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Link between energy investment in biosynthesis and cellular resistance to oxidative insult: Test the cost-quality hypothesis in insects

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Department:

LINK BETWEEN ENERGY INVESTMENT IN BIOSYNTHESIS AND CELLULAR RESISTANCE TO OXIDATIVE INSULT: TEST THE COST-QUALITY HYPOTHESIS IN INSECTS

by

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ABSTRACT

The metabolic cost of growth is the amount of energy invested to fuel the anabolic biochemical reactions of biosynthesis. It has been implicated in the efficiency of farm animals and fishes, thus, increased economic profit. The metabolic cost of growth in the cockroach nymph was reported to be 20-fold higher than that of the painted lady butterfly caterpillar. The cost-quality hypothesis was proposed to explain the extremely low biosynthesis energy cost in painted lady caterpillar when physiological and ecological processes the metabolic growth cost was attributed to could not. In this study, the cost-quality hypothesis was tested.

Midgut cells were isolated from the two insect species and the cell metabolic rates and growth rates were determined. Using the energy budget model, the metabolic cost of growth of the cells was estimated and compared to the values reported in the organisms. 7AAD viability staining and Annexin V apoptosis were used to determine cell viability and percentages of apoptotic cells in the insect species after exposure to various concentrations of oxidant. Cell viability and percentages of apoptotic cells were used as an index of cell quality.

The ratio of the estimated metabolic cost of growth between the caterpillar and the cockroach nymph at the cellular level is relatively equivalent to one obtained at the organismal level. The caterpillar cells were more vulnerable to oxidative damage than the cockroach, having lower cell viability and a higher percentage of apoptotic cells after treatment with oxidant. Therefore, the cost-quality hypothesis was established to be true.

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1. INTRODUCTION

The metabolic cost of growth is the amount of metabolic energy required to synthesize a unit of biomass (1). The metabolic cost of growth has been extensively studied in agricultural and aquacultural animals where its relevance in profit maximization and optimizing efficiency in animal production was underscored. The metabolic cost of growth is estimated in insects using the energy budget model, Equation (1):

$$B = E_m G + B_{MA} \quad (1)$$

where B, E_m, and G represent the metabolic energy, metabolic cost of growth, growth rate and B_{MA} is the amount of energy allocated for maintenance and activity (7,54). The energy cost of biosynthesis, digestion, and transportation of digested nutrients as well as the energy cost of foraging are the physiological and ecological processes to which metabolic cost of growth is attributed (4,8,26,27,29,30,65). The cost quality hypothesis was proposed (1) when these physiological and ecological processes were unable to explain the extremely low metabolic cost of growth in the painted lady butterfly caterpillar.

Oxidative stress develops when there is an imbalance between pro-oxidants (reactive oxygen species (ROS) and reactive nitrogen species) and antioxidants in favor of pro-oxidant (38). Hydrogen peroxide is a reactive oxygen species (ROS) that cause massive damage to cellular macromolecules at higher concentration. The low molecular weight of hydrogen peroxide enables it to pass through the cell membrane to oxidize susceptible intercellular components, resulting in apoptotic and necrotic cell death (51). The physiological actions of hydrogen peroxide include disruption of protein synthesis, lipid peroxidation and disruption of the cellular membrane, impaired energy production,

oxidation of oxygen scavengers, oxidation of nucleosides, enzyme inhibition, and ultimately cell death (51 - 53).

The two insect species used in this study: *Blatta lateralis* (Turkestan cockroach) and *Vanessa cardui* (painted lady butterfly) have very different life histories. Vanessa cardui (painted lady butterfly) is holometabolous: insects that undergo complete metabolism. This group of insects consists of approximately a million named species of insects (49) and represent about 50% of all animal diversity (50). The developmental stage of the life history of *Vanessa cardui* (painted lady butterfly) is completed in 5-6 weeks with a 2 week long feeding (larval) stage. The mode of development in the hemimetabolous is remarkably different from that of the holometabolous. Hemimetabolous insect species undergo a complete metamorphosis where the embryos hatch into nymphs that are a miniature resemblance of the adults. *Blatta lateralis* (Turkestan cockroach) is the hemimetabolous insect species used as a reference in this study. It lives up to 2 years and the nymphal stage ranges from 100 to 200 days (54). The metabolic cost of the growth of the cockroach nymph was found to be 20-fold higher than that of the caterpillar (1). Farrel et al. hypothesize that compared to the cockroach nymph the caterpillar achieves its fast growth rate by spending less energy on the biosynthesis of macromolecules which would cause its cells to be more vulnerable to oxidative stress (1). In this study tert-butyl hydroperoxide (t-BHP) was used to induce oxidative stress. t-BHP is widely used as a better alternative to hydrogen peroxide in oxidative stress studies because it is more thermodynamically stable and does not easily decompose into water and oxygen.

2. LITERATURE REVIEW

Holometabolous and hemimetabolous insects have sharply different life histories and physiologies. One of the most noticeable distinction is in their growth rates. Compared to hemimetabolous species, holometabolous insects generally grow fast during the larval stage. For example, the caterpillar of *Vanessa cardui* (painted lady) grows almost 30-fold faster than the nymph of *Blatta lateralis* (Turkestan cockroach) with the same dry body mass (1). From the energetic viewpoint, the growth rate depends on the total amount of metabolic energy allocated to biosynthesis and the energy required to synthesize one unit of biotissue (1-8). If the total amount is the same, the cheaper the unit cost is, the faster the growth is. The unit cost of biosynthesis, denoted as E_m, varies significantly among species (1, 8). We have found (1) that the value of the metabolic cost of the growth of the Turkestan cockroach is about 20-times higher than that of painted lady caterpillar, which results in the slow growth of cockroach.

The metabolic cost of growth is the amount of energy invested to fuel the anabolic biochemical reactions of biosynthesis. It is referred to as the respiratory cost of growth (8, 26 -29), appears as 'Organizational energy or the energy expended for the "work" of growth and morphogenesis' in Brody (1945, page 2) and Wieser (1994)(8) interpreted it as the implicit in the growth coefficient K3 of Ivlev (42). In some literature metabolic cost of growth is defined as the amount of metabolic energy required to synthesize a unit of biomass (1). The metabolic cost of growth has been extensively studied in agricultural and aquacultural animals where it has been reported to parallel economic profit in agricultural and animal husbandry via its connection to the efficiency of production (2,40,43,57). In

the estimation of this quantity, many studies used the simplified energy budget which was reported to suffer conceptual flaws. Farrel et al. employed models from Joblin,1985 (61) and Peterson et al, 1999 (7) to estimate the Em value, where the energy allocated to maintenance and activity was not considered negligible as other literature (39**,** 41).

