\begin{aligned} D(t) &= \int_0^t (B_{\text{rest}} - \varepsilon \times B_{\text{maint}}) d\tau \\ &= \int_0^t B_{\text{rest}} - \varepsilon \times (B_{\text{rest}} - B_{\text{syn}})] d\tau \\ &= (1-\varepsilon) \int_0^t B_{\text{rest}} d\tau + \varepsilon E_{\text{m}} \Delta m \,|_0^t \\ &= (1-\varepsilon) \times ME + \varepsilon \times SE \end{aligned}$$

where $ME = \int_0^t B_{\text{rest}} d\tau$ is the metabolic energy spent during growth (in units of joules); Δm is the increase in body mass during growth, and E_{m} is the energy required to synthesize one unit of biomass, so $SE = E_{\text{m}} \Delta m$ is the synthetic energy spent during growth (also in units of joules).

6.2. DISPROPORTIONALITY BETWEEN ROS PRODUCTION AND OXYGEN CONSUMPTION

In Assumption 1, we assumed a proportional relationship between ROS production and the oxygen consumption rate (metabolic rate). However, a natural leak of proton exists across the mitochondrial inner membrane. The fraction of respiration that drives the proton leak is not involved in ROS production. Thus, the proton leak may cause disproportionality between ROS production and metabolic rate (Brand, 2000). Moreover, due to a series of factors under certain conditions, even after taking the proton leak into consideration, the ROS production may still not be proportional to non-proton-leak dependent oxygen consumption (Gustavo Barja, 2007, 2013; A. J. Hulbert, R. Pamplona, R. Buffenstein, & W. A. Buttemer, 2007). Here, we make three points to address this issue.

First, under normal conditions the percentage of oxygen consumption that drives the mitochondrial proton leak in a series of cells and tissues from different animals with a wide range of body masses and taxons (rat, mouse, ferret, sheep, pig, horse, lizard, frog, and snail) is roughly a constant, ranging from 15-30%, and clustering around 20% (Brand, 2000). Therefore, a constant fraction (~80%) of the oxygen consumption is involved in ROS production. If a unit of proton-leak independent oxygen produces a constant amount of ROS, after taking consideration of proton leak, the ROS production is still proportional to the total oxygen consumption.

However, under some irregular conditions, proton leak can be abnormally high, and the ROS production will be lower for a given oxygen consumption rate, i.e., the linear proportionality no longer holds. In the context of our model, the low-ROS production due to proton leak means a low damage coefficient, δ. (Assumption 1: ROS/damage production, $H = \delta \times B$. coefficient δ is low, if H is low for a given B due to proton leak.) Recalling that repair efficiency is $\varepsilon = \eta / (\delta f)$, a lower δ means a higher ε. Now, back to the main point of this paper: if the repair efficiency ε is high, then the damage will be insensitive to metabolic rate, because the contribution of metabolic energy to damage is $(1 - \varepsilon) \times B$ (Eq.1), and when ε is high, no matter how B changes, this term is close to zero.

However, the nonlinear relationship between metabolic rate and ROS production does not always lead to the insensitivity of damage to metabolic rate. If, for some reason, the proton leak is abnormally low, then ROS production will be higher for a given oxygen consumption. This means, the damage coefficient, δ, will be higher, which will in turn lead to a low repair efficiency, $\varepsilon = \eta / (\delta f)$. In this case, the metabolic term in the

damage equation, $(1-\varepsilon)\times B$ suggests that if ε is lower than normal, the damage will be relatively more sensitive to the changes in B.

Second, the main purpose of our model is to disentangle the effects of growth and the metabolic rate that fuel growth. The oxygen consumption that drives the proton leak does not produce ATP (energy), so it is not entangled with growth, and it is not even included in our energy partition equation. Thus, the model suggests that, even after considering the proton-leak, the variation in proton-leak-independent metabolic rate is still not the major cause of the variation in cellular damage, as opposed to conventional thinking.

The non-constant percentage of respiration that drives the mitochondrial proton leak can cause variation in the metabolic rate, but not all the variations in the metabolic rate can be attributed to the variation in the proton-leak. A series of environmental and physiological factors can alter the metabolic rate without changing the percentage of the proton leak. In this study, the variation in metabolic rate is mainly induced by food supplies. Therefore, we assume that, in this study even if there is a variation in the proton leak across individual caterpillars, the observed variation in metabolic rate induced by this factor is negligible compared to that induced by food supply.

Third, even after considering the proton-leak, ROS production may still not be proportional to the proton-leak independent respiration. Some researchers have summarized five situations, in which the non-proportionality may occur (Gustavo Barja, 2007, 2013; A. J. Hulbert et al., 2007), namely: (1) between different states of mitochondrial respiration; (2) under diet restriction; (3) in cross comparison taxon; (4) between different exercise statuses; and (5) between different tissues.

Assumption 1 of our model is made for species within a taxon (as opposed to a bird-mammal comparison), is for lifelong normal status (as opposed to short term intensive exercise status), and is at the whole organismal level (as opposed to tissue-specific). We have also discussed the case of chronic exercise training in the discussion section. Thus, we do not consider situations 3-5 in our model. For situations 1 and 2, we recommend a detailed discussion in one of our previous publications (Chen Hou, 2013). Briefly, we discussed a series of evidence, which shows that, under normal conditions, mitochondria operate in the respiration states where ROS production is neither extremely high nor low, and generally proportional to oxygen consumption. We also discussed that, under diet restriction, the low ROS production is the result of enhanced maintenance efforts, because diet restriction suppresses growth, and channels more energy from biosynthesis to maintenance (Chen Hou, 2013).

