BEHAVIORAL AND PHYSIOLOGICAL RESPONSE OF *TRIBOLIUM CASTANEUM* (HERBST) EXPOSED TO HYPOXIA

by

Kabita Kharel

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THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL

Dr. Linda J. Mason, Co-chair
Department of Entomology
Dr. Dieudonne Baributsa, Co-chair
Department of Entomology
Dr. Larry L. Murdock
Department of Entomology
Dr. Michael E. Scharf
Department of Entomology
Dr. Lisa J. Mauer
Department of Food Science

Approved by:

Dr. Stephen Cameron
Head of the Graduate Program

To my late grandmother Chandra Kala Kharel

&

my parents Mr. Jib Nath Kharel and Mrs. Kamala Kharel

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TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

Author: Kharel, Kabita, Ph.D.
Institution: Purdue University
Degree Received: December 2018
Title: Behavioral and Physiological Response of *Tribolium castaneum* (Herbst) Exposed to Hypoxia.
Committee Chair: Dr. Linda J. Mason and Dr. Dieudonne Baributsa

Hermetic storage systems represent viable pest control methods for postharvest crop storage. Nevertheless, there is limited understanding of how insect respond to hypoxic conditions inside hermetic storage. This study was conducted to investigate: i) the oxygen consumption behavior of *T. castaneum* under hermetic conditions, and effects of hypoxia exposure on: ii) the survival of different life stages after hypoxia iii) their feeding and movement (activity), and iv) the cellular energy as a measure of adenylate energy charge (AEC). *T. castaneum* consumed 6.80 ± 0.45 mL of oxygen during egg-to-adulthood while adults consumed 5.37 ± 0.30 mL of oxygen during 21-d at 27°C. The daily rate of oxygen utilization increased with increasing temperature and in larger volume containers. Eggs and young larvae were the most susceptible stages, experiencing total mortality when exposed to 2% oxygen level for 3d compared to larvae and pupae (required ≥10 d), and adults (required ≥15 d). At 4% oxygen level, total mortality was achieved for eggs and other life stages at 5 d and ≥15 d, respectively. By contrast, the 8% oxygen level was not lethal except for eggs, but it caused significant developmental delays in immatures. Likewise, the ultrasonic device revealed that hypoxia exposure could affect insect activity within the first 30 minutes of treatments. Furthermore, the AEC index of *T. castaneum* adults at normoxia control was 0.70 ± 00, while the AEC at <1%, 2, 4, 8% oxygen levels were 0.18 ± 0.0, 0.19 ± 0.01, 0.25 ± 0.02, and 0.58 ±0.01, respectively suggesting that hypoxia diminish energy production in insect cells. Complete mortality of *T. castaneum* adults was achieved when

exposed to <1% oxygen levels for 96 h. In conclusion, exposing *T. castaneum* to <2% oxygen levels for 15 d produce total mortality; however, even the higher levels of oxygen at 4-8% can subtly affect insect population development.

Keywords: Modified atmosphere, hermetic storage, hypoxia, stored product pest control, *Tribolium castaneum*,

CHAPTER 1. REVIEW OF LITERATURE

1.1 Introduction

Modified atmosphere (MA) is a practice of modifying the composition of the atmospheric air of grain storage structures or facilities to create an environment which is not optimum for the growth, development, and reproduction of the insect pests (Adler et al. 2000). The available oxygen inside hermetic containers is depleted naturally due to the respiration of the insects and microbes present in the grain or by the grain itself. The system, being air-tight, greatly slows down or prevents the infiltration of oxygen back into the system. Internal oxygen levels in hermetic crop storage bags such as Purdue Improved Crop Storage (PICS) and GrainPro bags can go below 2% within 3-4 days to several weeks of sealing depending upon the levels of infestations and the ambient environment (Baoua et al. 2012, Murdock et al. 2012, Kharel et al. 2018). Generally, oxygen levels of 2% and below is considered unsustainable for growth, development, and survival of storage insect pests.

At present, millions of smallholder farmers and traders in Asia and Africa have adopted the PICS and GrainPro hermetic bags (Jones et al. 2011, De Bruin et al. 2012, Mutambuki et al. 2015). These technologies provide cheap, viable and chemical free means to address insect-related postharvest crop losses. There are numerous studies from different countries to support the effectiveness of PICS and GrainPro bags in controlling primary pests of a wide range of stored commodities. Nevertheless, little is known about the mechanisms of action underlying the effects of hypoxia on the behavior and physiological functioning of the storage insects. Knowledge of the insect's respiratory behavior and their developmental, behavioral, and physiological response to hypoxia can provide insight into efficacy against major pests.

1.2 Insect pests of stored crop commodities

Various beetles, weevils, and moths are the primary pests of stored crop commodities. Broadly, storage insects can be divided into two groups: internal feeders and external feeders. Internal feeders spend most of their life cycle within the kernel of grain, while external feeders spend their life cycle on the surface of the grains or feeding on grain particles. When designing an effective pest control measure, it is critical to identify the internal and external feeder insects targeted for control. Generally, the internally feeding insect species are more challenging to control with conventionally used insecticidal dusts and contact insecticides. Common internal feeders of stored crop commodities include rice weevil *Sitophilus oryzae* (L.), lesser grain borer *Rhizopertha dominica* (F.), pulse beetles *Callosobruchus maculatus* (F.), groundnut bruchid *Caryedon serratus* (Ol.), and Angoumois grain moth *Sitotroga cerealella* (Olivier).

