Runaway Metabolism in Crickets: Analysis of Anomalous CO_2 Release after Heat-induced Death

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Abstract

Ten to fifteen minutes following death, a large release of CO_2 is produced in many species when killed by high temperature. Studied in mosquitoes, hissing cockroaches, grasshoppers, and desert harvester ants, this post-mortal peak (PMP) appears to be temperature-dependent and, to our knowledge, does not occur in insects killed by means other than high temperature. Four effects were applied to common house crickets (*Acheta domestica*) to analyze the origin and properties of the PMP. First, it was shown that the PMP does not occur without oxygen. Second, post-mortal CO_2 release was studied as a function of temperature-exposure following death and it was established that the phenomenon is dependent on extreme temperatures and runs to completion when exposed to temperatures above 60°C. Third, basic and buffered solutions were employed to assess the possible involvement of dissolved HCO_3^- (bicarbonate), the dissolved form of CO_2, in production of the peak. Hemolymph factors like bicarbonate did not appear to have an effect on the PMP. Finally, exposure to hydrogen cyanide inhibited the PMP, demonstrating the involvement of mitochondria and cytochrome c oxidase in particular. Together, these results rule out any effect of hemolymph or possible CO_2 stores in the body of an insect on the PMP. The PMP occurs as an aerobic mitochondrial reaction that requires high initiation temperatures. We believe that this underlying cause may be mitochondrial breakdown at high-temperatures. More specifically, fluidity of the mitochondrial membranes likely increases with high heat, disabling the established proton gradient and ATP production. The resultant accumulation of electron carriers allows for cyclic, but futile operation of the citric acid cycle and electron transport chain with remaining pyruvate stores.

Introduction

Within the past decade, researchers have made headway in understanding the breakdown of cellular metabolism following death. In 2004, Lighton and Turner provided a method to directly measure metabolic rates of insects in changing temperatures, aptly named Thermolimit Respirometry. This technique measures rates of released CO_2 against changing temperature in real time with great accuracy, but exposed a novel phenomenon on its maiden voyage: release of CO_2 long after death.

Lighton and Turner described their findings in their 2004 paper, which aimed to better describe the Critical Thermal Maxima (CT_{max}), using two species of desert harvester ants (*Pogonomyrmes rugosus* and *P. californicus*). Ramping temperatures up from 25°C, the authors observed an increase in the ants' metabolic rate, illustrated by CO_2 release, as expected. As temperature rose to near-lethal levels, CT_{max}—the temperature at which muscle control is lost—was reached soon after a 'pre-mortal plateau' in the insects, marking the loss of muscle-control, which included spiracle opening (Klok et al, 2004). This benchmark closely approximates insect death (Vorhees, 2012). Following CT_{max}, CO_2 release rate rapidly decreased, forming a sharp "post-mortal trough" in the waveform data recording; yet, within ten to fifteen minutes a large release of CO_2 was seen (Lighton and Turner, 2004). This "post-mortal peak" (PMP) replicated many times in many species since, can be seen often magnitudes greater than CT_{max} following insect death (Figure 1).

The PMP is a relatively new phenomenon and little is known about its cause. A few characteristics of the PMP have been described in past research. Although measured in the same way as metabolism, the PMP does not share all of its qualities. The PMP is independent of mass, but exhibits a positive correlation with CT_{max} magnitude (Lighton and Turner, 2004). Lighton and Turner also noted the peculiar nature of the delayed release, as spiracle control ceased and spiracles most likely opened long before the peak of CO_2 release was seen. CO_2 would be expected

to rapidly fall after spiracle opening, but the delayed release of the PMP suggests it results from a biochemical process that occurs after death. The authors thought this process to be either a release of dissolved CO_2 in hemolymph or a metabolic reaction caused by high-heat, leaving much to question.

The hypothesis of hemolymph CO_2 release has since been tested and discredited. Put simply, CO_2 stored within the body would be expected to release upon spiracle opening. Although sometimes contested, it is thought that spiracles remain closed until oxygen is needed and tracheal PCO_2 is high (Hetz and Bradley, 2005). Spiracle opening at CT_{max} allows for diffusion of any remaining CO_2 before death. Nonetheless, this hypothesis required a detailed exploration before it could be discredited. Molich et al (2012) provided the first evidence against hemolymph CO_2 release by testing the oxygen-dependency of PMPs in fruit flies (*Drosophila melanogaster*). By switching input air from atmospheric gases to pure nitrogen after the appearance of CT_{max}, the authors observed that post-mortal peaks did not appear in the absence of oxygen (Molich et al, 2012). Furthermore, water-vapor analysis revealed a significant release of water during CT_{max}, confirming spiracle-control to fail before death.

Gas exchange in insects lacks many confounding pathways found in higher animals. Without significant presence of cooperatively-binding oxygen-carriers like hemoglobin (Molich et al, 2012), metabolic gases travel to and from cells with few enzymatic interactions, providing the observed release of CO_2 to be a reliable representation of metabolism. The PMP may appear in vertebrates, but presence of complex cardiopulmonary systems and O_2-binding proteins could mask direct release of metabolic products (Vorhees, 2012). However, the Pill Bug (*Armadillidium vulgare*), a crustacean, does not produce an observable PMP (Klok et al, 2004). Incidentally, Pill Bugs lack trachea and should be further researched as their anatomy or physiology may correlate with the observed anomaly. This indicates the PMP is not necessarily a universal phenomenon and must be replicated before it can be viewed as a broad occurrence among many differing species.

Only 14% of CO_2 produced by metabolism dissolves as a gas, the remainder reacts with H_2O by enzymatic catalysis with carbonic anhydrase to form HCO_3^- (bicarbonate) and H^+, which easily dissolve as ions and revert to CO_2 and H_2O when in high concentration before exhalation (Berg et al., 2002). O_2 does not directly influence CO_2, HCO_3^-, or H^+ dissolved in hemolymph, thus its absence should not alter any release of CO_2 from hemolymph HCO_3^-. This finding suggests the release of CO_2 following death to be in fact metabolic, and that it relies on an aerobic reaction. Glucose enters a eukaryotic cell and is broken down into two molecules of pyruvate, which then enter the citric acid cycle and produce CO_2 by a series of redox reactions. This cycle is the only reaction in catabolism that produces CO_2, as it reduces NAD^+ and FAD to NADH and $FADH_2$ (electron carriers), respectively. However, as the citric acid cycle is linked to many metabolic reactions, additional pathways may play a role in post-mortal metabolism. While only speculation, the authors hypothesized the PMP to be a result of mitochondrial breakdown and subsequent 'decoupling' (loss of) of oxidative phosphorylation, referred to as "hyperthermic overdrive."