The physiological and ecological processes the value of the metabolic cost of growth has been associated with are: the energy cost formation of peptide bonds during deposition of new protein, RNA transcription, mitosis, and lipid biosynthesis and metabolism; energy cost of ingestion, digestion, transportation and absorption of nutrients and energy cost of foraging (4,5,7,8,26, 27, 28, 30,31, 65). Animal body mass has a direct influence on the energy cost of foraging and physiological cost inherent to growth (7). In animals with the same growth rate but different body mass, the energy requirement for the supply of molecular components of the same amount of bio-tissues per unit time should be higher in larger body sized animals than the one with smaller body size. Likewise, in the same spatial distribution, the energy requirement for obtaining food would higher in the larger animal than the smaller one. This was different from the observation of Ferral et al. (1) where the painted lady caterpillar was reported to have an extremely metabolic cost of growth despite a similar body size as the cockroach nymph (1). Furthermore, the efficiencies of the biochemical transformation of diet ingredients to body tissue are different. Energy costs associated with protein biosynthesis have been demonstrated to be higher than the energy of lipid synthesis. Based on diet composition, animals with high protein and low lipid content in their bio-tissue and a low protein and high lipid diet should require a higher amount to energy for protein synthesis and biochemical transformation to lipid. However, the caterpillar diet contained a low protein and lipid content in their bio-

tissue and its diet is of low protein and high lipid content, yet has an extremely low metabolic cost of growth.

Ferral et al. proposed the cost-quality hypothesis to explain the extremely low metabolic cost of growth in the painted lady butterfly caterpillars (1). It suggests that the unit cost of biosynthesis largely determines the "cellular quality" of biotissues, including the number of errors in protein and DNA sequences, resistances to stresses, and rate of senescence. Taking protein homeostasis as an example, the value of metabolic growth cost depends on amino acid compositions (9-11), which affect the protein stabilities (12, 13), and the proofreading efforts (14, 15), which are tightly associated with protein fidelity (16). Most importantly, the value of metabolic growth cost is largely determined by the degree of tolerance to mistakes in protein synthesis. A species with low error tolerance would spend more energy (high growth cost) on making one unit of protein, if newly synthesized proteins are quickly unfolded and refolded via the chaperon activities, and/or degraded and resynthesized via the proteasomal activities. These activities, on one hand, slow down the net gain of biomass, and therefore increase the value of metabolic growth cost; on the other hand, they also slow down protein aggregation and improve protein homeostasis (17-20).

Considering their life histories, this hypothesis explains the great difference in the values of metabolic growth cost between holometabolous and hemimetabolous insects. In the former, a large portion of biomass synthesized during the larval stage serves as energy storage for reproduction, instead of functional structures, during the adult stage. Such tissues are disintegrated and remodeled during the pupal stage (21-25). Thus, synthesizing high-quality bio-tissues during the larval stage, which requires a high amount of energy, would be economically wasteful for these species.

Theoretically, the cost-quality hypothesis agrees with these biochemical and life history observations, but it has never been tested directly with dedicated experiments. The goal of this study is to test the hypothesis by comparing energy investments of cell replication (the cost) and cellular resistance to oxidative insult (quality) between cultured midgut cells from painted lady caterpillar and Turkestan cockroach. We choose midgut cells, because it is a typical tissue that is degraded during the painted lady's pupal stage, and therefore a good candidate tissue to test the hypothesis.

Farrel et al., have shown (1) that the values of E_m are ~300 and ~6000 Joules per gram of dry mass in painted lady caterpillar and Turkestan cockroach nymph, respectively. But these values were measured in living animals, averaging over all types of tissues. At the whole organismal level, this difference in metabolic growth cost may stem from the differences in the network efficiencies of digesting and transporting metabolites, the ratios of weights of tissues that have different energy costs, and locomotion activities (4, 5, 7, 8, 26-31). None of these factors is directly connected to protein homeostasis. Thus, to test the hypothesis, the evidence at the whole organismal level is not sufficient. In this study, we investigated the energy cost of replication of cells from the same tissue cultured in the same media at the cellular level and to investigate the difference in tissue qualities between the species, we assay the cellular resistance to oxidative insult using cell viability and percentages of apoptotic cells at multiple levels of oxidant concentrations as the index of resistance.

3. MATERIALS AND METHODS

3.1. INSECTS

Painted lady caterpillars and cockroach nymphs were reared at 25 ± 1 ºC. painted lady caterpillars were fed ad libitum with sucrose and protein-based diet (Carolina biological supply, NC. 80% moisture; per unit dry food has 13 – 15 % protein content and a negligible amount of lipid content). Cockroaches were supplied with Wardley Pond Pellets (Hartz Mountain Corp., Secaucus, NJ; the protein and lipid contents of the dry mass are 33% and 5.5%, respectively). The water supply was limited.

3.2. REAGENTS AND SOLUTIONS

Grace insect medium, p-hydroxybenzoic acid methyl ester, and fetal bovine serum were from Thermofisher Scientific, Rockford, IL. FITC-Annexin V/7AAD was generously supplied by Dr. Yue -wern Huang's lab. Insect physiological solution (NaCl 178 mM, KCl 4.3 mM, $CaCl_2$ 4.3 mM, $NaHCO_3$ 3.8 mM, 0.5% gentamicin, 0.01% antibiotic antimycotic PH 6.5) and PBS buffer (8.00 g NaCl, 0.20 g KCl, 1.29 g Na2HPO4·3H_2O, 0.20 g KH_2PO4, 1000 mL ddH_2O, pH 7.4) was prepared and sterilized using 0.22 µm filter. Septisol, Sodium hypochlorite, antibiotic antimycotic solution, gentamycin, and vitamin mixture were from Sigma, St. Louis, MO.