Similarly, the common external feeders of stored crop commodities are red flour beetle *Tribolium castaneum* (Herbst), saw-toothed grain beetle *Oryzaephilus surinamensis* (L.), rusty grain beetle: *Cryptolestes ferrugineus* (Stephens), flat grain beetle: *Cryptolestes pusillus* (Schonherr), rice moth: *Corcyra cephalonica* (Staint), and grain mite: *Acarus siro* (L.). However, the immature stages of most storage insects spend a significant amount of their life cycle inside damaged grain kernels or buried beneath the flour surface as in the case of *T. castaneum* (Mason et al. 2012). In such locations inside grain storage bags, oxygen is limited because compactly filled stored commodities retard oxygen inflow and the grain itself presents a barrier to oxygen movement (Williams et al. 2016).

1.3 *Tribolium castaneum* (Herbst), the red flour beetle

T. castaneum is one of the most important cosmopolitan pests of stored grain and pulses (Arbogast 1991, Arnaud et al. 2005, Ahmad et al. 2012). The life period from egg to

adult is 35 days at 30°C (Good 1936). The eggs are white, microscopic and about 0.60 mm in length. They hatch within 5 to 12 days. The larvae are slender, about 0.3 inches long when fully grown, and creamy yellow to light brown. Larvae are moderately active but avoid the light and hide inside the food material. There are generally four larval instars which can range from 22 to 100 days depending on the environmental conditions (Good 1936, Howe 1956). Pupae are white to light brown, and the stage lasts for about eight days (Howe 1956). Gender can be determined during the pupal stage by observing genital appendages at the ventral surface of the terminal abdominal segments. Adults are brown, active 3.5 mm in length, and remain concealed inside their habitat (Good 1936). They are long-lived and may live for more than three years (Walter 1990, Rebecca and Thomas 2003).

Infestation of *T. castaneum* is evident by the appearance of adults on the surface of the grain. Larvae and adults both can cause damage by feeding as well as through contamination by broken body parts, excrement, and metabolic byproducts. The metabolic secretions can produce pungent odor and displeasing taste in the crop commodities (Bakuła et al. 2011). However, both larvae and adults are also tolerant to starvation and can survive 14 to 40 d depending on temperature and relative humidity (Good 1936).

1.4 Pest control tactics for *T. castaneum*

Control of *T. castaneum* populations in stored grain relies heavily on synthetic insecticides, including slow-release grain fumigants such as phostoxin and essential oils. The grain may be treated with insecticidal dust or residual sprays before storage (i.e., diatomaceous earth, Deltamethrin, Chlorpyrifos-methyl) (Zettler and Cuperus 1990, Lee et al. 2002, Epidi and Odili 2009). Occasionally, repeated applications of those insecticides are necessary to achieve the control (Zettler and Arthur 2000). However, insecticidal treatments and fumigants are not optimum measures to the smallholder farmers who store a small volume of grains for offseason trading or consumption. There are several issues regarding the

use of chemical protectant in the stored grain. For instances: *T. castaneum* has developed resistance to 33 different insecticide active ingredients, with 337 documented cases worldwide (Whalon et al. 2015 as cited in Zhu et al. 2016). Likewise, the safety concern related to insecticide use during its preparation, application, as well as the residue in the environment and the food materials are high (Damalas and Eleftherohorinos 2011). Furthermore, in recent times, the preferences for chemical-free foods is increasing due to awareness among consumers and other stakeholders (Arthur 1994, Hartmann and Klaschka 2017).

1.5 Hermetic grain storage

Hermetic storage provides viable, and chemical-free pest control means to control *T. castaneum* for the smallholder farmers. The low oxygen environment inside the hermetic container is achieved mainly due to (i) respiration of insects and other organisms present, and (ii) making the storage system airtight to restrict the movement of air significantly from outside (Navarro et al. 1994). Studies show that up to 100% insect mortality can be achieved typically within 2-weeks of sealing the hermetic bags if the environmental conditions and the integrity of the system are optimum (Navarro et al. 2006). When the hermetic bags are stored at or above the room temperature, crop losses can be reduced up to <1% (Villers et al. 2010). Examples of hermetic storage technologies include the conventional methods such as clay pots, plastic containers, and metal silos along with multi-layered crop bagging system such as Purdue Improved Crop Storage (PICS) bags.

PICS technology utilizes two layers of polyethylene plastic bags covered by an outer polypropylene woven bag to maintain the airtight environment. Purdue University, Indiana, USA has been promoting the PICS technology in developing countries through the establishment of the PICS program since 2007 with funds from the Gates Foundation (Baributsa et al. 2010). Since its establishment, the PICS program has been able to implement

the technology in 29 countries in Africa and Asia with sales of more than 14 million bags to farmers and other users (PICS Overview, 2018). Studies conducted in ten countries in West and Central Africa in 2010 and 2012 showed that a farmer storing 100 kg of cowpea in a PICS bag (price range $ 1.5 to 2.0 per bag) for a single season could get an average return of $27 (Moussa et al. 2014). Likewise, GrainPro bags are produced by GrainPro Inc. in the Philippines and have been marketed worldwide (Bern et al. 2013). The bag consists of a single high-density polyethylene bag fitted inside a standard polypropylene bag. The bags are successfully used for storing a wide variety of crops and seeds (Villers et al. 2010, Jonfia-Essien et al. 2010, Bern et al. 2013).