Aside from the likely metabolic explanation, other sources of this CO_2 were still possible after the PMP was demonstrated to rely on oxygen. The post-mortal peak of CO_2 may be released from active bacteria within an insect's gut. To test this hypothesis, Vorhees (2012) inoculated mosquitoes *(Culex tarsalis)* with strong antibiotics to kill gut bacteria, but found no significant difference in PMP magnitudes. Gut bacteria do not appear to be a valid explanation for the PMP. Furthermore, in replicating Molich's anoxia study, the absence of oxygen again reduced PMP magnitude to near-negligible levels. Vorhees' paper supports the hypothesis of a "hyperthermic overdrive," but necessitates further replication.

These results, albeit preliminary, suggest that the post-mortal peak arises from aerobic metabolism of the insect. The mitochondria of the eukaryotic cell consume oxygen and produce CO_2 through energy-producing reactions. Specifically, the citric acid cycle within the mitochondrial matrix is solely responsible for CO_2 production during aerobic metabolism, but coincidentally, does

not consume oxygen. This suggests PMP originates within the mitochondria, but is additionally linked to an oxygen-consuming pathway. Within the mitochondria, only the electron transport chain (ETC) consumes oxygen as it oxidizes electron-carriers produced by the citric acid cycle and glycolysis. As NADH and $FADH_2$ are oxidized by the ETC and O_2 is consumed, the four ETC complexes actively transport H^+ from the mitochondrial matrix to the intramembrane space, building a H^+ gradient that is exploited by ATP Synthase to power phosphorylation of ADP to highly-energetic ATP. This process of ATP production yields the highest amount of ATP in catabolism. Adding to Molich's hypothesis, Vorhees (2012) postulates that an influx of H^+ due to heat-induced mitochondrial permeability allows for ATP synthase decoupling and overdrive of the citric acid cycle in order to replenish the electron-carriers consumed by the ETC complex, creating a futile cycle of redox reactions and CO_2 release with no ATP production.

Heat-stress related mitochondrial breakdown has been demonstrated in rat cells. Mitochondrial deformation is observed as temperatures reach 39°C in isolated cardiomyocytes, including loss of cristae-folds, disruption of the outer-membrane, and overall swelling of the organelle (Qian et al, 2004). Oxidative phosphorylation is seen to diminish as temperatures increase past 39°C, and ATP synthase activity significantly decreases. Furthermore, study of Ca^{++} concentrations shows that calcium, normally retained in the mitochondria, diffuses outwards at high temperatures (Qian et al, 2004). These results suggest that mitochondrial membranes denature at high temperatures, allowing loss of Ca^{++} and release of cellular apoptotic-agents, as well as H^+ influx. Influx of H^+ is supported by the significant loss of ATP synthase activity, the final step of oxidative phosphorylation that produces maximal ATP via the established H^+ gradient. With these results, all research to date (Lighton and Turner, 2004; Molich et al, 2012; Qian et al 2004; Vorhees, 2012), points to an aerobic process of metabolism behind post-mortal CO_2 release. Yet, PMP research is only in its infancy and this hypothesis requires further testing.

This research set out to identify the possible mitochondrial explanation to the PMP, all the while assessing the validity of alternative hypotheses. i/Is this release of CO_2 truly a product of aerobic metabolism? Already studied twice by Molich et al (2012) and Vorhees (2012), the oxygen-dependency of PMP will again be assessed through replication in a new and larger species. ii/Does hemolymph play a role in the release of CO_2 following death? Many would argue that CO_2 is stored in the hemolymph due to its conversion to soluble bicarbonate. The equilibrium between CO_2 and HCO_3^- will be altered with pH, the common ion effect, and an enzyme inhibiter to examine the reaction's participation in the PMP. iii/ Is the post-mortal peak temperature-dependent? If the PMP is in fact resultant of mitochondrial enzymes, temperature at death should dictate rate of CO_2 production, regardless of hemolymph effects. iv/Finally, is hyperthermic overdrive a valid explanation of PMP? Decoupling of embedded mitochondrial enzymes and subsequent unrestricted aerobic metabolism between the citric acid cycle and the electron transport chain is a likely explanation of the delayed generation of CO_2 following death. The role of the mitochondria, particularly the electron transport chain will be tested through inhibition to assess the mitochondria's contribution to the PMP as well as the linkage between O_2-dependency and CO_2 release. Crickets were used in this study as they are larger than previous species studied and have yet to be researched. A move to a larger insect should replicate and hopefully support previous findings.

Methods

Insects

Five- and six-week old house crickets (*Acheta domestica*) were purchased from Fluker Farms (Port Allen, Louisiana) and kept at room temperature. Crickets were fed Fluker's Cricket Quencher and Fluker's Turtle Diet (Fluker Farms, Port Allen, Louisiana) as well as diced carrots. Crickets received food and water daily, which were consumed rapidly, suggesting the crickets to be

healthy. Crickets were chosen at random for experiments, but those appearing unhealthy, injured, or slow to react were discarded.

Thermolimit Respirometry

Insects were placed inside a 4.0 mL glass testing chamber within a PELT-5 temperature controlled chamber (Sable Systems, Las Vegas, Nevada). Atmospheric air travelled through a series of two silica columns followed by a column containing Ascarite (Thomas Scientific, Swedesboro, New Jersey) and Drierite (W.A. Hammond Drierite Company, Zenia, Ohio) to remove CO_2 and H_2O before it was supplied to the testing chamber. The input air was kept at a flow rate of 200 mL•min^{-1} using a Side-Trak flowmeter (Sierra Instruments, Monterey, California) controlled by a MF-2 mass flow-controller (Sable Systems, Las Vegas, Nevada). Temperature was monitored with a TC2000 Thermocouple meter (Sable Systems, Las Vegas, Nevada) receiving analog input from a thermocouple sensor inserted in the air-tubing upstream of the test chamber. CO_2 and H_2O signatures were measured with a LI-7000 infrared gas analyzer (Li-Cor, Lincoln, Nebraska) connected to the test-chamber's air output. All analog data output was collected and converted to a digital stream by a UI-2 universal interface (Sable Systems, Las Vegas, Nevada), and in conjunction with LI-7000 digital output, recorded and analyzed by ExpeData software (Sable Systems, Las Vegas, Nevada).