6.3. ESTIMATION OF ENERGY REQUIRED TO SYNTHESIZE ONE GRAM OF WET MASS, E_m

We took the approach described by (Peterson et al., 1999) and (Bennett, 1987) to estimate E_m. This is a common approach to estimate the metabolic cost of biosynthesis (Peterson et al., 1999; Webster, 1989; Wieser, 1994). We measured metabolic rate and body mass gain every day during the 5^{th} instar, and calculated the mass residual for each caterpillar from the regression equation for growth and metabolic rate on body mass. Upon regression of mass residuals of metabolic rate against mass residuals of growth rate, the calculated slope is interpreted as the metabolic cost of growth with units of Joule/gram.(Peterson et al., 1999).

The regressions yield $E_{\mathrm{m}} = 211.9$ Joules/gram from the 4-day experiment and $E_{\mathrm{m}} = 142.9$ Joules/gram from the 6-day experiment. The average value, 177.4 Joules/gram, is essentially the same as the value estimated by (Sears et al., 2012), 168 Joules/gram of wet mass.

6.4. ESTIMATE OF REPAIR EFFICIENCY, ε

We estimated ε in two ways. First, we derived an equation from Eq. 1, which was used to estimate the relationship between lifespan extension and suppression of growth by diet restriction and growth hormone interference:

$LS_{\mathrm{D/G}} \times B_{\mathrm{m,\,D/G}} - LS_{\mathrm{con}} \times B_{\mathrm{m}} = \frac{\varepsilon E_{\mathrm{m}} \mu_{\mathrm{con}}}{1-\varepsilon}(\frac{M_{\mathrm{con}}}{M_{\mathrm{D/G}}} - 1)$, where LS is lifespan, M is the adult mass, μ is the ratio of birth and adult mass, and B_{m} is the mass-specific resting metabolic rate ($B_{\mathrm{m}} = B_{\mathrm{rest}}/M$). The subscription, D/G stands for diet restriction and genetic interference and "con" stands for control. This equation predicts that the normalized lifespan extension (left-hand side) is proportional to the body mass reduction on the right-hand side $(M_{\mathrm{con}} / M_{\mathrm{D/G}} - 1)$ with a slope of $\frac{\varepsilon E_{\mathrm{m}} \mu_{\mathrm{con}}}{1-\varepsilon}$.

In the slope, E_{m} is a constant for a given species, μ (the ratio of birth/adult mass) is also a constant for a given species. We have collected 246 data points from published studies on rats and mice, whose lifespan (LS) and body mass (M) were given for both control groups and groups under diet restriction or genetic interference. We then plotted the lifespan extension versus body mass reduction, and obtained the fitted slopes for both mice and rats (Chen Hou, 2013).

The fitting is very good (R^2 = 0.82 and 0.87 for mice and rats, respectively, which suggests that the body mass reduction can explain more than 80% of lifespan extension by diet restriction and genetic interference). We then used the values of E_m and μ for mice and rats and used fitted slopes to estimate ε. We have ε = 0.998 and 0.999 for mice and rats respectively.

The second method is from the basic principle of biochemistry. To connect the damage level to lifespan, we assume that, when the lifespan (*LS*) is reached, the mass specific accumulated damage reaches a certain level (=*C*), i.e., a certain fraction (C) of the body mass is damaged when animals die. Eq. 1 can be used to obtain: $C/(\delta f) = (1-\varepsilon)LS \times B_m + \varepsilon E_m(1-\mu)$. Again, B_m is the mass-specific metabolic rate and μ is the birth/adult mass ratio. By solving this equation for ε, we have:

$$\varepsilon = \frac{LS \times B_m - C/(\delta f)}{LS \times B_m - E_m(1-\mu)} \qquad \text{(S1)}$$

It is difficult, if even possible, to have an accurate calculation of ε. Here, we make an approximate estimate as follows.

Three terms in Eq.S1 determine the value of ε, namely $LS \times B_m$, $E_m(1-\mu)$, and $C/(\delta f)$. Using a mouse as an example, whose $LS \approx$ 1000 days, $M \approx$ 50 gram, B_m = 720 Joules/day (Peters, 1983), $E_m \approx$ 4500 J/gram (Moses et al., 2008), and $\mu \approx 0.1$, we can estimate the first two terms: $LS \times B_m \approx 7.2 \times 10^5$ Joules/gram, and $E_m(1-\mu) \approx 4050$ Joules/gram. In the third term, $C/(\delta f)$, C is the critical fraction of the body that is damaged when animals die, and δf is the ratio of the damage rate and metabolic rate, i.e., $\delta f = H/B_{rest}$ (Assumption I in the main text). Therefore, δf indicates the amount of damaged body mass that is associated with one unit of metabolic energy.

To produce one joule of metabolic energy, 2.22×10^{-6} moles of oxygen molecules need to be combusted, on average. It is estimated that, under physiological conditions, approximately 0.2% of the oxygen consumed by cells is converted by mitochondria to reactive oxidative species (ROS) (R. Balaban et al., 2005; M. E. Harper, L. Bevilacqua, K. Hagopian, R. Weindruch, & J. J. Ramsey, 2004). Thus, for every joule of metabolic energy, $2.22 \times 10^{-6} \times 0.2\% = 4.44 \times 10^{-9}$ moles of oxygen molecules are converted to ROS.