3.3. CELL ISOLATION AND CULTURE

Gut cells were isolated using a modified protocol from Hakim et al. (32). Insects were surface sterilized by submerging consecutively in 20% Septisol, 0.1% *p*-hydrobenzoic acid methyl ester, and 1% sodium hypochlorite for 2 min. Surface sterilized

insects were transferred to the culture hood in sterile Milli Q water and dissected in sterile insect physiological solution. Guts were pooled in insect physiological solution containing 0.001% sodium hypochlorite and washed twice in sterile insect physiological solution before transferring to a well in 6-well plate (1483210, Thermofisher Scientific, Rockford, IL). Cells were maintained in Grace insect medium supplemented with 10% heat-inactivated fetal bovine, 0.1% gentamicin, vitamin mixture, and, 0.1% antibiotic antimycotic at 28 ºC.

After 24 h, primary cell culture was filtered using 70 µm cell strainers (CLS431751, Thermofisher Scientific, Rockford, IL) to remove gut explants. Cells were collected after gentle pipetting and washed twice in 0.1 M cold PBS buffer (8.00 g NaCl, 0.20 g KCl, 1.29 g $Na_2HPO_4 \cdot 3H_2O$, 0.20 g KH_2PO_4, 1000 mL ddH_2O, pH 7.4) to be used in further analysis.

3.4. CELLULAR RESPIROMETRY

Midgut cells maintained in complete grace insect medium were resuspended at 0.5 x 10^5 cells/µL in the same medium. An oxygen electrode (Mitocell 200A; Strathkelvin Instruments, Glasgow, UK) equipped with a fast-response fluorinated ethylene propylene membrane was used to measure metabolic rate at 27 °C. In brief, the electrode was calibrated with air-saturated water (high-point, 267 µM) and 2% (wt./vol) Na_2SO_3 in 0.01 M Na_3BO_3 buffer (zero point). Metabolic rates were measured in 5 min intervals.

3.5. CELL VIABILITY

Cells were collected after gentle pipetting and an initial cell density of 1 x 10^6 cells mL^{-1} was seeded in a six-well plate. After 30 min, fresh media containing t-BHP was added

to a final concentration of 3 mM, 6 mM, 12 mM, 15 mM, 50 mM, 100 mM, and 200 mM. After a 6-hour incubation at 27 °C, treated cells were collected directly in the 15 ml centrifuge tubes and washed twice at room temperature in 0.1 M cold PBS buffer. Cell pellets were resuspended in 5 µL 7AAD staining solution, incubated in dark at room temperature for 15 minutes, and cells were analyzed within an hour. Using the flow cytometer (Beckman Coulter Cytoflex), Forward scatter (FSC) vs Side scatter (SSC) gates were set appropriately to exclude debris and cell aggregate, untreated cells (negative control) stained with 7-AAD was used to define the basal level of dead cells and set up the necessary laser compensation and the 7-AAD fluorescence was collected at FL3 channel.

3.6. CELL POPULATION GROWTH

Cells were resuspended in fresh media to an initial cell density of 0.3 x 10^6 cells mL^{-1} were seeded in a 125 cm^2 culture flask. Cell counting was performed using the Nauber hemocytometer over 48 hours. Cell viability was obtained using 7AAD single staining and fluorescence was measured using the flow cytometer.

3.7. ANNEXIN V APOPTOSIS

Simultaneous staining of cells with Annexin V – FITC (green fluorescence) and 7-aminoactinomycin (7AAD) (red fluorescence) allows the discrimination of intact cells (Annexin V – FITC negative, 7AAD negative), early apoptotic cells (Annexin V – FITC positive, 7AAD negative), late apoptotic (Annexin V – FITC positive, 7AAD positive) and dead cells (Annexin V – FITC negative, 7AAD positive). Negative control with untreated cells was used to define the basal level of apoptotic and necrotic cells. To set up flow

cytometer compensation and quadrants, untreated unstained cells, untreated cells stained with Annexin V – FITC alone, and untreated cells stained with 7-AAD alone were used as additional controls. After exposure to t-BHP for 6 hours at 27 °C, cells were transferred directly to centrifuge tubes and washed once at room temperature in cold PBS. Cell pellets were resuspended in 100 µL of 1X binding buffer solution at a final concentration of 1 X 10^6 cells/ml. To each 100 µL of cell suspension, 5 µL of Annexin V - FITC and 5 µL 7AAD staining solution was added. The mixture was incubated at room temperature in the dark for 30 min. After incubation, 400 µL of 1X binding buffer solution was added and cells were analyzed within one hour. Using a flow cytometer (Beckman Coulter Cytoflex), FSC vs SSC gates were set appropriately to exclude debris and cell aggregate, the single-color controls were used to set up the necessary laser compensation, and the Annexin V – FITC and 7-AAD fluorescence were collected in appropriate channels.

3.8. DATA ANALYSIS

Experiments were performed at least twice using different isolations of midgut cells cockroach nymph and caterpillars. Statistical analyses of metabolic rates, oxidative damage, and apoptotic data were performed by Student's t-test and by two-way ANOVA followed by Tukey's post hoc test. Log and arcsine transformation were performed on data to satisfy normality and constant variance assumptions. In metabolic rate analysis, the null hypothesis was that there was no difference in the mean metabolic rate of the caterpillar and the cockroach while the alternative hypothesis was that the mean metabolic rate of the caterpillar was less than that of the cockroach. The t-test with unequal variance was run and the p-value was obtained. In the Student's t-test analysis of oxidative damage and

apoptosis data, for each of the t-BHP concentrations, the null hypothesis was that there was no difference in the means of the percentages of viable cells and apoptotic cells between the cockroach and caterpillar while the alternative hypothesis was that the means of the percentages of viable cells and apoptotic cells in the caterpillar was less than that of the cockroach nymph. The t-test with unequal variance was run and p values obtained. In the two-way ANOVA analysis of the oxidative damage and apoptotic data, the interaction effect of the oxidant concentration and insect species on cell viability and apoptosis were determined. From the interaction plot, there appears to be an interaction between the oxidant concentration effect and the insect species effect, hence, the main effects of the oxidant concentration and insect species were not analyzed. The null hypothesis for the global F test was the average of the percentages of viable and apoptotic cells was equal to that of the cockroach while the alternative the percentages were not equal between the two insect species. After the null hypothesis was rejected for the global F-test, the Tukey pairwise comparison was performed. $p < 0.05$ was considered as statistically significant.

Population growth data was statistically analyzed using exponential regression. Using Excel, a scatterplot of the cell count as the response variable and time in hours has explanatory variable was plotted. The exponential trendline and equation were then added. Thereafter, the correlation coefficient was obtained.