Ramping Protocol

Baseline data were recorded for at least one minute before and after every trial, and CO_2 and H_2O values zeroed beforehand. Upon initiation, each trial maintained a constant 25°C for twenty minutes to allow the crickets' metabolic rate to equilibrate. Although a ramping rate of 0.25°C•min^{-1} has been established as the most accurate rate for smaller insects (Lighton and Turner, 2004), a rate of 0.5°C•min^{-1} was determined fast enough to prevent acclimation, but slow enough to ensure metabolic changes. A ramping rate of 0.5°C•min^{-1} was then established for sixty minutes to achieve a final temperature of 55°C. The temperature at 55°C was held for ten minutes

to ensure observation of CT_{max}, death, and the PMP before being rapidly ramped-down to 25°C. Ramp rate conditions have been proven to alter outcomes for values like RMR, MMR, and CT_{max}; thus, ramp rate may directly correlate to the magnitude of CO_2 produced by the post-mortal peak (Terblanche et al, 2007). To minimize this effect, ramp rate was kept at 0.5°C•min^{-1} throughout all trials.

Control Group

To assess the characteristics of the normal PMP in crickets, 12 male and 12 female crickets were ramped to 55°C without prior treatment. The cricket's CO_2 release rate and change in temperature were recorded and graphed as a function of time without any interruption. The recorded PMPs often took up to fifteen minutes.

Hemolymph alterations

To test the role of hemolymph in modifying and transporting CO_2, insects were injected with: i/a basic solution that would favor more [HCO_3^-] and [H^+] in equilibrium, reducing CO_2 release; ii/ a bicarbonate solution that would favor more [CO_2] in equilibrium, increasing release of CO_2; and iii/ a carbonic anhydrase inhibitor that would halt or slow CO_2 release if the gas-ion conversion were a significant factor. Insect saline was made from 175.3 mg NaCl, 44.8KCl, 29.0 mg $CaCl_2$•H_2O, and 264.0 mg $MgCl_2$•$6H_2O$ per 100.0 mL and titrated to 320 mOsm, the approximate osmolarity of cricket hemolymph. This solution was used as a control to assess any adverse effects of inoculating crickets. A solution of 320 mOsm NaOH was prepared and used to test the effect of increasing pH on hemolymph CO_2 release. Sodium Bicarbonate was dissolved in ddH_2O to create 332 mOsm bicarbonate solution to test the common ion effect.

Insects were sometimes anesthetized with CO_2 before injection (for the researcher's sanity) and allowed to recover for ten minutes before testing. This was not expected to cause error as CO_2 is limited only by diffusion and reaches equilibrium within minutes and injectable solutions do not take effect until fifteen minutes after inoculation (Gulinson and Harrison, 1995). All injections were

carried out using a 10.0 µL Hamilton Syringe (Hamilton Company, Reno, Nevada); males received 1.2 µL of solution, and females, 1.7 µL. These doses were calculated to produce averages of 7.2 $\mu L \bullet g^{-1}$ of body mass in females, and 5.9 $\mu L \bullet g^{-1}$ in males per the protocols of Gulinson and Harrison (1995).

Finally, 99% Acetazolamide (LOT#:10112594, Alfa Aeser, Great Britain) was titrated with equimolar NaOH to produce soluble Sodium Acetazolamide, then diluted to prepare a 10.0 mL stock solution of 324 mOsm for injection. Insects were injected before being tested to examine the effect of carbonic anhydrase inhibition on hemolymph CO_2 release. It should be noted that sodium acetazolamide was not as soluble as was desired. The prepared solution was gently warmed before injection to increase solubility of the drug in solution and was compared to the non-alkylated solution, which was warmed in the same manner. The original drug remained a white crystalline in solution (even at high temperatures), while the prepared solution fully dissolved with light heat.

Temperature dependency

To test the post-mortal peak's dependence on temperature, two experiments were designed to assess the effect of temperature on kinetics and possible activation energy (E_A) of the reactions causing PMP. In the first experiment, ice-baths were prepared and the testing chamber quickly submerged following death. If enzymatic, exposure to cold temperatures should greatly slow, if not halt, release rates of CO_2 during the post-mortal phase. In many trials, the cold challenge was kept for up to forty-minutes before rapid rewarming with 80°C water.

In the second experiment, water baths were prepared at 55°C, 60°C 65°C, 70°C 75°C, 80°C, and 90°C and used to envelope the testing chamber following cricket death. If enzymatic, PMP release rate should correlate with temperature. Both subgroups were recorded and analyzed by ExpeData to decipher possible reaction rate changes.

Oxygen dependency

To test the dependency of the PMP on atmospheric oxygen, crickets were exposed to anoxic air immediately after death and the change in CO_2 release analyzed. To do so, a second line of tubing connected medical-grade N_2 gas (Airgas, Cleveland, Ohio) to the air input upstream of the testing chamber via a three-way valve used to select between atmospheric air and the pure nitrogen input. Following death, the input line was quickly switched from atmospheric air to N_2 to flush the chamber of any oxygen during the post-mortal fall and the results observed and analyzed in ExpeData. The N_2 input met the testing apparatus upstream of the flowcontroller, in order to standardize nitrogen input to 200 $mL \bullet min^{-1}$.

Mitochondrial inhibition

Potassium Cyanide (Lot#:085978; Fisher Scientific, Mapton, New Hampshire) was loaned from the Colorado College Chemistry Department to study the participation of the mitochondria, specifically cytochrome c oxidase, in producing post-mortal peaks. Hydrogen Cyanide made from KCN acts as a strong chelating agent—despite being a weak acid—binding metals with high affinity (Harris, 2010). Much like carbon monoxide, HCN's triple bond allows it to bind to Iron (II) in cytochrome c oxidase with an affinity for the ligand orders of magnitude greater that oxygen's, displacing oxygen from its natural enzymes. The only two metabolic oxygen-binding proteins are hemoglobin and cytochrome c oxidase. Hemoglobin and similar proteins are not found in substantial numbers within insects (Nation, 2002), leaving only cytochrome c oxidase to be inhibited by hydrogen cyanide.