ROS cause damage in proteins, lipids, and DNA (G. Barja, 2004). It is not possible to accurately calculate how much biomass (including all these macromolecules) is damaged by one mole of ROS. Here, we focus on the protein oxidation, a typical molecular damage caused by ROS, to carry out an approximate estimate. It has been demonstrated (Stadtman, 2004) that amino acids, peptides, and proteins, react with $^{\bullet}OH$ and $HO_2^{\bullet}$, "forming a carbon-center radical, which may react with molecular oxygen to form a peroxyl radical. The peroxyl radical is then converted to the alkyl peroxide, which reacts with superoxide and yields an alkoxyl radical and the hydroxyl derivative. The alkoxyl radical derivatives of proteins are capable of undergoing peptide bond cleavage" (Stadtman, 2004). It takes 1 mole of oxygen atoms to react with 1 mole of amino acid and a total of 7 moles of oxygen atoms to produce 1 mole of hydroxyl derivative (Stadtman, 2004). We assume that, on average 4 moles of oxygen atoms in ROS would cause damage in one mole of amino acid (Morowitz, 1978), (Calow, 1977) estimated that there are approximately 0.002 moles of amino acids in one wet gram of average metazoan biomass. Therefore, the value of *δf*, which, again, indicates the biomass damaged by ROS associated with one joule of metabolic energy, is about

$4.44 \times 10^{-9} \times 2 / 4 / 0.002 = 1.11 \times 10^{-6}$ grams/Joule.

Putting all these relationships into Eq. S1, we have:

$$\varepsilon = \frac{7.2\times10^{5} - C/(1.11\times10^{-6})}{6.8\times10^{5}}.$$

Now, if we assume that the value of C is about 5~10%, meaning that 5~10% of the proteins in the body are damaged before death, we have $\varepsilon = 0.9926$ for $C = 5\%$, and $\varepsilon = 0.9265$ for $C = 10\%$. We need to emphasize that the calculation above only provides a rough approximate range of ε. The exact calculation and accurate value of ε require more quantitative empirical information on damages to proteins, lipids and DNA by ROS.

6.5. EXPERIMENTAL METHOD DETAILS

6.5.1. Animal Rear and Food Supply Level. In each experiment, approximately 80 hornworms were raised from eggs (Carolina Biological Supply) on a long day cycle (17 hours light: 7 hours dark) at 25 ºC. Animals were fed *ad libitum* and checked for molting each day until the 5th instar. On the first day of the 5th instar, larvae were randomly separated and treated with four levels of food supply with wheat germ-based diet (Carolina Biological Supply, NC, 20160 Joules/gram, dry weight). The four cohorts were free-feeding (AL), short-term food restriction-A (SFR-A), short-term food restriction-B (SFR-B), and long-term food restriction (LFR).

All larvae were sacrificed on the sixth day and fourth day in the 6-day and 4-day experiments for MDA measurement. For larvae under food restriction, the amount of food was given based on individual's body mass, as $F = 0.27 \times m + 0.44$, where F and m are the wet mass of food and body in grams. This restriction level was designed based on our previous result (M. Hayes et al., 2015) so that the food uptake rate of the food restricted cohorts was about 50% of that of the AL cohort.

6.5.2. Metabolic Energy Spent During the Experimental Period. The same method described in our previous publication was used to measure the metabolic rate of hornworms (M. Hayes et al., 2015). The rates of O_2 consumption and CO_2 production ($\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$) of each larva were measured for 7-10 minutes time intervals every day using Sable System International (Las Vegas, U.S.A.) CA-10 CO_2 and FC-10 O_2 analyzers (incurrent flow-through respirometry) in a temperature-controlled chamber set at 25 ºC. The rates of $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$, both in units of ml/min, were calculated as $\dot{V}_{CO_2} = \mathrm{FR} \times [CO_2] / 100$, and $\dot{V}_{O_2} = \mathrm{FR} \times (20.95 - [O_2]) / (100 - [O_2])$, where FR is the flow rate in unit of ml/min, and $[CO_2]$ and $[O_2]$ are the concentration of CO_2 and O_2 in the respirometry chamber (Lighton, 2008). We assumed that the metabolic rate of each caterpillar would increase linearly between two successive measurements (approximately 24 hours apart). Based on this assumption, we calculated the metabolic energy consumed in a particular day as 24 hours multiplied by the mean value of the rates measured at beginning and end of the 24-hr period in units of Joules. Overall the metabolic energy consumed each day was estimated as $(10.34 - 5.38 \times RQ) \times \dot{V}_{CO_2} \times 4.18 \times 1440$, where $RQ = \dot{V}_{CO_2} / \dot{V}_{O_2}$ is the respiratory exchange ratio, the factor (10.34 – 5.38×RQ) converts the emission rate of CO_2 (in unit of ml/min) to metabolic rate (calorie/min) , and the factors 4.18 and 1440 convert calories to joules and minutes to days, respectively. The metabolic energy (*ME*) was defined as the sum of the larval metabolic energy expenditure each day during the experiment in units of Joules.

6.5.3. MDA Assay. The MDA-HPLC method described by (Lin et al., 2006) was optimized and validated for haemolymph samples. A well separated peak was produced in both standard and haemolymph samples with a retention time of 2.5 min. All chemicals and reagents used were HPLC or analytical grade. Acetonitrile, methanol, ethanol, n-butanol, tetrahydrofuran (THF), and trichloroacetic acid (TCA) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Butylated hydroxytoluene (BHT), 1, 1, 3, 3-tetramethoxypropane (TMP), and 2-thiobarbituric acid (TBA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Ultra-pure water was used to prepare mobile phase and other aqueous solutions.