4. RESULTS

4.1. POPULATION GROWTH RATES AND METABOLIC RATES OF THE TURKESAN COCKROACH AND PAINTED LADY BUTTERFLY CELLS

From the regression analysis, the growth rate of caterpillar cells was not significantly different from the cockroach nymph cells. Cockroach growth rate = 19 cells/min, $R^2 = 0.9888$ and caterpillar growth rate is 18 cells /min, $R^2 = 0.9941$. Metabolic rates of cockroach (0.27 µg O_2/min/million cells) is higher than caterpillars (1.075 µg O_2/min/million cells), p-value < 0.001.

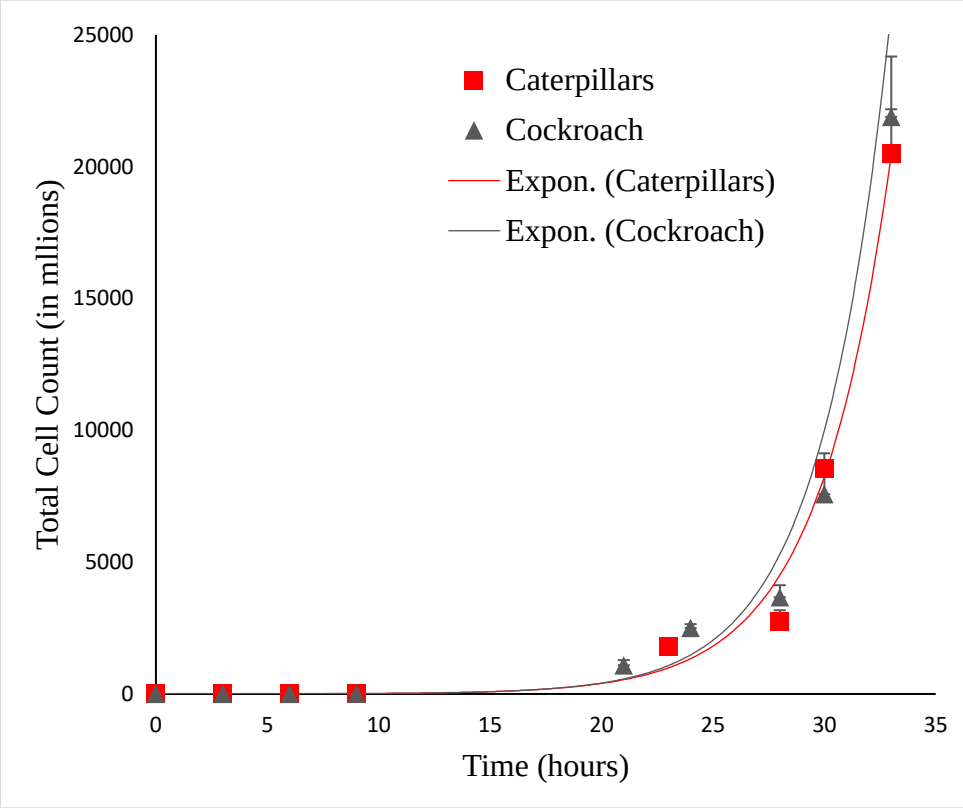

Figure 4.1 Population growth curve of cockroach nymph and painted lady butterfly caterpillar cells

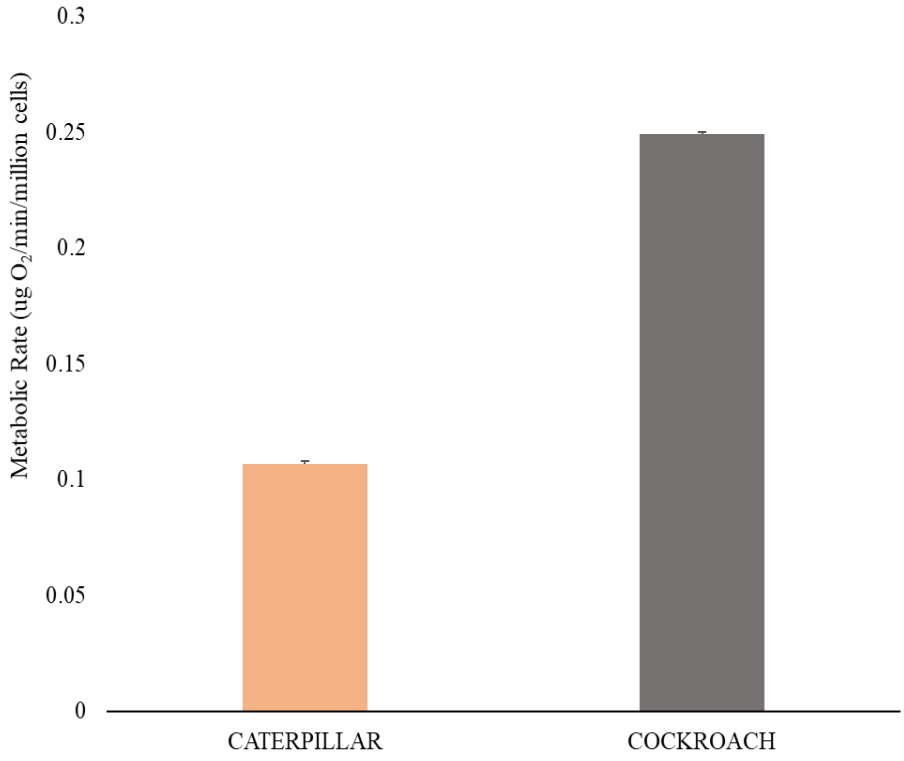

Figure 4.2 Metabolic rate of cockroach nymph and painted lady butterfly caterpillar cells

4.2. ESTIMATE OF THE METABOLIC COST OF GROWTH IN THE CATERPILLAR AND COCKROACH NYMPH

At the cellular level, we can assume energy spent on maintenance and activity (energy expended on locomotion, feeding, and other activities) is negligible. Thus Equation (1) becomes:

$$B = E_m G \qquad (2)$$

The estimated values of the metabolic cost of growth from the measured metabolic rates and growth rates for the caterpillar and cockroach are 0.015 μO_2/million cells and 0.056 μO_2/million cells respectively. The metabolic rate of the Turkestan cockroach is 3.94-fold higher than that of the caterpillars (B = 1.0757/B = 0.2727). Similarly, the metabolic cost

of the growth of the cockroach nymph is 3.75-fold higher than the caterpillars' (0.056/0.015) (Figure 4.1 & 4.2).