A separate chamber, in parallel to the testing chamber, was filled with the KCN and a syringe filled with 3.0 M H_2SO_4 was attached and sealed to the top of this chamber. Upon subject death, 1.0 mL of H_2SO_4 was injected to make roughly 0.05 M Hydrogen Cyanide gas and three-way valves at either end of the chamber were activated so that only HCN was allowed into to the chamber. It should be noted that a downstream vent was used to clear the line of HCN to prevent

damaging the gold-lined LI-7000, producing a thirty to forty second data gap in recording during HCN exposure. To ensure accuracy, several trials were conducted without the use of HCN. Upon first appearance of a PMP upslope LI-7000 input was disrupted for 45 seconds or more. In each trial, it was found that a gap even as large as 60 seconds in the recording obstructed only a fraction of the post-mortal peak profile (Figure 2).

Data Collection

The total fraction of CO_2 released in each post-mortal peak was extrapolated from ExpeData by 2-point drift correction and integration of the area underneath the PMP curve. This value was converted to Total Release Volume (TRV) in milliliters, a product of the total volume of air to flow through the test chamber at 200 $mL \bullet min^{-1}$ multiplied by the parts per-million of released CO_2 represented by the area underneath the curve. The total duration of the PMP release was recorded in seconds, and Average CO_2 Release Rates (ARR) were calculated for each data point in $mL \bullet S^{-1}$. To later compare data subsets, both TRV and ARR were weight-corrected with that cricket's mass in grams.

Statistical Analysis

Data were compiled in Microsoft Excel 2011 (Microsoft Corporation, Redmond, Washington) and arranged by subset. StatPlus (AnalysisSoft, Vancouver, British Columbia) was used for basic two-tailed t-tests assuming equal variances, and IBM SPSS (IBM, Armonk, New York) was used to analyze normal distribution, homogeneity of variances, general linear models and two-way ANOVAs. For each statistical comparison, the weight-correct ARR and TRV values were logarithmically-transformed to return normal distribution in each case. Interestingly, gender showed a significant influence in normal distribution, but did not contribute to significant differences in later ANOVA tests. Using SPSS, potential outliers were selected in distribution histograms, but none were removed, as no Grubbs-tests expressed significance. Any obviously odd or irregular CO_2-waveforms recorded were intentionally left out of the data prior to analysis. All

linear models and t-tests were performed again in Excel 2011 to ensure analyses were conducted properly.

Injections trials were compared to the control group by a two-tailed t-test assuming equal variances between the control group and saline-injection group to assess for confounding factors associated with the injection procedure. After identification of any confounding factors involved the injection process, all injection trials were compared to one-another and the control to assess for any of the chemicals' effects. A two-tailed ANOVA assuming equal variances was performed for every injection group, including both saline and the non-inoculated control, and followed by a Turkey HSD post-hoc multiple-factor analysis in the case of significant difference.

Temperature dependency

During six cold-temperature trials, PMP returned with rapid rewarming via introduction of 80-90°C water following 40 minutes of cold exposure (Figure 3). In these delayed warming trials, initial PMP volume released before freezing was recorded and analyzed in summation with PMP volume released during rewarming. As well, duration of both release events were summed to determine the combined ARR. Two-tailed t-tests assuming equal variances were performed on these summed PMPs in comparison to the control group.

Waterbath trials were replicated and collected in groups of 55°C (n=2), 60°C (n=10), 65°C (n=11), 70°C (n=10), 75°C (n=8), 80°C (n=9), and 90°C (n=9), however; both the 55°C and 90°C groups were removed for their outlying spreads when compared to median groups. A third dependent variable was assessed: duration of PMP in seconds, and log-transformed for normalcy of fit (Table 3). PMP Duration was then tested with a general linear model.

Mitochondrial inhibition

Hydrogen cyanide ARRs and TRVs were compared to those of control group's by two-tailed t-test assuming equal variances.

Results

Hemolymph alterations

Twelve crickets were first injected with saline and their metabolic pattern and PMP were compared to non-injected control crickets. Crickets were then injected with NaOH (N=6), $NaHCO_3^-$ (N=7), and sodium acetazolamide (N=7) to test the role of hemolymph CO_2 in the PMP. There were no significant differences between injected and a non-injected crickets (ARR *P*=.661, TRV *P*=.563). Analysis of variance found no significant differences among all injection trials and the control (Table 1).

Temperature dependency

Seven crickets were submerged in an ice bath during the post-mortal valley. The resulting post-mortal peak profiles closely resembled those seen in nitrogen-gas trials: there was no observable PMP. Upon introduction of the cold challenge, escaping CO_2 rates rapidly decreased and reached near-zero values. Even during late exposure to the ice-baths, the crickets' PMPs were cut short, regardless of whether PMP was approaching its peak or bottoming-out. In trials where PMP returned after re-exposure to warm water, The TRV did not significantly differ from that of the control group (*P* =0.001) and neither did the ARR (*P* =0.001) in the combined PMPs. This indicates that while PMP can be halted by exposure to ice, and is not only a temperature-dependent reaction, it is predetermined in volume once it has begun.

Sixty-eight crickets were submerged in waterbaths at different temperatures after death. Both TRV and ARR were significantly effected by exposure to these waterbaths (*P*= 0.013 and *P*= 0.047, respectively) and gender had no significant effect. However, both TRV and ARR, were negatively correlated to temperature (R^2= 0.175 and 0.128, respectively) (Figures 4 and 5). A general linear model within SPSS reveled that temperature had a significant effect on duration (*P*=0.003) with a good-fit linear regression (R^2=0.210), but this relationship was in fact positive.

Oxygen-dependency

In six trials, airstream was switched from room air to pure nitrogen gas during the post-mortal plateau. In all six cases, no PMP was seen. However, in 95% of the control trials involving no switch, a PMP was clearly visible. There was a significant difference between the N_2 group and the control group in occurrence of the PMP (TRV: P=0.0269 and ARR: P=0.003).

Mitochondrial inhibition

Exposure to hydrogen cyanide gas during the PMP's initial upslope wiped the recording clean of any traces of CO_2, and later exposure during the peak's apex or downslope quickly reduced initial PMP CO_2 release to near-zero values. Crickets were left in the chamber for at least twenty minutes following return of normal air to assure death had occurred. No traces of PMP were seen after the release of hydrogen cyanide (Figure 6). Again, both data subsets of total PMP volume and release rate were compared with those of the control group. The rates of cyanide-poisoned crickets were significantly decreased in comparison to the control group (TRV P<0.001, ARR P=0.017).