Hemolymph samples from the larvae were collected into tubes containing an EDTA solution (Grotto et al., 2007) after clipping the third proleg. After centrifugation at 6000g for 10 min at 4^0C, the supernatant was stored at -80 ^{0}C until analysis. Protein bound MDA was hydrolyzed by adding 25 µL of 3N NaOH into 100 µL plasma and incubating at 60 ºC for 30 min (Moselhy, Reid, Yousef, & Boyle, 2013; Pilz, Meineke, & Gleiter, 2000). A 100 µL of 500 ppm BHT, 1mL of 0.1 N HCl, and 1mL of 10% TCA solutions were then added to the mixture. After centrifugation at 3,000 rpm for 10 min, 500 µL of supernatant was mixed with 500 µL of TBA and boiled for 10 min. A 1 mL of n-butanol was added and vortex-mixed for 30 seconds. After centrifugation, the top layer was filtered through a 0.45 µm filter and analyzed with an Agilent 1100 series HPLC system (Santa Clara, CA, USA) and a fluorescence detector set at 515 nm (excitation) and 553 nm (emission). The MDA-TBA adduct was separated on an Alltima C18 column (250 × 4.6 mm, 5 µm particle) using 1 mL/min isocratic mobile phase consisting of 5 mM sodium phosphate buffer (pH 7.0), 30% (v/v) Acetonitrile, and 0.6% (v/v) THF.

SECTION

2. TEST THE MODEL'S PREDICTIONS BY MANIPULATIVE EXPERIMENTS IN HORNWORMS (*Manduca sexta*) LARVAE

2.1. INTRODUCTION

In our preliminary study, we induced variations in metabolic energy (ME) and synthetic energy (SE) in 5th instar hornworms, and assayed the corresponding plasma malondialdehyde (MDA) level, as a surrogate of cellular damage. MDA is a specific end-product of phospholipid oxidative damage, and has been commonly used as a biomarker of oxidative stress (Del Rio, Stewart, & Pellegrini, 2005; Monaghan et al., 2009). We assume that the level of MDA is proportional to the total cellular damage (variable, D, in Eq. 1) with a factor of g, as $MDA = g \times D$, and, therefore, Eq. 1 becomes Eq. 2. Comparing the theoretical Eq. 2 and the regression Eq. 3, we made three predictions. First, the fitted regression coefficient of the metabolic term, $\hat{\beta}_1$ of $ME_{residual}$, would be smaller than that of the biosynthetic term, $\hat{\beta}_2$ of $SE_{residual}$. Second, the regression of the metabolic term would have a large P-value, indicating its insignificant contribution to the MDA level after accounting for SE. Third, the repair efficiency (ε) estimated from the ratio of the regression coefficients, $\hat{\beta}_1 / \hat{\beta}_2 = (1 - \varepsilon) / \varepsilon$ would be close to 0.99. The statistical results presented in the paper (Table 3, Model C) strongly support the theoretical predictions.

We must emphasize that our preliminary study presented in the paper was observational. The food treatment only induced broad variations in the metabolic energy (ME), synthetic energy (SE), and MDA level, so that the linear regression could be conducted. However, there was no difference in the MDA level between treatments with

ME and SE being the covariates (ANCOVA, $P > 0.05$). In other words, we did not control either ME or SE in the preliminary study.

We then designed a manipulative experiment. Compared to the observational study, the manipulative experiment offered better understanding of and more direct evidence for the separate effects of biosynthesis and metabolism on cellular damage. In that way biosynthesis and metabolic rates were controlled in four separate cohorts, which yielded two levels of *ME* and two levels of *SE*.

2.2. METHOD

2.2.1. Animal Rearing. We induced ME and SE in hornworms in four cohorts by changing the food supply, temperature and controlling the target of rapamycin (TOR) pathway. Larvae were randomly selected on their first day of the 5th instar and allocated to the 4 treatment groups described in Table 2.1. On their 5th day, larvae were sacrificed for MDA measurement. When hornworms are free fed, increasing temperature increases both metabolic and growth rates. However, when the food supply is limited, there is a tradeoff between growth and metabolism. Under food restriction (FR), high temperature increases metabolic rate, but decreases growth rate, as opposed to what was observed under free feeding (M. B. Hayes et al., 2014). Under 50% FR, a 10 ^{O}C difference in temperature increases metabolic rate by 1.5-fold, but decreases growth rate by 1.07-fold (M. B. Hayes et al., 2014). We used slightly higher than 50% food restriction so that the difference in growth rate would be smaller than 1.07, and close to 1. The cohorts reared at a higher temperature would have a higher metabolic rate. To keep the growth rate low while maintaining the higher metabolic rate, one cohort was treated with rapamycin. The

TOR signal pathway controls growth and reproduction. Inhibition of TOR signaling extends the life span in *C.elegans* (Lapierre & Hansen, 2012) and rapamycin treatment in diet inhibits growth in horn worm larvae (Kemirembe, Liebmann, Bootes, Smith, & Suzuki, 2012).

The rapamycin treatment described by (Kemirembe et al., 2012) was slightly modified. One gram of rapamycin (Guangzhou Puho Pharmaceutical Co., Limited) was dissolved in 1ml of DMSO, and 20µl of this solution was mixed with 180µl of PBS (pH 7.4). The solution was homogenized, and a quantity of 200µl was evenly pipetted to 1 g of diet. Worms were free fed with a rapamycin treated diet for four days, starting from the 1st day of the 5th instar. The details of animal rearing are available in Table 2.1.

Table 2.1. Description of treatments.

Group	Description	N	Treatment
1	AL 30 rapamycin treatment	12	Temperature 30 ^{O}C, rapamycin in diet
2	AL 20	14	Temperature 20 ^{O}C, Ad libitum
3	AL 30	17	Temperature 30 ^{O}C, Ad libitum
4	FR 30	15	Temperature 30 ^{O}C, Food restricted slightly >50%

For larvae under food restriction the amount of food was given based on individual body mass, as $F=m^{0.21}$, where F and m are the wet mass of food and body mass is in units of grams. The restriction level was designed based on our previous results (M. B. Hayes et al., 2014).

2.2.2. Synthetic Energy Spent During Experimental Period. Body mass was measured in each larvae approximately the same time every day during experimental period. Biosynthetic energy (SE) was calculated in units of Joules using equation $SE = \Delta m \times E_m$ where, E_m in 5th instar hornworm was taken to be 168 Joules/gram, Δm was the weight gain during experiment period.