4.3. COCKROACH NYMPH CELLS ARE MORE RESISTANT TO OXIDATIVE DAMAGE THAN CATERPILLAR CELLS

7AAD viability staining showed that t-BHP significantly decreased cell viability in caterpillar cells at 50 mM concentration while the cockroach cells were less affected (Figure 4.3 and 4.4) (*p-value = 0.0046).

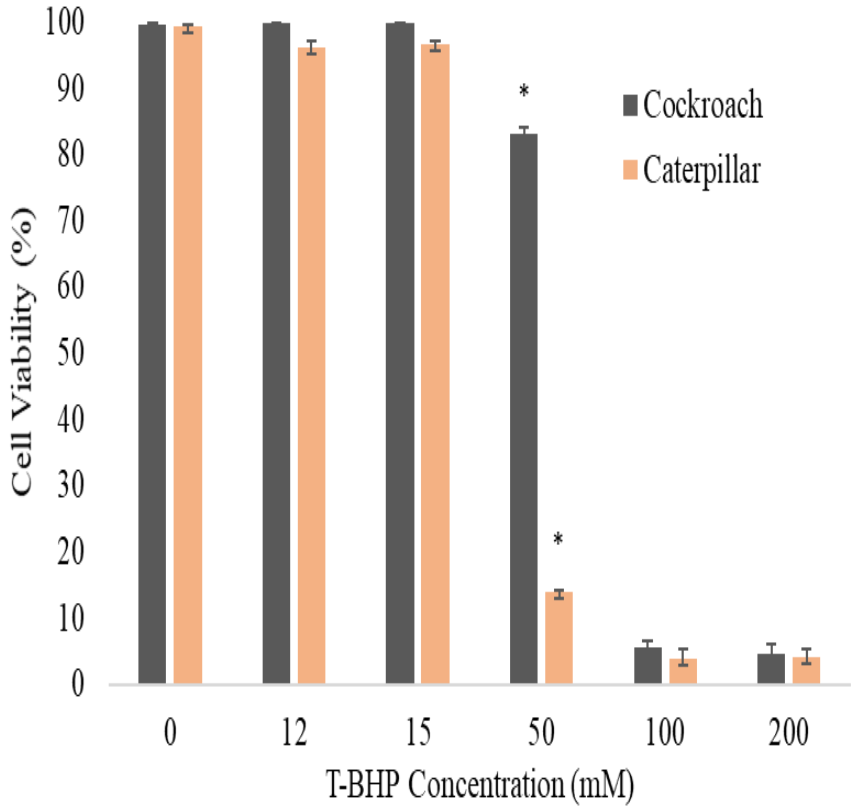

Figure 4.3 Cell viability of cockroach nymph and painted lady butterfly cells after six-hour exposure to t-BHP

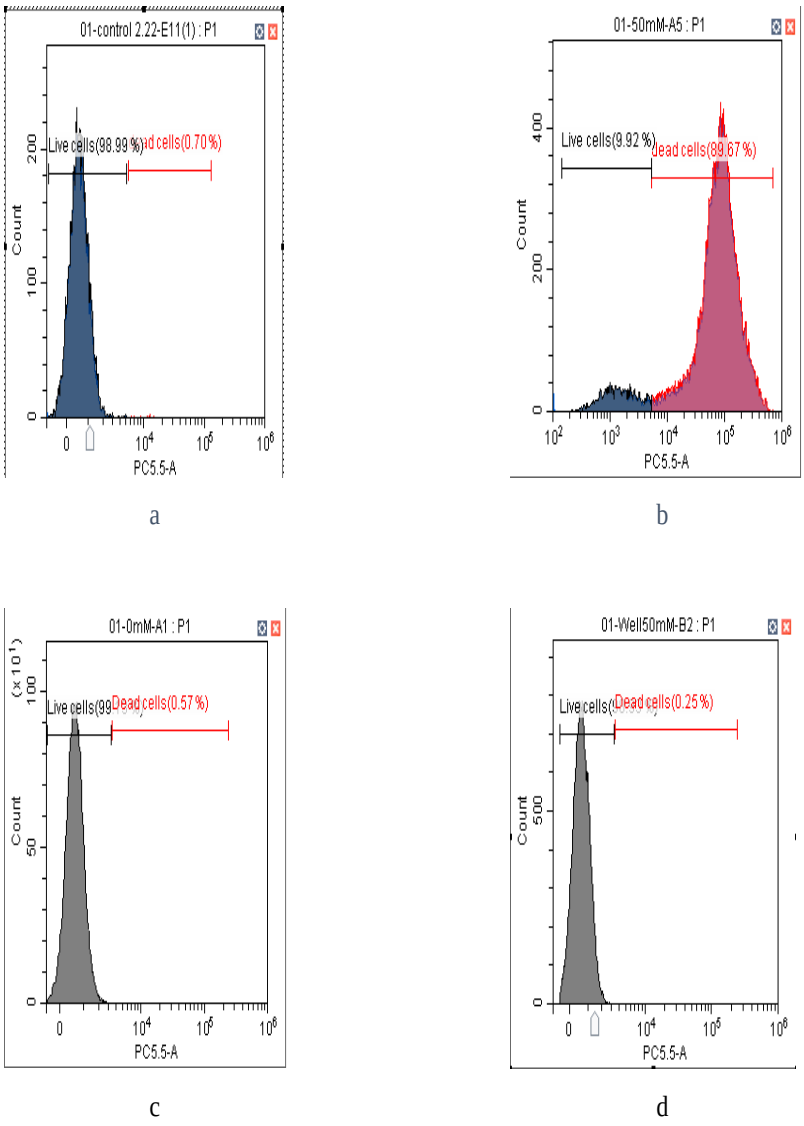

Figure 4.4 Flow cytometry analysis of cell viability of painted lady caterpillars' cells (a = control and b = 50 mM t-BHP) and cockroach nymph cells (c = control and d = 50 mM t-BHP)

4.4. T-BHP INITIATED APOPTOSIS IN THE CATERPILLAR CELLS BUT NOT IN COCKROACH CELLS

Using Annexin V and 7AAD double staining, the percentages of apoptotic cells at low concentrations of t-BHP was obtained. At lower concentrations (12mM and 9mM) t-