1.6 Effects of hypoxia

Studies have shown that hypoxia affects various aspects of insect population development such as reproduction, developmental time, feeding, oxygen use behavior, and their survival. Murdock et al. (2012) utilized an ultrasonic device called Purdue Insect Feeding Monitor (PIFM) (Shade et al. 1981) to detect and record the feeding behavior of *Callosobruchus maculatus* at different levels of oxygen. They reported that *C. maculatus* essentially stops feeding when the oxygen levels reach below 5% levels. Furthermore, exposure to hypoxia leads to a reduction in oxygen supply to tissues and cells which can produce effects at the cellular level. For example, it affects the carbohydrates catabolism that involves the breakdown of glucose molecules into carbon dioxide, water, and energy. The energy produced through the oxidation of carbohydrates is used for regulating various biochemical pathways such as oxidative phosphorylation and biosynthesis. The primary functions of oxidative pathways are to regenerate ATP from ADP, which puts energy into the adenylate system, while the biosynthesis and physical work in the cells converts ATP to ADP, thereby removing energy from the adenylate system (Baker et al. 2010). Regulation of these two oppositely directed pathways is critical to meet the metabolic needs of the cell

(Raffin and Thebault 1996). Furthermore, the adenylate system is responsible for providing energy for the chemical activities of the cell, including directing substrate into synthetic sequences, transportation of ions across membranes, and for other biological activities, including mechanical movement. The synthetic reaction rates are more strongly affected by the ratio of adenylate nucleotides in the adenylate system than their absolute concentrations (Swedes et al. 1975). For instance, a mutant strain of *Escherichia coli* that was unable to synthesize adenylate nucleotides grew on adenine-limiting media at normal rates when the intracellular concentrations of ATP, ADP, and AMP were half the normal levels, however, that mutant did not grow well when the energy charge fell below 30% of its usual value of about 0.9 (Swedes et al. 1975).

1.7 Adenylate energy charge

The energy status of the adenylate system in living cells can be measured with the index called adenylate energy charge (AEC). The AEC index was developed by Atkinson (1968), which is expressed as below.

$$AEC = \frac{ATP + \frac{1}{2}\,ADP}{ATP + ADP + AMP}$$

The AEC concept can be used to assess the impact of environmental stresses such as nutritional stress, oxygen depletion, desiccation, heat and chemical stresses on an organism (Ivanovici and Wiebe, 1981, Hochachka et al. 1996, Milusheva et al. 1996, Mitcham et al. 2006). Chapman et al. (1971) demonstrated that the AEC level of 0.5 could cause stress in *Escherichia coli* as the synthesis of ATP is insufficient to maintain an adequate energy charge. Similarly, Ridge (1972) suggested that the AEC levels below 0.5 can cause irreversible viability losses in the vertebrates. However, invertebrates could survive relatively

low AEC value, even though the AEC value below 0.3-0.4 is critically low for their functioning and survival (Ivanovici and Wiebe 1981).

1.8 Research Objectives

The overall goal of this dissertation was to understand the response of T. castaneum adult and immature stages exposed to the hypoxic conditions inside the hermetic storage system, thereby to provide practical recommendations to the stakeholder on improving the performance and efficacy of the hermetic grain storage technology.

1.8.1Specific Objectives

This study consisted of five specific objectives to achieve the overall research objective. The specific objectives are as following:

1) To investigate the daily and cumulative oxygen utilization behavior of *T. castaneum* immature (egg-to-adulthood) and adult stage at different headspace and temperature levels,
2) To understand the life stage-specific response of *T. castaneum* exposed for different times (1, 3, 5, 10, and 15 days) to artificially-created hypoxic conditions as seen in hermetic containers (2, 4, 8% oxygen levels) as well as at ambient oxygen (20.9%),
3) To study the effects of hypoxia on the immature developmental time,
4) To characterize individual activity (movement plus feeding) of adults and immatures of *T. castaneum* (Herbst) exposed to different levels of hypoxia (2, 4, 8% oxygen level) as well as at normoxia control (20.9%), and
5) To determine the adenylate energy charge (AEC) in the hypoxia treated adults of *T. castaneum* to determine the effects on cellular energy through the estimation of ATP, ADP, and AMP utilizing the high-performance liquid chromatography (HPLC) technique.

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CHAPTER 2. ESTIMATION OF DAILY AND TOTAL OXYGEN USED BY *TRIBOLIUM CASTANEUM* (HERBST) TO COMPLETE ITS LIFE CYCLE

2.1 Abstract

Hermetic grain storage technologies have been shown to suppress population growth and survival of insect pests in a wide range of stored crop commodities. However, more information is needed as to how insects are affected by depleted oxygen levels inside hermetic storage systems. Understanding the daily and cumulative oxygen use by insects through their life stages is a critical step towards understanding the performance and mode of action of hermetic storage. In the present study, we placed a 4-d old *Tribolium castaneum* (Herbst) egg together with 0.5g wheat flour in each of fifteen 60 or 125 ml containers. The containers were sealed and placed inside insect growth chambers maintained at 20, 27 or 34° C and 60 RH%. Using an Oxysense 5250i® device we monitored the average daily oxygen level in each container until an adult emerged. Similarly, to assess oxygen consumption by *T. castaneum* adults we added single unmated adults to 60, 125, and 250 mL containers containing 0.5 g of wheat flour. After sealing the containers, we monitored oxygen levels for the next 30 days. Over their juvenile lifetime, egg-to-emerging adult stage, *T. castaneum* consumed 10.52 ± 0.56, 6.80 ± 0.45, and 5.81 ± 0.41 mL of oxygen per insect at 20, 27, and 34°C, respectively. By contrast, *T. castaneum* adults consumed <2.23 ± 0.09, <5.37 ± 0.30, and <7.56 ± 0.40 mL of oxygen at 20, 27, and 34°C, respectively during 21-d. Additionally, total oxygen utilization of adults increased with container sizes at 20 and 34°C but not at 27°C. This suggests that the extreme temperatures affected the metabolic rate and respiratory behavior in *T. castaneum* and the available oxygen may have been limiting in the smaller containers. These results will be helpful in understanding how *T. castaneum* performs in hypoxia and may be useful in improving the efficacy of hermetic grain storage technology.