Discussion

These results have answered many specific questions about the post-mortal peak while also better illustrating the general nature of the PMP. Four questions were asked in designing this research, and the above results offer answers to all. i/Is this release of CO_2 truly a product of aerobic metabolism? The dependency of the PMP on oxygen indicates that it is aerobic and metabolic, this is further demonstrated by inhibition of the mitochondria through cyanide poisoning. ii/Does hemolymph play a role in the release of CO_2 following death? Hemolymph does not appear to account for or alter PMP expression in crickets, as squish tests revealed no release of PMP and this has been replicated in other insect species as well. Furthermore, a lack of oxygen would not prevent dissolved CO_2 from escaping from the hemolymph. Finally, no injected chemicals or drugs intended to alter hemolymph dynamics produced significant changes in the PMP; however, this does not rule out confounding factors associated with the injection protocols.

iii/ Is the post-mortal peak temperature-dependent? While the differential temperature trials yielded confusing results, the ice-bath results clearly indicate that PMP is both a temperature-dependent and irreversible process. iv/Finally, is hyperthermic overdrive a valid explanation of PMP? These results fit the hypothesis of hyperthermic overdrive, however they do not confirm its validity. Hyperthermic overdrive appears to explain the PMP of CO_2 through a decoupling of two important pathways in ATP production, but further analysis of these pathways is required before the hypothesis can be confirmed. In an important ending note, no other alternative hypotheses to date have been proven plausible.

Hemolymph Alterations

The physical process of injection and the use of CO_2 in anesthesia did not alter the cricket's post-mortal peak in any way. Crickets injected with saline did not differ in their PMP signatures compared to their control counterparts, even though some crickets were anesthetized with CO_2 before injection. The injection protocols established therefore did not contribute to any error in the experimental design and were used for all injection trials once these results were reported.

CO_2 dissolved in the hemolypmh as either a gas or its ionic counterparts did not contribute or account for the PMP seen in insects. Alterations of a cricket's hemolymph with chemical compounds did not significantly alter the PMP. NaOH would be expected to decrease CO_2 release, as neutralization of H^+ would cause consumption of CO_2 to replace H^+ in equilibrium. On the other hand, introduction of bicarbonate would push equilibrium towards CO_2 (away from excess HCO_3^-) and cause greater release of CO_2 during the PMP. As neither of these solution appeared to affect equilibrium, it seems that hemolymph does not store quantities of CO_2 sufficient to yield the PMP. Also, sodium acetazolamide had no significant effect on CO_2 release, and it can be understood that carbonic anhydrase activity does not play a significant role in the PMP.

However, this lack of significant effect may also indicate the amounts injected were insufficient. The dosages used in this experiment were retrieved from Gulinson and Harrison's

1995 paper, in which they altered grasshoppers's tracheal gases with the same volume to weight ratios for each compound used. While effects were significant within grasshoppers and one would expect that same effect to carry to a smaller species at the same weight-based dosage, it is possible that these dosages were not high enough for crickets. Thus, the injection results cannot be taken as definitive until studied further.

Temperature dependency

The PMP peak shows indications of a chemical reaction. Firstly, freezing-cold water halted the PMP entirely. Its absolute dependence on high-temperature indicates that it possesses an activation energy which must be overcome with energy input. Secondly, the return of a normal PMP after reintroduction to warm temperatures occurs as if though PMP was paused and later resumed. This suggests that there is a precursor to PMP reactions, a molecule that is definite in amount and is not consumed by any other pathways. This notion of a precursor fits with a metabolic hypothesis. Metabolism is comprised of a collection of committed and usually non-reversible enzymatic pathways that share a common macromolecule: acetyl-CoA derived from pyruvate. Pyruvate is created through glycolysis and used in citric acid cycle and electron transport chain reactions. In ectotherms, metabolism is directly proportional to external temperature. With increasing external temperature, aerobic metabolism rates increase, requiring higher glycolysis rates and yielding more pyruvate. As pyruvate, able to enter any of the anabolic or catabolic pathways, would not enter a biosynthesis pathway at times of high metabolic demand, it continues to enter the citric acid cycle, as the possible precursor.

However, further temperature trials offered confusing and almost contradictory results. At death, insects were submerged in water at 55°C, 60°C 65°C, 70°C 75°C, 80°C, or 90°C to analyze temperature's effects on possible reaction kinetics. One would expect volume to stay constant through all temperatures, as a metabolic reaction would be limited by precursor reactants available. However, volume stayed constant below 60°C but decreased with increasing

temperature. This could be due to fast recovery of the seeping mitochondrial membrane when brought to temperatures below that of its critical maxima, halting PMP production and trapping products within the matrix. At exceedingly high temperatures, metabolic enzymes may denature as well, reducing the cellular machinery available in the mitochondria. Further research into enzymatic interactions should be conducted to establish specific enzymes responsible and their heat of denaturing.

Oxygen dependency

The PMP comes from an oxygen-dependent pathway that occurs after death, indicated by significant loss of PMP volume (TRV) and release rate (ARR) in nitrogen gas trials. Across all previous insect species tested, lack of oxygen after death inhibited even the smallest fraction of the PMP release; this was replicated in all the crickets tested. As oxygen was not displaced until after death, the underlying oxygen-consuming reaction must occur following death. Moreover, this oxygen-dependency suggests that the process is aerobic and metabolic, as oxygen is consumed strictly by metabolism in eukaryotes. However, oxygen consumption and carbon dioxide production do not occur in the same metabolic pathway. Previously illustrated, only the citric acid cycle produces CO_2 in response to aerobic metabolism that consumes O_2 in the electron transport chain. Normally, these pathways occur in the mitochondria and are linked to one another to produce maximum metabolic energy. The citric acid cycle consumes pyruvate made from glucose to produce the high-energy electron carriers NADH and $FADH_2$ the ETC than uses the electrons of these carriers to produce a proton (H^+) gradient across the internal mitochondrial membrane. This gradient is later used for ATP production by ATP synthetase. Yet, as this phenomenon is seen after death, the assumption that these two pathways are still directly linked cannot be made.