BHP induced apoptosis in painted lady butterfly caterpillars' cells but not in Turkestan cockroach cells. Cockroach cells has significantly higher resistance to apoptosis than caterpillar cells (*p value= 0.004892, **p value= 0.021)

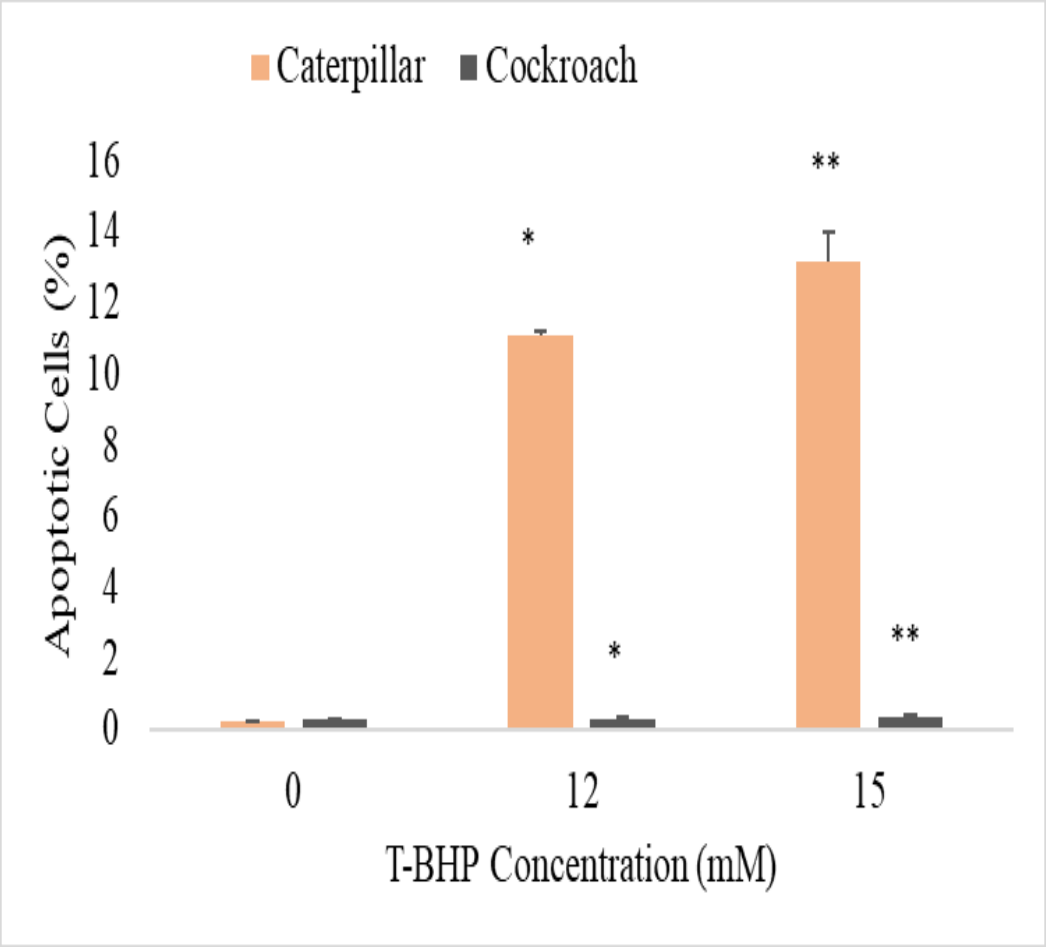

Figure 4.5 Caterpillar cells are more vulnerable to apoptosis than cockroach cells