Keywords: Oxygen utilization, hermetic storage, *T. castaneum*, temperature, oxygen availability

2.2 Introduction

Ambient air contains about 20.9% oxygen. When the oxygen level of the environment goes below 15%, the condition is known as hypoxia (Jensen 2006). Hypoxic conditions affect the growth and development of most organisms (Stamati et al. 2011). Hypoxic conditions have been used since prehistoric times to control pests of stored crop commodities and referred to as hermetic technology. The two crucial aspects of hermetic grain storage technology are: 1) the storage system is designed to be airtight to reduce oxygen infiltration, and 2) the respiration of any living insect, microbes, as well as the stored grain will gradually deplete the oxygen level inside the container to the level that is unsustainable for the population development and survival of pests.

Hermetic grain storage is safe, simple, and economical alternative to chemical control methods in grain storage and food processing industries. Many portable hermetic grain storage bags such as GrainPro, PICS, ZeroFly, GrainSafe, IRRI Super Bag or higher capacity storage system such as cocoons are commercially available. A considerable number of studies conducted in the last two decades have shown that hermetic technology is successful in controlling storage insects such as *T. castaneum*, *T. confusum* Jacquelin du Val, *Callosobruchus maculatus* (F.), *Sitophilus zeamais* Motschulsky, *S. oryzae* (Li.), *Rhyzopertha dominica* (Fab.), *Cryptolestes ferrugineus* (Stephens) and *Oryzaephilus surinamensis* (L.). However, very little is understood of the underlying mechanisms behind how insects respond to declining oxygen levels inside the hermetic storage system. Additionally, different insects and their life stages exhibit different levels of sensitivity to hypoxic environments. Consequently, growing interest in hermetic technology have necessitated understanding the mode of action of this system.

Understanding the respiratory and oxygen use behavior of insect living inside a hermetic container is a most important step towards understanding the mode of action of hermetic technology. It might also help predict population growth under given hermetic conditions. Additionally, it is known that ambient temperature affects nearly all physiological and biochemical processes in organisms (Neven 2000, Huey and Berrigan, 2001, Khaliq et al. 2014). According to the Van 't Hoff equation, for every 10°C increase in temperature, the rate of chemical reaction is increased from two to three times. The biochemical processes in organisms as reflected in the metabolic rate can be measured by oxygen consumption, carbon dioxide production, or heat generation (Huey and Berrigan 2001). Additionally, the respiration rate of insects inside hermetic containers can be influenced by the available headspace or air (Williams et al. 2016). Previously, Murdock et al. (2012) and Swathi et al. (2017) estimated the lifetime oxygen utilization by *C. maculatus* and *Caryedon serratus* (Olivier), respectively, and to date, they are the only studies to determine the oxygen consumption of post-harvest insects. However, previous investigators did not take into account the inevitable effects of ambient temperature on oxygen utilization under hermetic conditions.

The red flour beetle (RFB), *T. castaneum* (Herbst) is one of the most important pests of grain storage and food processing industries. It is also an important model insect for many physiological and molecular studies. We hypothesized that increased temperature and increased headspace of the sealed container (thus higher availability of oxygen) increase the oxygen utilization rate for immature and adults of *T. castaneum*. To test this hypothesis, we measured (i) oxygen utilization of immature of *T. castaneum* (egg-to-adulthood) at 20, 27 and 34°C inside 60 and 125 mL size containers, and (ii) the daily and cumulative oxygen utilization of *T. castaneum* adults at 20, 27 and 34°C inside 60, 125, and 250 mL sealed containers for 30 days.

2.3 Methods

2.3.1 Insects

T. castaneum were obtained from colonies maintained on a mixture of whole wheat flour and brewer's yeast (95:5) by the Stored Product Insect Rearing Facilities at the Department of Entomology, Purdue University. The colonies were reared in a CARON Insect Growth Chamber (CARON model 6025-1) (Caron Products & Services, Inc., Ohio, USA) at 27 +1°C, 60% R.H. and at 16:8 light:dark photoperiod.

2.3.2 Estimation of the volume of oxygen in the empty glass container

Three sizes of clear glass septum bottles 60, 125, and 250 mL (The Lab Depot Inc., Georgia, USA) were used. The oxygen content in the empty containers was estimated by the liquid displacement method. First, the containers were filled entirely with water. Then the water was poured into a calibrated container to determine the volume of water. The volume of displaced water was assumed to be equal to the volume of the air in the empty bottle. After that, the total oxygen in the empty containers was estimated by using a 20.9% rule in the total air (Bugbee and Blonquist 2006) (Table 2.1).