Mitochondrial inhibition

The PMP results from both the citric acid cycle and the redox reactions of the electron transport chain. Hydrogen cyanide only inhibits cytochrome c oxidase in insects, the fourth enzyme

in the electron transport chain. As suspected in oxygen-dependency trials, the citric acid cycle and ETC are still linked to one another with electron-carriers and play some role in the production of the PMP. The CO_2 seen is from the citric acid cycle linked to the ETC for NAD^+/FAD regeneration as per usual mitochondrial activity. Without doubt, cyanide inhibition of cytochrome c oxidase indicates that the process of PMP release originates within the mitochondrial matrix and is associated with the ETC. Anaerobic processes do not occur in the mitochondria, and no non-metabolic processes are linked to the ETC.

Conclusion

Upon exposure to upper critical temperatures, insects release a large bolus of CO_2 ten to fifteen minutes after death. Many hypotheses have been proposed to explain this post-mortal peak. The PMP occurs long after any tracheal gases release from the dead insect (Lighton and Turner, 2004). By squishing insects, Vorhees (2012) showed that release of CO_2 dissolved in the hemolymph was not sufficient to account for the PMP. Oxygen dependency infers the PMP originates in metabolism. Moreover, antibiotic trials have shown that gut bacteria are not a plausible source of the PMP (Vorhees, 2012). Oxygen dependency, temperature-dependency, and cyanide inhibition in summation infer the PMP originates within the mitochondria due to a process normally seen in aerobic metabolism. There have not been any additional sources of stored CO_2 identified to possibly play a role in the PMP.

The post-mortal peak results from some form of coupling of the citric acid cycle to the electron transport chain. This reaction appears to fully consume pyruvate and does not occur until after death. As well, the skewed results of temperature trials infer that high temperatures may cause other changes within the insect cell, possibly denaturing any enzymes involved and reducing the CO_2 released by this process. Further research should be pursued to better illustrate the metabolic pathways that include the citric acid cycle and the electron transport chain and cause this

massive release of CO_2 after death. Yet, there is a hypothesis that fits, but does not conclusively explain, the presence of the PMP at extreme heats.

"Hyperthermic overdrive" is believed to occur due to mitochondrial breakdown (Molich et al, 2012). High heats may increase the fluidity of the mitochondrial membrane and exceedingly allow the accumulated H^+ to seep back inward. As the H^+ gradient is lost, ATP production halts, but NADH and $FADH_2$ produced in the citric acid cycle can enter the electron-transport chain and repetitively recycle H^+ outward, cyclically producing NAD^+ and FAD^+, the cyclic coupling of the ETC and citric acid cycle continues to renew NAD^+ and FAD^+ until glucose-derived pyruvate remaining in the cell in consumed. Further research is required to confirm this hypothesis. Particularly, differing glucose stores should produce differing PMP magnitudes, and trials comparing starved and overfed insects should be attempted.

As the PMP is a mitochondrial phenomenon, one would expect it to occur across all eukaryotes. Future research should aim to capture the post-mortal peak in animal species other than insects. Vertebrates are expected to produce the PMP when killed by high heats, though it may be masked by respiratory machinery much more complex than that of an insect. Study of isolated vertebrate cells will show a better picture of a possible PMP in vertebrates, by removing these confounding factors. As well, study of isolated mitochondria would better describe the mitochondria's involvement in the post-mortal peak and the reaction pathways that may occur after death.

In higher vertebrates, cardiac ischemia and failure has been linked to mitochondrial membrane degradation at high-heats. Mitochondria not only provide cellular energy, but are a major source of calcium in sarcomeres and have been identified as an important checkpoint in cell cycle regulation (Qian et al, 2004). In humans, serious conditions like heat stroke, heat exhaustion, and febrile seizures are often fatal due to heart failure after long-exposure to high-temperatures. It has been thought, but unconfirmed, that mitochondrial dysfunction in cardiac muscle cells results

in cellular apoptosis and heart tissue infarction through inadequate metabolism (Qian et al, 2004). Study of the post-mortal peak may elucidate further explanations of vertebrate responses to temperature extremes.

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Appendix I: Figures and Tables

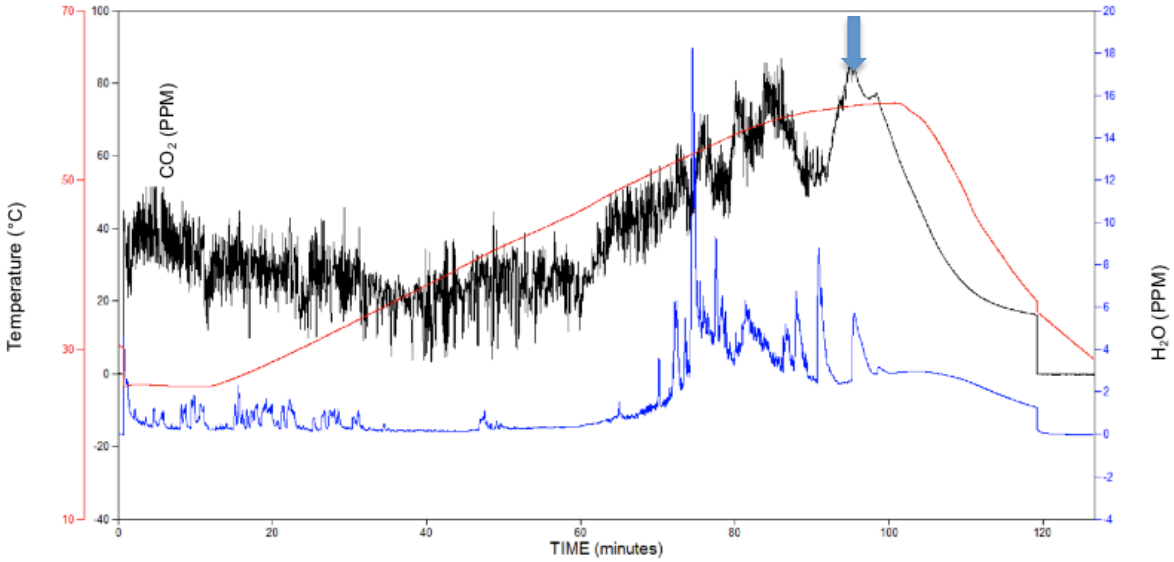

Figure 1. A normal presentation of the post-mortal peak in a male cricket. The waveform marked CO_2 represents VCO_2 in PPM; H_2O, the VH_2O in PPT; and Temperature, the temperature in °C. CT_{max} is marked by an arrow. Before and after the introduction of a cricket, baseline measurements in all three channels can be seen at each end of the recording.