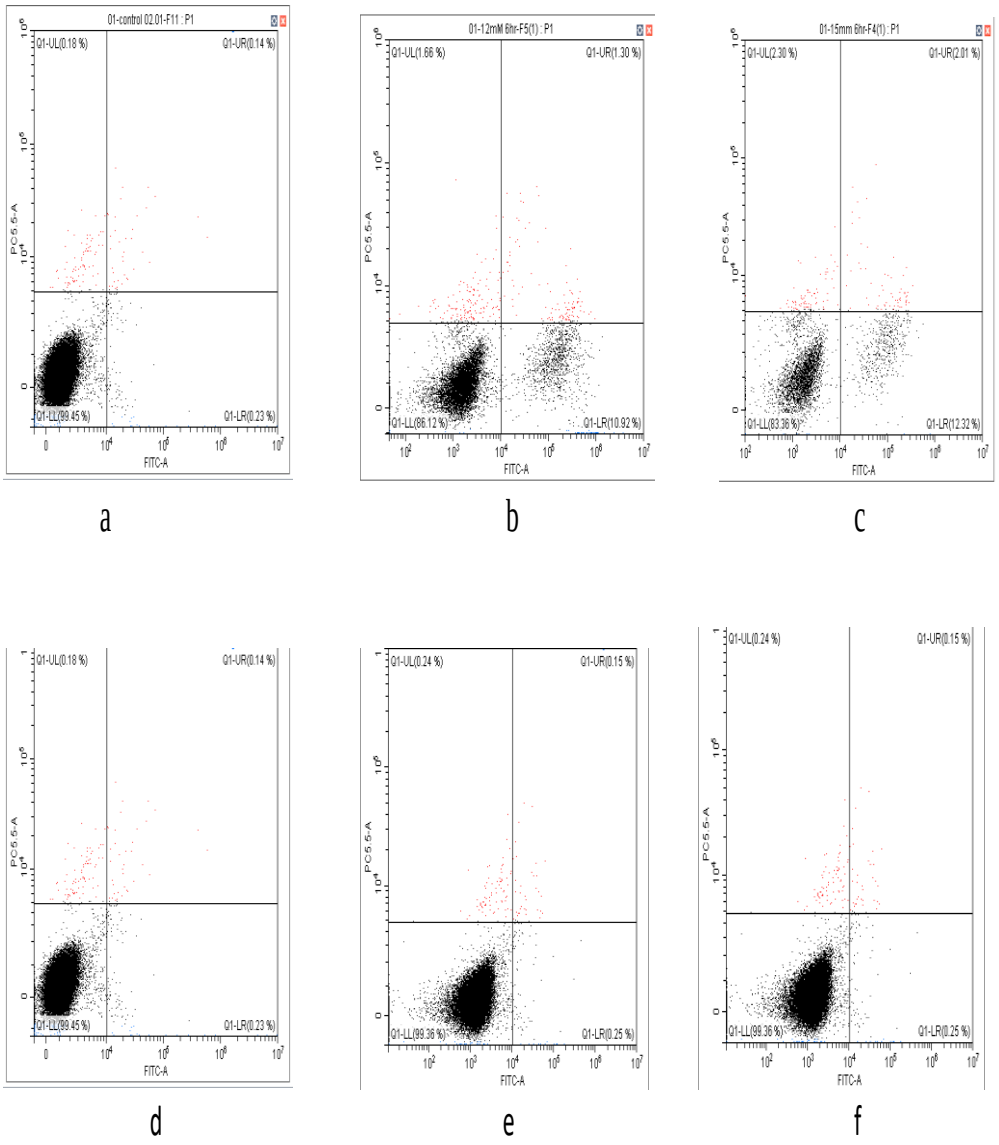

Figure 4.6 Flow cytometry analysis of apoptosis of painted lady caterpillars' cells (a = control, b = 12 mM t-BHP and c = 15 mM t-BHP) and Turkestan cockroach cells (d = control, e = 12 mM t-BHP and f = 15 mM t-BHP)

5. DISCUSSION

The metabolic cost of growth is an important component of an animal's ontogenetic energy budget and is relevant in understanding the physiology and ecology of insects. The extremely low metabolic cost of growth estimated in holometabolous insect larvae in comparison to the hemimetabolous insect species could not be explained by the physiological and ecological factors associated with growth cost. In this study, we were able to establish that the painted lady caterpillar achieved its high growth rate (14 – 21 days) at the expense producing high quality while the cockroach nymph slow growth rate (100 – 200 days) was compensated with the production of a high-quality cell as the cost-quality hypothesis (1) proposed. First, to compare the biosynthesis energy cost in the cellular and organismal level of organization, the metabolic rates and growth rates in the midgut cells of the two insect species was estimated. Surprisingly, we found the growth rate of the caterpillar was approximately equivalent to that of the cockroach (0.3028 for caterpillar and 0.3178 for cockroach, Figure 4.1). This differed from the values reported at the organismal level by Farrel et al. (1) where the growth rate (dry mass gain per day) was 0.354M and 0.0130M dry body mass (M is the dry body mass that varies between 0.002 to 0.15g) for the caterpillar and cockroach respectively and the ratio the growth rate of the caterpillar was 27-fold higher than that of the cockroach nymph. A significant difference in the metabolic rate of the two insects was also observed (Figure 4.2). In previous literature (1), the metabolic rate of the insects at the organismal level reported was $2976.5M^{0.794}$ and $1101.9M^{1.068}$ for the caterpillar and cockroach and the ratio of the metabolic rate of the caterpillar to the cockroach nymph reported was 6.1 ± 1.87 but here we estimated this value

to be 3.9. In the estimation of the metabolic of the Turkestan cockroach and the painted lady butterfly caterpillar, the energy expended on activity and maintenance ($B_{M, A}$) included in the energy budget model (1) and was reported to be $2982.4M^{0804}$ and $1261.0M^{1.165}$ for the caterpillar and the cockroach respectively. The caterpillar was spending 7.06 ± 3.06-fold energy on maintenance and activity than the cockroach nymph. Here, we estimated the metabolic growth cost assuming that the energy allocated to maintenance and activity in cells is negligible since cells expend a negligible amount of energy on maintenance, locomotion, feeding, and other activities compared to whole organisms. Using the simplified energy budget model, equation (2) (39, 41), the estimated values of the metabolic cost of growth from the measured metabolic rates and growth rates for the caterpillar and cockroach are 0.015 μO_2/million cells and 0.056 μO_2/million cells respectively and the ratio of the metabolic cost of growth in the caterpillar and cockroach was estimated to be 3.75. The growth cost ratio we estimated is approximately 5-folds less than the value reported at the organismal level (1). This difference in metabolic growth cost in the cellular and organismal levels may stem from the differences in the ratios of weights of tissues that have different energy costs in different tissues and organs and locomotion activities in whole organisms (4, 5, 7, 8, 26-31). Besides, differences in metabolic rates in individual organs and tissues have been reported (67,68) and considering that the metabolic and biosynthesis cost estimated was for a tissue (midgut), we can assume that difference in the ratio of growth cost estimated at the cellular level gives a snapshot of the overall metabolic cost through the five levels of organization (cells to the organismal level) in the two insect species. Taken together, the higher metabolic cost of growth of the

Turkestan cockroach compared to the caterpillar at the cellular level is reflective of the estimated value at the organismal level.

To explore the quality of the Turkestan cockroach and caterpillar cells, oxidative damage was induced using different concentrations of t-BHP. The cell membrane is among the most vulnerable cellular component to oxidative stress. T-BHP oxidizes membrane phospholipid initiating lipid peroxidation resulting in loss of plasma membrane integrity and permanent plasma membrane permeabilization (65). 7-Aminoatinomyin D (7AAD) is a fluorescent cell viability dye that is excluded from cells with an intact membrane but penetrates dead or damaged cells and binds to the double-stranded DNA by intercalating between the cytosine and guanine bases of the DNA. It allows discrimination of viable cells using flow cytometry. From flow cytometry analysis, 7AAD viability staining showed that the cockroach cell was unaffected on exposure to the oxidant at 50 mM t-BHP concentration while there was a decrease in viability of the caterpillars' cells (Figure 4.3 & 4.4). At low concentrations of oxidant (3mM - 15 mM), the viability of cells was over 90% and there was no significant difference in the viability of the cockroach and caterpillar cells. Similarly, very high concentrations of t-BHP (100 mM and 200 mM) were severely lethal to both cell types and no significant difference in viability was observed.

Another interesting observation is the resistance of both insect species cells to low concentrations of t-BHP compared to mammalian cells. Concentrations as low as 50 μM t-BHP and a shorter incubation period was reported to induce oxidative damage in endothelial cells (66) and rat hepatocytes (37). This corroborates Kumar et al.'s study where the Lepidopteran insect cells were demonstrated to have higher extensive resistance to membrane damaging agents compared to mammalian cells (36).