2.3.3 Experiment 1: Estimation of egg-to-adulthood oxygen consumption

Fifteen 60 or 125 mL capacity containers received a single 4 d old egg of *T. castaneum* + 0.5 g wheat flour. The containers were then sealed with a cap which was covered by a double layer of a parafilm to make it airtight. The control treatment containers had only 0.5 g of wheat flour to estimate oxygen consumption by the flour itself. Containers were held inside CARON Insect Growth Chambers maintained at 20, 27 and 34°C and 60% R.H. The temperature and percent relative humidity (R.H.) inside the chamber were monitored every six hours using USB data loggers (Lascar, Erie, PA, United States). Target

temperatures of 20, 27, and 34°C were maintained ± 0.5°C and target R.H. of 60% ± 5% inside CARON growth chambers (Appendix A and B). Oxygen use in each container was monitored using a non-invasive Oxysense 5250i® oxygen reader (Oxysense, Dallas, TX). The Oxysense system consists of two components: fluorescent yellow Oxydots, which were placed inside the sealed containers, and an ultraviolet light pen which is directed onto the Oxydots from outside the container to measure the internal oxygen levels. Oxydots can withstand the temperature up to 150 °C. The volume of the oxygen was determined by multiplying it by the volume of the container times 20.9. Finally, the total volume of oxygen consumed was determined by subtracting the volume of oxygen measured in the container from the initial volume of oxygen in the empty container.

Previous literature shows that *T. castaneum* requires about fifteen, seven, and three weeks to complete the life cycle at 20, 27, and 34°C, respectively. Therefore, the experiment was designed as a randomized incomplete block design in which the 20°C treatment was run once, 27°C twice, and 34°C three times.

2.3.4 Experiment 2: Estimation of oxygen consumption adult *T. castaneum*

Ten 60, 125, or 250 mL capacity container were used per trial (experimental run). Each container received a single 2 d old unmated adult *T. castaneum* + 0.5 g wheat flour. Individuals were selected and kept singly during the pupal stage to prevent mating. Containers were sealed with the caps and made airtight with the double layered parafilm over the caps. Control containers contained only 0.5 g of wheat flour and were sealed in a similar way. Containers were placed inside CARON Insect Growth Chambers maintained at 20, 27 and 34°C and 60% RH. Oxygen consumption in each container was measured daily for 30 d. The volume of oxygen consumed by each individual was determined by the procedure explained above for estimating oxygen used during egg-adulthood section. The experiment was replicated three times.

2.3.5 Statistical analyses

Statistical analyses were conducted with the Statistical Analysis System (SAS Institute Inc. 2013, SAS Institute, Cary, NC) (SAS, 2013). For the total oxygen utilization by the adults and by the immature stages (data analyzed separately), the data were subjected to a mixed model using PROC MIXED to determine the significance of the random effects of the experimental run on the total oxygen intake by the individuals in the presence of the fixed effects. If the random effects of the experimental run were not significant, the data were pooled over the experimental runs for further analysis. Then the data were subjected to a Two-Way-ANOVA with the use of PROC GLM with the fixed effects of temperature and container size. Subsequently, a one-way-ANOVA was performed for the treatment groups if applicable. The treatment means were separated using adjusted Tukey at $\alpha \leq 0.05$. For the daily oxygen utilization in adults and immature stages, data were transformed using log(abs(y+0.005)) to correct the residuals. Then a mixed model was used with experimental run as a random effect and the repeated measures ANOVA. If the random effects of the experimental run were not significant, the data were pooled over experimental runs for further analysis. Subsequently, a repeated three-way ANOVA was performed to determine the effects of temperature, container size and days on overall daily rate as well as at different time period of observations. The data were back transformed for reporting.

2.4 Results

The oxygen levels inside the containers for the flour only control treatments in both immature and adult stage experiments remained in the range of 20 to 21.27% (v/v) during the entire study period. Thus the wheat flour supplied as a food source for T. castaneum did not significantly influence the oxygen levels in the containers. Therefore, no adjustments were

necessary for treatment data and flour only controls were eliminated from the statistical analysis.

2.4.1 Oxygen consumption from egg-to-adulthood

For the egg-to-adult stage emergence, the mixed model procedure showed that the estimated random effects from the experimental run were zero, i.e., the experimental runs were not significantly different. The two-way ANOVA showed that the oxygen used was significantly different for different temperatures ($F_{2, 138}$= 549.74, P<0.01) but not significant for the container ($F_{1, 138}$= 1.54, P=0.22), or the interaction of the container and temperature ($F_{2, 138}$= 0.02, P=0.98). Therefore, data were pooled over for the effects of container sizes and compared for the effects of temperatures. Results showed that the total oxygen consumed was affected significantly by temperature ($F_{2, 141}$= 554.48, P <0.01). The total oxygen consumption during the developmental period of *T. castaneum* increased as temperature decreased (Figure 2.1 and 2.2).

However, the daily oxygen utilization rate increased with increasing temperature (Table 2.2). For instance, daily oxygen utilization rate increased by 2.9 times at 27°C compared to 20°C, and it further increased by 1.5 times at 34°C compared to 27°C (Table 2.2). Furthermore, the immature stages grew slowly at 20°C (135.05 ± 0.49 d) and 27°C (38.60 ± 0.28 d) compared to 34°C (23.13 ± 0.23 d) (Table 2.2).

2.4.2 Oxygen consumption by adults

Mortalities were observed in the 60 mL containers at 34°C towards the end of the observation period of 30 d. This might be because the oxygen in the 60 mL container may have depleted to very low level, too low for *T. castaneum* to sustain life. Therefore, we consider data only up to 21 days (d) to estimate cumulative oxygen use in *T. castaneum* adult for three weeks.