2.2.3. Metabolic Energy Spent During Experimental Period. The exchange rate of CO2 and O2 was measured for 7-10 minutes every day using Sable System International (Las Vegas, U.S.A) CA-10 CO2 and FC-10 O2 analyzer and changing the temperature according to the cohort temperature. The details of resperiometric data collection are explained in Section 6.5.2.

2.2.4. MDA Assay. On the 5th day of the experiment, the hemolymph was drawn from the larvae and MDA was measured using the HPLC method described in Section 6.5.3.

2.2.5. Data Analysis and Statistics. Statistical analysis was performed using Minitab17. The damage accumulate through entire growth period and a fraction of MDA is accumulated during first fourth instars. Since the experiment manipulations started on the first day of 5th instar, the accumulated damage in first 4th instars should be removed. This was corrected by linear regressing the MDA level with the initial body mass (Mo) on the 1st day of 5th instar. According to our previous study (Figure.1) the ME, SE and

MDA level during experimental period are linearly proportional to the final body mass (M). This confounding effect of final body mass was removed by performing separate linear regressions of MDA, ME, SE on M and calculating the residuals in each of the three analysis. Then, linear regression was conducted with mass residual of MDA (MDA $_{residual}$) on the mass residual of ME (ME $_{residual}$), mass residual SE (SE $_{residual}$), Group as a categorical variable and including interaction terms; ME $_{residual}$ x Group and SE $_{residual}$ x Group. The model is as follows;

$$\widehat{MDA}_{residual} = \hat{\beta}_0 + \hat{\beta}_1 ME_{residual} + \hat{\beta}_2 SE_{residual} + \hat{\beta}_3 Group + \hat{\beta}_4 (ME_{residual} \times Group) + \hat{\beta}_5 (SE_{residual} \times Group)$$

The model allowed the possibility of obtaining a separate estimated regression for each of the four groups while using all of the data in the estimation. The regression equation for each group is of the following form:

$$\widehat{MDA}_{residual} = \hat{\beta}_0 + \hat{\beta}_1 ME_{residual} + \hat{\beta}_2 SE_{residual}$$

Then, the significance of SE $_{residual}$ and ME $_{residual}$ were studied within group and between groups.

2.3. RESULTS

The initial analysis between groups for ME after correcting for M showed, group 1 and 4 had medium ME, group 2 had low ME whereas group 3 had high ME (Figure 2.1 B). SE between groups showed, medium SE in group 1, 2, 4 and high SE in group 3 (Figure 2.1 A).

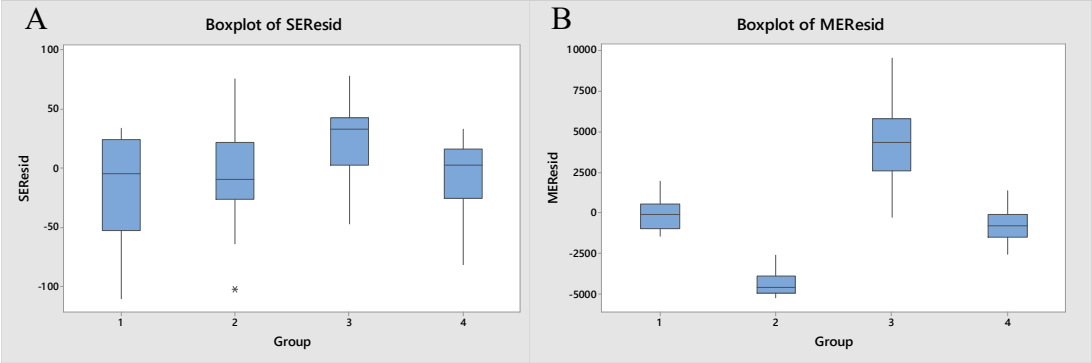


Figure 2.1. Boxplots for $ME_{residual}$ (A) and $SE_{residual}$ (B) for groups.

To test for a statistically significant linear relationship between the predictor variables and the response, a hypothesis test for whether the regression coefficient for each predictor is equal to zero after accounting for the other predictor is conducted. P-values for these tests in the within group analysis (Table 2.2) show $MDA_{residual}$ and $ME_{residual}$ are not significantly correlated ($P>0.05$) for all four groups after accounting for $SE_{residual}$, but SE is significantly correlated to $MDA_{residual}$ ($P<0.05$) in groups 1, 3 and 4 whereas group 2 is insignificant ($P>0.05$).

A between group analysis was conducted to determine if there were differences in the intercept and/or slopes of $ME_{residual}$ and $SE_{residual}$ between the four experimental groups. Between group analysis (Table 2.3) shows that the intercepts between group 1 and group 2, group 1 and group 3, group 1 and group 4, group 3 and group 4 are significant ($P<0.05$). However, the intercepts between group 2 and group 3, group 2 and group 4 are insignificant ($P>0.05$). The slope of $ME_{residual}$ is significantly different for group1 and 2 ($P<0.05$). The slopes of $SE_{residual}$ between groups are insignificant ($P>0.05$).

Table 2.2. Regression analysis within groups.

Group	Coefficient of β1	P value for β1 $ME_{residual}$	Coefficient of β2	P value for β2 $SE_{residual}$
Group 1	1.75	0.168	91.4	0.001
Group 2	-2.20	0.147	27.7	0.290
Group 3	0.413	0.271	83.0	0.005
Group 4	0.72	0.505	93.7	0.009

Table 2.3. Regression analysis between groups.