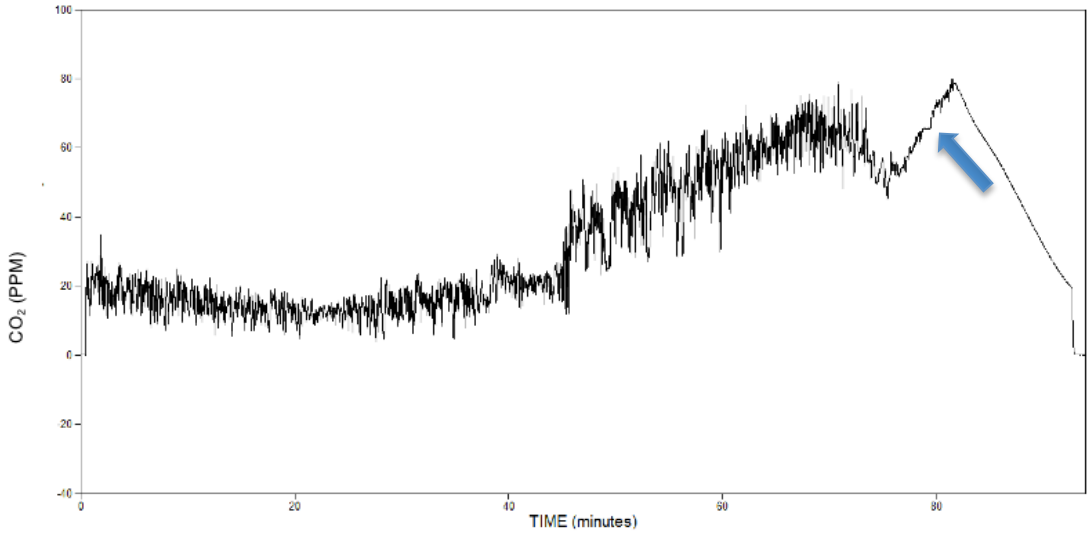

Figure 2. Interruption PMP recording in a female cricket. Marked by the arrow, the input to the LI-7000 was stopped for 45 seconds, demonstrating its minimal effect on summation of the PMP.

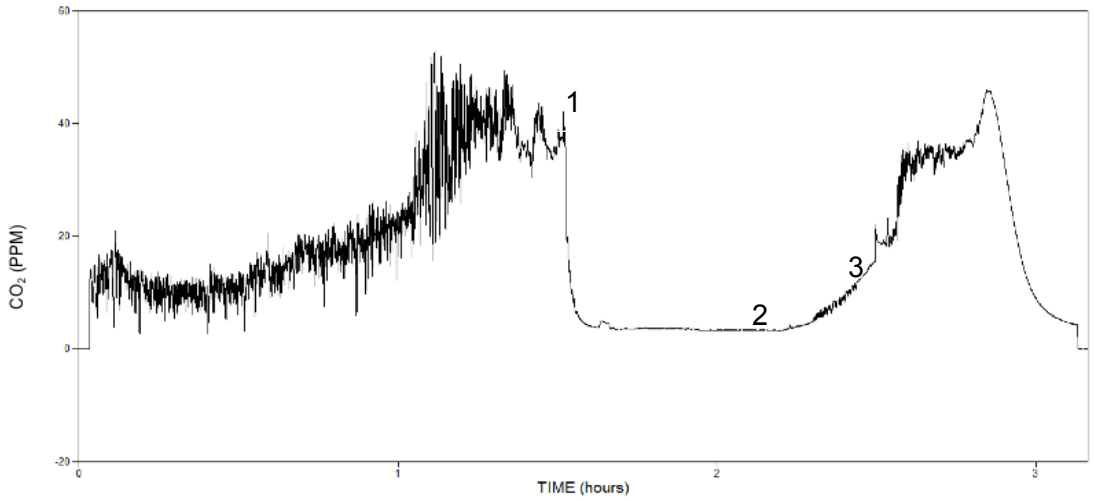

Figure 3. **Return of PMP following exposure to ice water in a male cricket.** Number 1 marks the starting upslope of the PMP and delivery of the cold-water challenge. Number 2 represents the removal of the cold-water and introduction of water at or above 80°C. Number 3 represents the return of the PMP, which when added to its starting upslope at number 1 did not significantly differ from control group PMPs.

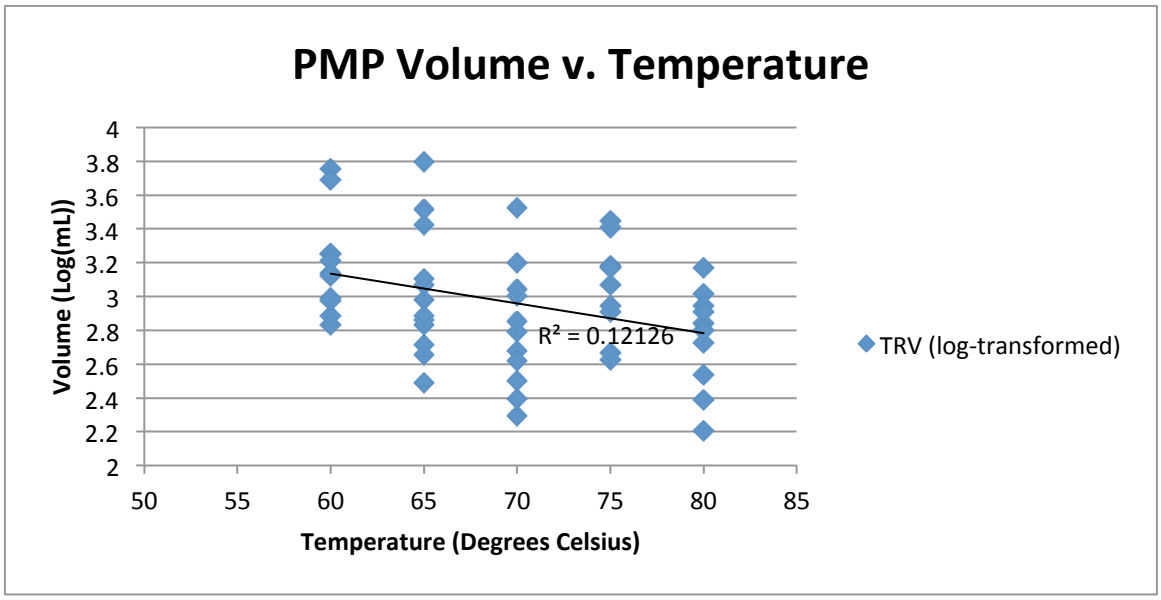

Figure 4. Linear regression analysis of the effect of exposure to waterbaths at different temperatures on log-transformed PMP volume. Deviation is seen below 60°C, but temperature shows a negative relationship to temperature above.