For the 21-d period, cumulative oxygen use data, the mixed model procedure showed that the effects of the experimental runs were not significantly different ($Z = 0.39$, $P = 0.35$). Therefore, the data were pooled over the experimental runs for further analysis. Two-way ANOVA showed that the oxygen use by the individuals was significant for main effects of temperature and container ($F_{2, 261} = 9.26$, $P < 0.01$, $F_{2, 261} = 174.12$, $P < 0.01$, respectively) but not for interactions ($F_{4, 261} = 0.97$, $P = 0.43$). Subsequent One-way ANOVA to compare the effects of container size within temperature treatments showed that cumulative oxygen use was significantly different among containers at 20°C ($F_{2, 87} = 14.18$, $P < 0.01$) and 34°C ($F_{2, 87} = 3.32$, $P < 0.05$) but not at 27°C ($F_{2, 87} = 1.41$, $P = 0.24$). Cumulative oxygen use increased significantly with increase in the size of the container (higher total amount of oxygen) at 20 and 34°C and followed a similar pattern at 27°C though the difference was not significant among the containers. Similarly, oxygen use was affected by temperature at all 60, 125 and 250 mL containers ($F_{2, 87} = 81.15$, $P < 0.01$, $F_{2, 87} = 62.34$, $P < 0.01$, $F_{2, 87} = 45.77$, $P < 0.01$, respectively). Total consumption of oxygen increased with the increasing temperature (Figure 2.3).

For the daily oxygen use in adults, the mixed model procedure showed that the estimated random effects from the experimental run were zero. Therefore, data were pooled over experimental run for further analysis. The Three-way Repeated ANOVA showed that the daily oxygen consumption rate was significant for temperature, container, observation days, and the interactions of temperature x container ($F_{2, 1666} = 728.69$, $P < 0.01$, $F_{2, 1666} = 40.97$, $P < 0.01$, $F_{28, 7056} = 3.85$, $P < 0.01$, $F_{4, 1666} = 8.54$, $P < 0.01$). Cumulative and daily oxygen consumption rate increased with increasing temperature and container size (Figure 2.3 and 2.5). However, the rate decreased after a few days at 34°C in 60 mL containers (Figure 2.4). Only 46.7 ± 3.3% of the adult at 34°C and 60 mL containers survived until the end of the experiment (30 d). Therefore data were considered only for 21 d for that specific treatment.

2.5 Discussion

2.5.1 Oxygen consumption from egg-to-adulthood

T. castaneum consumes about 10.46 ± 0.22 mL of oxygen at 20°C (135 d), 6.72 ± 0.53 mL at 27°C (39 d), and 5.76 ± 0.40 at 34°C (23 d) to complete its immature developmental period. Previously Murdock et al. (2012) reported that the total oxygen consumption by a single *C. maculatus* during larval to the pupal stage was 8.9 ± 0.4 mL at 27°C. Adults of *C. maculatus* are 2.0-3.5 mm long (CABI 2018) and their developmental period is about 21 days at 32°C (CABI 2018) which is comparable to the *T. castaneum* biology and life cycle. However, a more recent study by Swathi et al. (2017) showed that a single *C. serratus* consumed a total of 39.97 mL of oxygen for its development from the egg to the pupal stage. There might be several plausible reasons behind why the total oxygen consumption by *C. serratus* was very high compared to *T. castaneum* despite both being Coleopterans. First, the average developmental period for *C. serratus* is 65-75 days at 25°C (Calderon and Navarro 1967), and the average length of the adult *C. serratus* is 4-5 mm (Gerson 2015) while the *T. castaneum* beetles are about 3 mm long. The difference in the fully developed adult size might influence the metabolic rate and overall oxygen consumption during the immature stage of development. Furthermore, Swathi et al. (2017) monitored the oxygen levels inside their container using the Mocon head space analyzer device which is an invasive method to record the oxygen content in the sealed container. In our study, we used Oxysense device which is a non-invasive method and does not influence the oxygen content inside the sealed container. At the lower temperature, the immatures of *T. castaneum* developed slowly and consumed the oxygen inside the sealed containers over a longer period of time (about twenty weeks at 20°C and about six weeks at 27°C) compared to 34°C (about 3.5 weeks) thus resulting in the increased utilization of total oxygen. However, the daily rate of oxygen use increased with the increasing temperature (0.06 ± 0.0, 0.17 ±

0.01, and 0.25 ± 0.01 mL at 20, 27, and 34°C, respectively. The elevated temperatures, within the thermal tolerance limit of organisms, accelerates the biochemical processes or the rate of enzymatically catalyzed reactions in organisms accordant to the rules of thermodynamics (Clarke and Fraser 2004, Schulte 2015). The shorter period for egg-to-adulthood at higher temperatures is due to accelerated biochemical processes and cellular metabolism at elevated temperatures. The metabolic rate in insects is mechanistically related to oxygen consumption or carbon dioxide production (Neven 2000). Faster depletion of oxygen inside the hermetic crop storage bags at the warmer region is evident from the field. For example, Baoua et al. (2012) conducted an experiment in Maradi, Niger where they showed that the oxygen levels in hermetic PICS bags filled with cowpea grain naturally infested with *C. maculatus* dropped to about 2.7% levels within 24 h of sealing the bags. In a different experiment conducted with similar conditions in Indiana, USA, Murdock et al. (2012) found that the oxygen levels inside the hermetic PICS bags remained above 3% levels even after 96 h of sealing. These two experiments suggest that ambient temperate of the region can influence the oxygen utilization rate of insects inside hermetic storage.