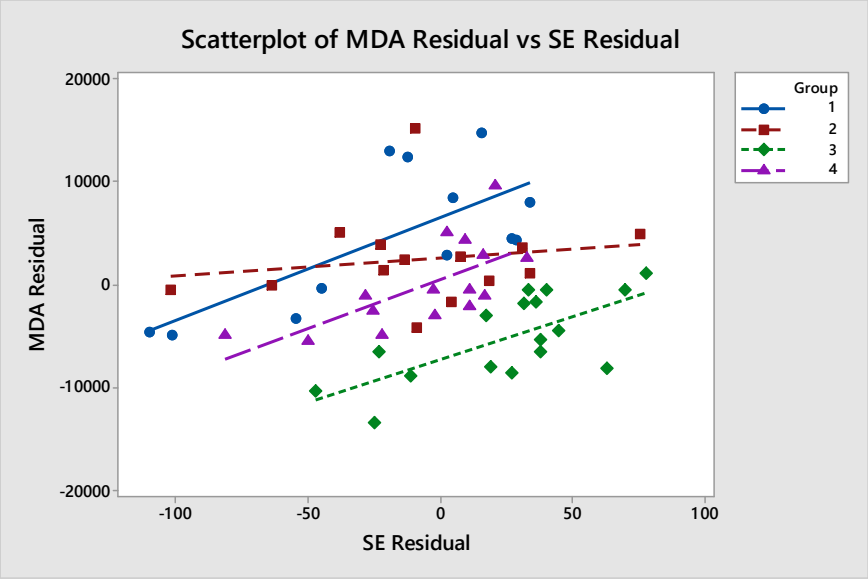
Comparison	P value for β0	P value for β1 $ME_{residual}$	P value for β2 $SE_{residual}$
Group 1 and Group 2	0.050	0.048	0.083
Group 1 and Group 3	0.000	0.310	0.827
Group 1 and Group 4	0.004	0.532	0.957
Group 2 and Group 3	0.760	0.096	0.158
Group 2 and Group 4	0.241	0.119	0.133
Group 3 and Group 4	0.000	0.789	0.813

These results are also explained by the scatter plots of $MDA_{residual}$ vs $SE_{residual}$ and $MDA_{residual}$ vs $SE_{residual}$ accounting for the effect of $ME_{residual}$ in Figures 2.2 and Figure 2.3

respectively. $MDA_{residual}$ and $SE_{residual}$ show positive correlation between all four groups and group 2 doesn't show a very strong relationship (Figure 2.2 A). This matches the earlier results that the $SE_{residual}$ was significant for all groups except for group 2. When the $ME_{residual}$ is accounted for in the model, similar results were observed (Figure 2.2 B). All the intercepts between groups are significantly different from each other except between group 2. However, differences in slopes between groups are insignificant as observed in the nearly parallel lines. $MDA_{residual}$ vs $ME_{residual}$ (Figure 2.3 A) is more varied, with groups 1, 3, 4 showing positive correlation with varying degrees of strength. Group 2 is different from other three groups as it shows negative correlation. With $SE_{residual}$ is accounted for in the model, (Figure 2.3 B), a significant difference in slopes is seen only between groups 1and 2.

The regression analysis of ME between groups shows highest MDA to lowest MDA level in group 1 (Rapamycin, 30 ^{O}C), group 4(FR 30 ^{O}C), group 3 (AL 30 ^{O}C) respectively. Since the slopes between group 1 and 2 are significantly different, a conclusion regarding MDA level cannot be drawn for group 2 (AL 20 ^{O}C) as it is the highest at low values of $ME_{residual}$ and lowest at high values of $ME_{residual}$. Based on regression results of SE between groups, the ranking of MDA from highest to lowest is seen in group 1 (Rapamycin, 30 ^{O}C), group 4 (FR 30 ^{O}C), group 2 (AL 20 ^{O}C) group 3 (AL 30 ^{O}C) respectively. Overall the treatments had altered the ME and SE in larvae resulting in different MDA production between groups and SE had a greater effect on MDA rather ME within group.

A.



B.

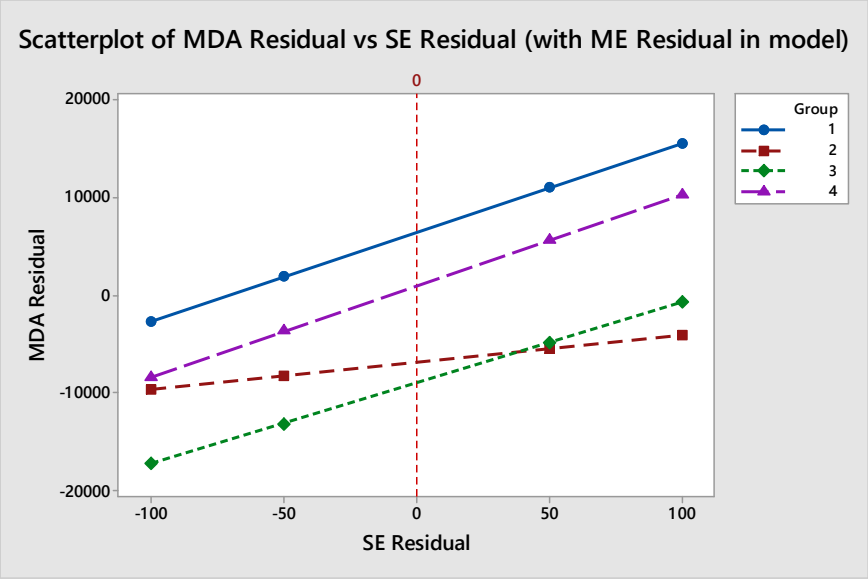
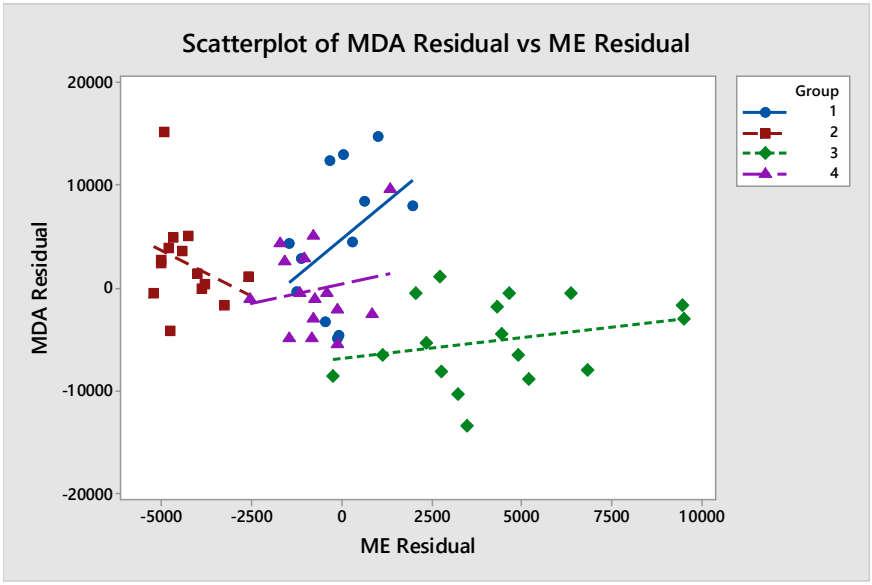