Finally, we explored the resistance of Turkestan cockroach and painted lady caterpillar cells to apoptosis at low concentrations of t-BHP. Cell death could either be regulated cell death (apoptosis, autophagy, entotic) or accidental cell death (necrosis) (54). Apoptosis, programmed events by specialized cell machinery that ultimately lead to cell death, is crucial to maintaining tissue homeostasis (33). Reactive oxygen species cause DNA double-strand breakage, lipid peroxidation, mitochondria dysfunction, cell membrane disruption, and disruption of protein biosynthesis which may induce apoptosis and ultimately cell death (51 - 53). At lower concentrations, Annexin V/7AAD double staining showed that t-BHP initiated apoptosis in the caterpillar cells but not in cockroach cells (Figure 4.5 & 4.6). The initiation of apoptosis indicates the vulnerability of the caterpillar's cell macromolecules to oxidative stress. For instance, DNA strand breakage may have occurred in the caterpillar cell as a result of oxidative damage which sequentially initiated apoptosis. When DNA double-strand breakage occurs, the cell surveillance pathways arrest proliferation in G1, S, or G2 checkpoint in response to damage (34). The cell cycle checkpoint is dependent on the induction of p53, tumor suppressor protein, which induces cell cycle arrest to promote DNA repair or initiates apoptosis when the DNA damage is serious and impossible to repair (48). Lipid peroxidation of the caterpillar cell membrane could as well play an important role in inducing apoptosis. The product of lipid peroxidation destroys DNA, protein, and enzyme activity (55), damage phospholipids directly, and act as an inducing signal for programmed cell death (56). Mitochondria dysfunction caused by the opening of permeability pore due to damage and crosslinking of membrane thiols (44-47) is another possible apoptotic inducer in the caterpillar cell. Oxidation of proteins causes changes in protein structure resulting in protein denaturation,

reduced solubility, and loss of biological functions (58). The most error-prone step in gene expression is protein folding. Oxidative stress leads to the accumulation of unfolded or misfolded proteins, a condition called stressed ER. Stressed Endoplasmic reticulum causes disruption of disulfide bond or inhibition of Ca^{2+} ATPase resulting inn inactivation of enzyme or important signaling molecules. When ER stress is too severe, the proapoptotic signaling pathway is activated in the cell (35,60,62). ER stress also causes mitochondria dysfunction and increase mitochondria reactive oxygen species production.

The higher vulnerability of the caterpillar cells to oxidative stress shows that the caterpillar cells have a poor-quality bio-tissue compared to the Turkestan cockroach has a result of its extremely low energy biosynthesis cost. Taking protein synthesis as an example, amino acid synthesis and efficiency proofreading in have been demonstrated to differ in energy cost (9 – 14) and protein stability is dependent on the presence susceptible amino acid chains side chains containing aromatic side chains (phenylalanine, tryptophan, tyrosine) or sulfur e.g. cysteine and methionine (56,57) or less efficient proof-reading during protein synthesis. So, to achieve its high growth rate, the painted lady butterfly expended a low amount of energy in amino acid synthesis and efficient proofreading resulting in the production of less stable proteins susceptible to oxidative damage.

6. CONCLUSION

In this study, the metabolic cost of growth of the Turkestan cockroach and painted lady butterfly was estimated at the cellular level and the estimated ratio of the growth cost between the two insect species was found to be relatively equivalent to the obtained values at the organismal level of organization. The cost quality hypothesis was established to be true based on the higher vulnerability of the caterpillar cells to oxidative insults than the Turkestan cockroach cells. It would be interesting to test this hypothesis in the caterpillar's cellular macromolecules. If one species has a higher value of biosynthetic energy cost and better tissue quality than the other, the hypothesis suggests that it may have some features of its proteins, such as amino acid composition that leads to better protein stabilities, more efficient proofreading of protein synthesis, a higher turnover rate of newly synthesized proteins, higher chaperon and proteasomal activities. As a test of the hypothesis, one of these mechanisms may be chosen, and the proteasomal activity, which costs a considerable amount of ATP and directly determines protein homeostasis may be investigated.

APPENDIX

RAW DATA

The average population growth of the caterpillar and cockroach cells incubated at 27ºC taken over a 48-hour period and the standard mean error (SEM) (Table A.1. and Table A.2.).

Table A.1. Population growth of caterpillar cells

Time (hours)	Caterpillar
	Total cell count ± SEM
0	1000000
3	2333333 ± 333333
6	4333333 ± 333333
9	15333333 ± 881917
23	1791333333 ± 246745708
28	2754000000 ± 422014612
30	8554666666 ± 573101018
33	20492000000 ± 1686830262

Table A.2. Population growth of cockroach cells

Time (hours)	Cockroach
	Total cell count ± SEM
0	1000000
3	1210000 ± 140000
6	4650000 ± 150000
9	14000000 ± 1000000
21	1090000000 ± 199000000
24	2500000000 ± 146000000
28	3670000000 ± 457000000
30	7580000000 ± 753000000
33	21900000000 ± 2290000000

Mean metabolic rates of the caterpillar cells and cockroach cells measured at 27ºC and the standard mean error (SEM) (Table A.3.).

Table A.3. Metabolic rates of cockroach and caterpillar cells

Caterpillar	Cockroach
Respirometry ± SEM (ugO2/min/million cells)	Respirometry ± SEM (ugO2/min/million cells)
0.1065 ± 0.0015	0.249 ± 0.001

The average of the flow cytometry analysis of cell viability of caterpillar cells and cockroach cells treated with various concentrations of t-BHP and the standard error of mean (SEM) (Table A.4.).

Table A.4. Cell viability of caterpillar and cockroach cells after treatment with t-BHP

t-BHP concentration (mM)	Caterpillar	Cockroach
	Cell viability ± SEM (%)	Cell viability ± SEM (%)
0	99.357 ± 0.175	99.705 ± 0.065
12	96.17 ± 0.865	99.81 ± 0.01
15	91.24 ± 1.185	99.77 ± 0.13
50	13.9 ± 0.31	95.24 ± 0.96
100	3.89 ± 1.35	5.46 ± 1.09
200	4.235 ± 1.06	4.56 ± 1.46

The average flow cytometry analysis of Annexin V apoptosis of caterpillar cells treated with different concentrations of t-BHP and the standard error of mean (SEM) (Table A.5.).

Table A.5. Annexin V apoptosis analysis after treatment with t-BHP

t-BHP concentration (mM)	Caterpillar	Cockroach
	Apoptotic cells ± SEM (%)	Apoptotic cells ± SEM (%)
0	0.25 ± 0.05	0.28 ± 0.065
12	11.07 ± 0.15	0.3 ± 0.07
15	13.165 ± 0.845	0.325 ± 0.075

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