Furthermore, the oxygen consumption during egg-to-adulthood peaked immediately at 34°C while it took about five weeks to peak at 20°C. At higher temperatures, the eggs might have hatched earlier, and the respiration and metabolic rate in the immature stages might have increased. Many previous studies have shown that the oxygen consumption in insects increases as temperatures increase (Rogers 1929, Tonapi et al. 1979). The daily oxygen consumption rate during egg-to-adulthood was highest over 20-30 d at 27°C and 10-15 d at 34°C. Consumption rates were slow during the latter part of the developmental period which may correspond to the pupal developmental phase. Increased activity prior to pupation could be due to the need of relatively higher amount of glucose and energy for chitin synthesis (Arrese et al. 2010) which consequently increased the rate of oxygen consumption.

Murdock et al. (2012) also found different levels of feeding activities at different stages of the developmental period in *C. maculatus*.

2.5.2 Oxygen consumption by adults

We found that the adult *T. castaneum* adults consumed about 2.23 ± 0.09 to 3.58 ± 0.27 mL of oxygen at 20°C, about 5.37 ± 0.30 to 6.04 ± 0.30 mL at 27°C, and 7.56 ± 0.40 to 9.55 ± 0.64 mL at 34°C during the three week-experimental periods. The total oxygen consumption, as well as daily rate, increased with the increasing temperature.

Our findings are consistent with Quellhorst et al. (unpublished) who found that the oxygen consumption of *C. maculatus* doubled when the temperature was increased from 25 to 35°C. Additionally, we observed that the oxygen consumption of *T. castaneum* adults was higher in the bigger size containers at 20 and 34 °C suggesting more utilization of oxygen with better availability at below as well as above the ambient conditions. This may be attributed to the energetic cost of adaptation or compensation metabolism at extreme temperatures (Clarke 2003). The changes in ambient temperature also affect enzyme-substrate binding, substrates oxidization, ATP synthesis, and energy utilization. All these effects in the biological processes in the cell further alter the stoichiometry of oxygen utilization at extreme temperatures (Clarke and Fraser 2004). However, oxygen consumption in *T. castaneum* adult decreased after a few days in the 60 mL container at 34°C. The total available oxygen in the 60 mL size container may have fallen below the optimum level, and it might have restricted metabolism and thus slowed down the utilization. Sondersom et al. (1992) also demonstrated that anoxic conditions make *T. castaneum* more sensitive to high temperatures producing greater mortality. Additionally, high mortality of *T. castaneum* adults was recorded in the 60 mL container at 34°C after three weeks of sealing the containers. Our results are in agreement with the previous findings in which authors have shown rapid mortality of the insects inside the sealed hermetic grain storage container in warmer regions of Africa

(Yakubu et al. 2009, Baoua et al. 2012).

This study has increased our knowledge regarding the response of *T. castaneum* adults and immature stages exposed to hypoxia at different temperature levels. Additionally, understanding the total and daily oxygen consumption of immature and adult stages of *T. castaneum* might be helpful in predicting the population development of *T. castaneum* under various oxygen levels or hermetic storage systems. Furthermore, our findings support that the hermetic grain storage technology may be of greater efficacy in the tropical and subtropical regions due to rapid internal oxygen decline at an elevated temperature. Overall, our study will hopefully generate interest in finding ways to improve hermetic storage technology still further.

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Table 1.1. Estimation of the total initial oxygen in the empty bottles

Bottle size (mL)	Liquid displaced (mL)	Volume of oxygen (mL)*
60	66	66 x 20.9/100 =13.80
125	128	128 x 20.9/100 = 26.75
250	253	253 x 20.9/100 = 52.8

*The total amount of oxygen in the empty containers were calculated using the liquid displacement formula. According to the formula, the total volume of liquid displaced is equal to the volume of air in the empty containers. Oxygen constitutes approximately 20.9% of the total air (Bugbee and Blonquist 2006). Therefore, the total volume of liquid displaced was multiplied with 20.9%

Table 2.2. Egg-to-adult eclusion of *T. castaneum*: length of developmental period and daily oxygen consumption rate (Mean + SE)

Particulars		20 ºC	27 ºC	34 ºC
N		17	24	54
Developmental time (d)		135.05 ± 0.49a	38.60 ± 0.28b	23.13 ± 0.23c
		Daily oxygen consumption rate (mL) (Mean ± SE)		
Day	5	0.00 ± 0.03c	0.07 ± 0.03b	0.26 ± 0.06a
	10	0.06 ± 0.03c	0.12 ± 0.03b	0.29 ± 0.04a
	15	0.10 ± 0.05c	0.20 ± 0.04b	0.45 ± 0.04a
	20	0.00 ± 0.04c	0.40 ± 0.05a	0.20 ± 0.03b
	25	0.03 ± 0.03b	0.31 ± 0.04a	
	30	0.00 ± 0.03b	0.24 ± 0.04a	
	35	0.13 ± 0.04a	0.17 ± 0.03a	
	40	0.03 ± 0.02b	0.14 ± 0.03a	
To complete life cycle		0.06 ± 0.00 a	0.17 ± 0.01 b	0.25 ± 0.01 c

The daily rate of oxygen consumption was calculated for five days intervals at each temperature levels. Data were pooled over experimental run and container size for analysis. Means within the row followed by the same letter are not significantly different based on adjusted Tukey.