Figure 2.2. $SE_{residual}$ show positive correlation with $MDA_{residual}$ in all four groups. A) Scatterplot of $MDA_{residual}$ vs $SE_{residual}$: Differences in slopes are insignificant between groups. B) Scatterplot of $MDA_{residual}$ vs $SE_{residual}$ with $ME_{residual}$ in the model; the intercepts are insignificant only between group 2 and 3, group 2 and 4. Slopes are insignificant between all groups.

A.



B.

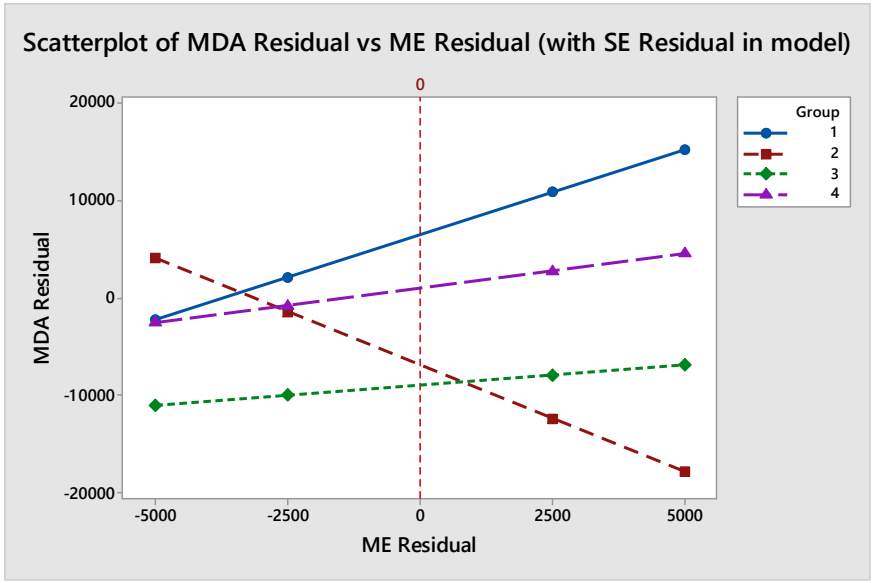


Figure 2.3. $ME_{residual}$ show positive correlation with $MDA_{residual}$ in group 1, 3, and 4 while group 2 shows a negative correlation. A) Scatterplot of $MDA_{residual}$ vs $ME_{residual}$: Slopes are insignificant between groups except for group1 and 2. B) Scatterplot of $MDA_{residual}$ vs $ME_{residual}$ with $SE_{residual}$ in the model; the intercepts are insignificant only between group 2 and 3, group 2 and 4. Slopes are significant between groups 1 and 2.

2.4. DISCUSSION

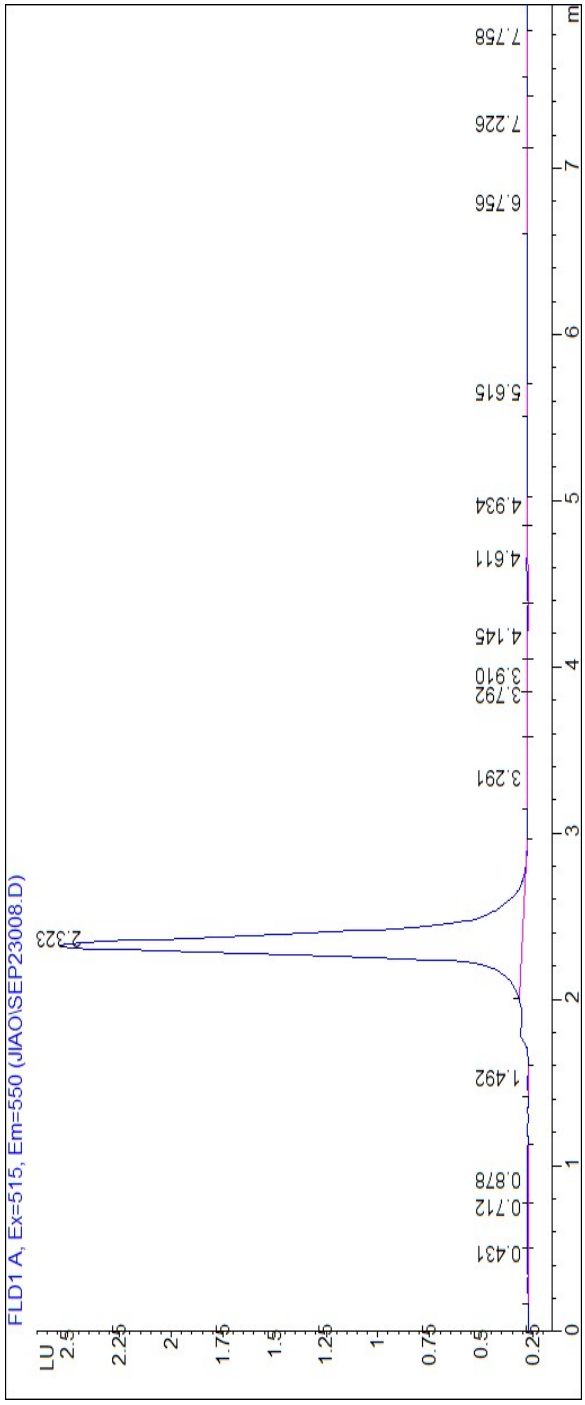
This study shows that cellular oxidative damage is more sensitive to synthetic energy compared to metabolic energy, in agreement with our previous study. Within each of the treatment groups, the synthetic energy is more influential to oxidative damage of lipids. Thus, our theory is supported by the results.