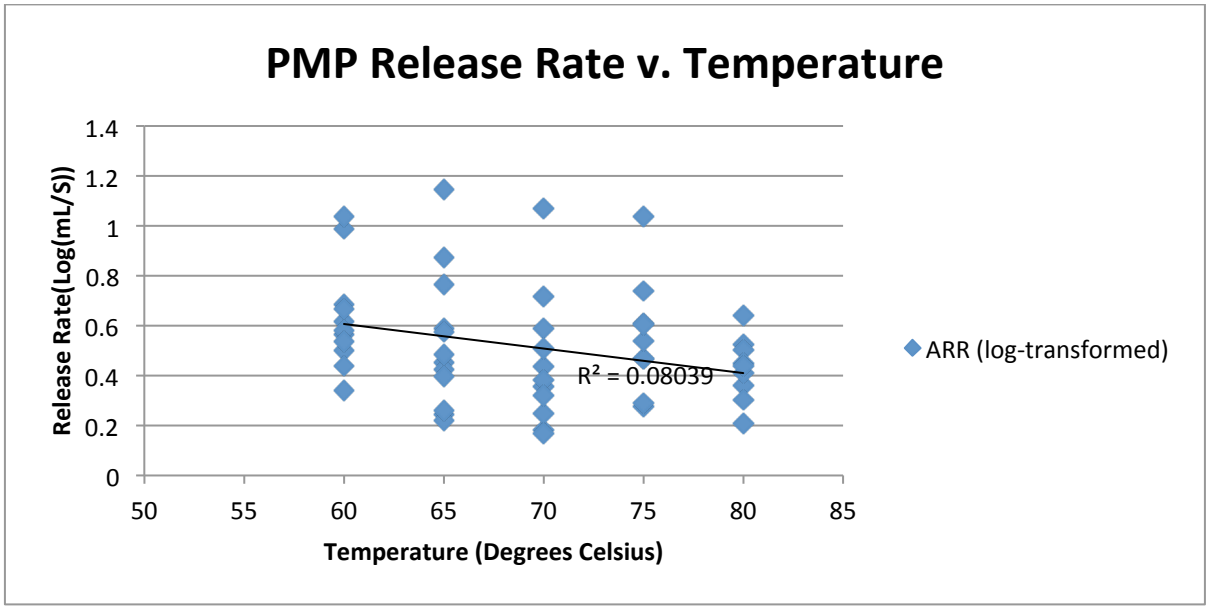

Figure 5. Linear regression analysis of temperature' effect on (log-transformed) release rate. Many outliers are again seen below 60°C, but release rate decreases with temperature as it increases over 60°C. This may be misleading as release rate is dependent on volume of the PMP, which has already been shown to change with temperature.

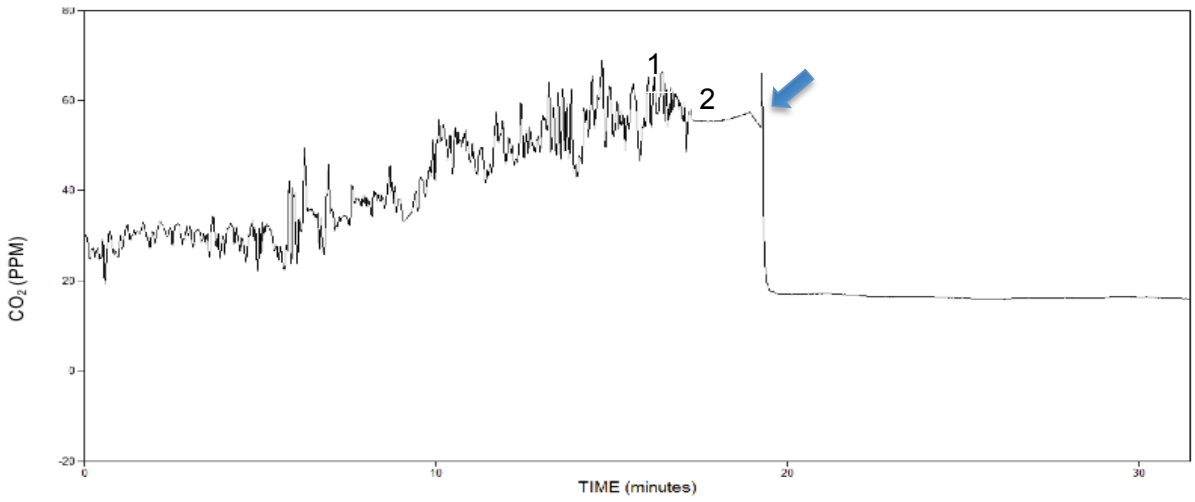

Figure 6. Exposure of a female cricket to HCN immediately prior to the PMP. CT_{max} is noted by the number 1; death by the number 2, and administration of $HCN_{(g)}$ by the arrow. A sharp increase is seen in CO_2 dues to input switch followed by immediate loss of waveform and no return of CO_2 release following reintroduction of atmospheric air.

Table 1. Two-way ANOVA assuming equal variance for all injection groups.

Multivariate Tests[a]				
Effect		**F-value**	**Error degrees of freedom**	**Significance**
Gender	Pillai's Trace	1.446	39.000	.248
	Wilks' Lambda	1.446	39.000	.248
Group	Pillai's Trace	.751	80.000	.646
	Wilks' Lambda	.735	78.000	.661
Gender*	Pillai's Trace	.64	80.000	.735
Group	Wilks' Lambda	.647	78.000	.736

Table 3. General linear model of temperature v. PMP duration

Tests of Between Subject Effects			
Source	**Degrees of freedom**	**F-value**	**Significance**
Temperature	1	10.131	**.**003
Gender	1	2.476	.122
Error	50		