However, we did not observe similar results between groups. Highest cellular damage was seen in medium metabolic rate, medium biosynthetic groups which are Rapamycin treated and FR 30 ^{O}C. Although rapamycin reduces growth rate (Kemirembe et al., 2012) it can lead metabolic changes including hyperlipidemia, decreased insulin resistance and glucose intolerance (Lamming, Ye, Sabatini, & Baur, 2013) and hyperlipidemia can increase the MDA levels (Li et al., 2014). Rapamycin can also change total and resting metabolic rate (Zhang et al., 2014). Thus, high cellular damage is seen when there is medium level synthetic rate and medium metabolic rate. The lowest oxidative damage was observed in high metabolic, high synthetic group namely ad libitum 30 ^{O}C larvae. Ad libitum 20 ^{O}C group behaved differently from other three groups where metabolic rate had an insignificant but a negative correlation with MDA level.

Our study for between groups must be interpreted with great care. In our analysis we did not conduct adjustments for multiple testing between groups and further statistical analysis should be done for better understanding of the predictors. Future research should be designed to understand the cellular damage with rapamycin treatment and Ad libitum 20 ^{O}C group.

APPENDIX

HPLC chromotogram for MDA standard 67nM



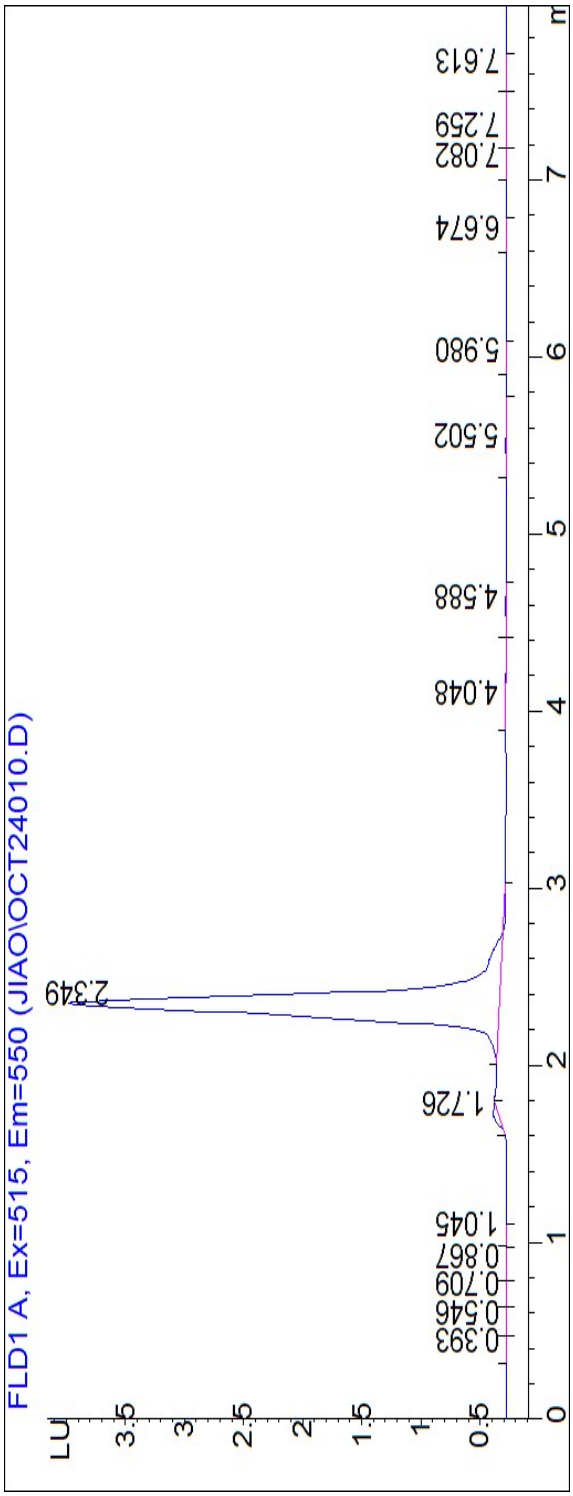
Method: MDA

Analysis time: 7.992 min

Sampling rate: 0.0072 min

Time	Area	Height	Width	Area%	Symmetry
2.323	20.2	2.2	0.1357	98.568	0.716

HPLC chromotogram for a sample



Method: MDA

Analysis time: 7.992 min

Sampling rate: 0.0072 min

Time	Area	Height	Width	Area%	Symmetry
2.349	30.4	3.7	0.1187	98.337	1.139

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