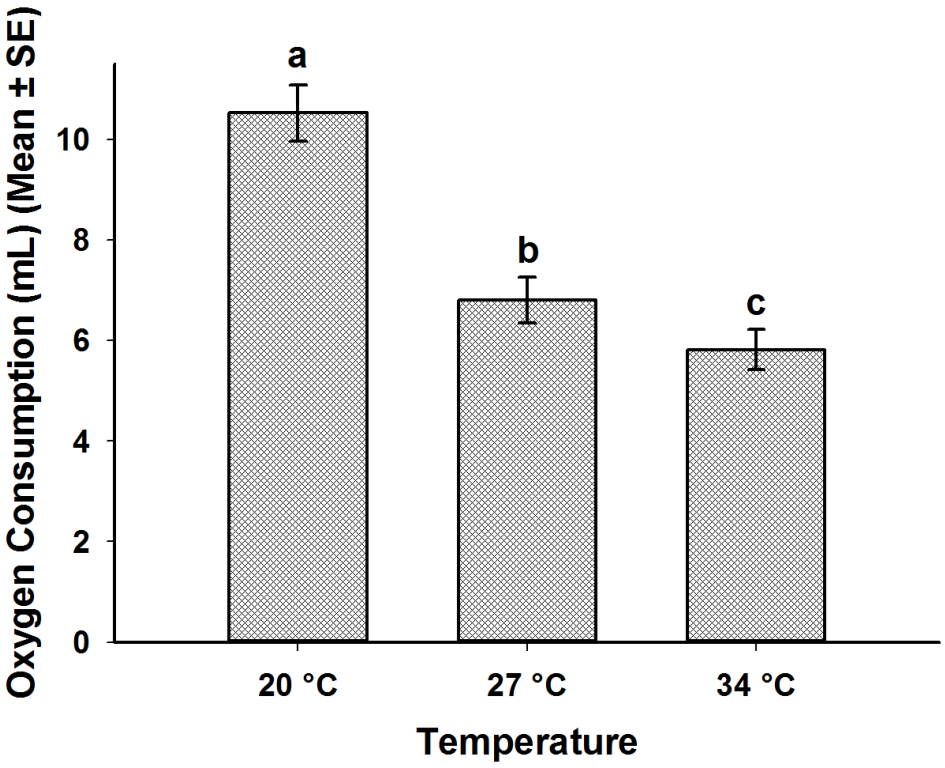

Figure 1.1. Average total oxygen consumption during egg-to-adult emergence by *T. castaneum* at different temperatures. Means among temperature levels with the same letter are not significantly different based on adjusted Tukey (alpha = 0.05)

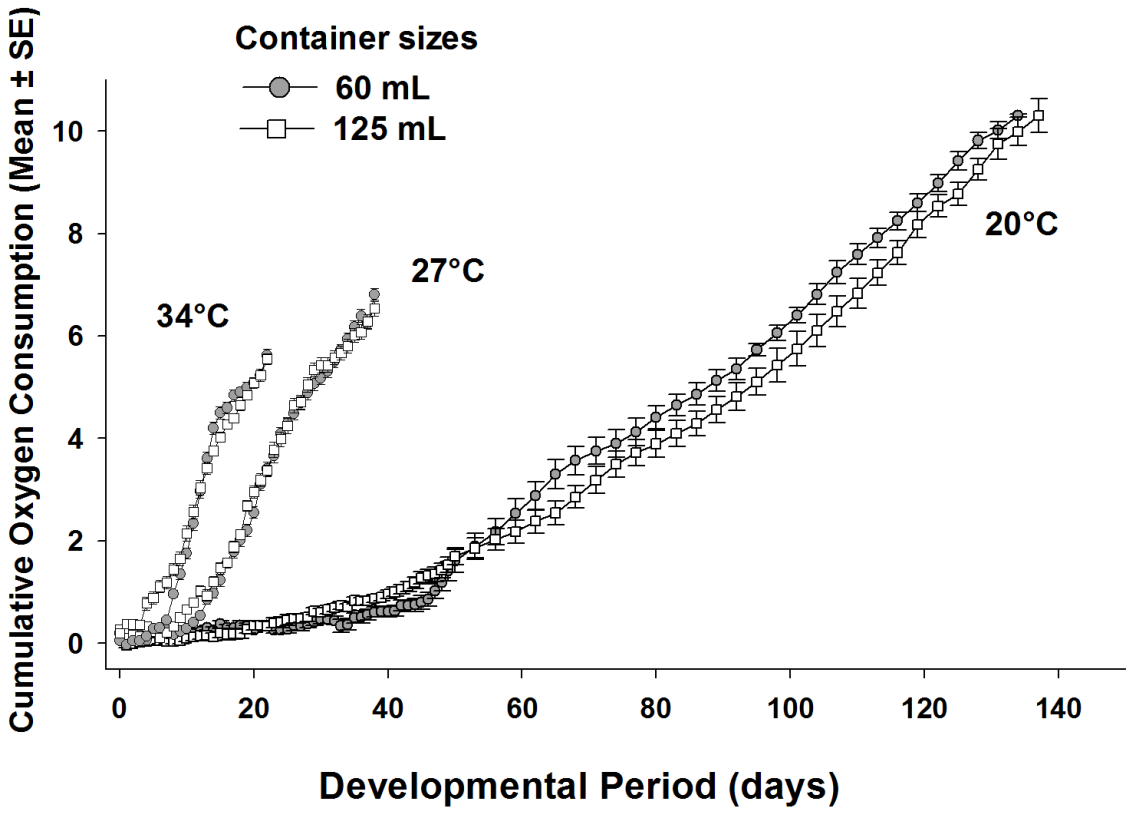

Figure 2.2. Cumulative oxygen consumption during egg-to-adult emergence by *T. castaneum* held in difference sized sealed containers at different temperatures.

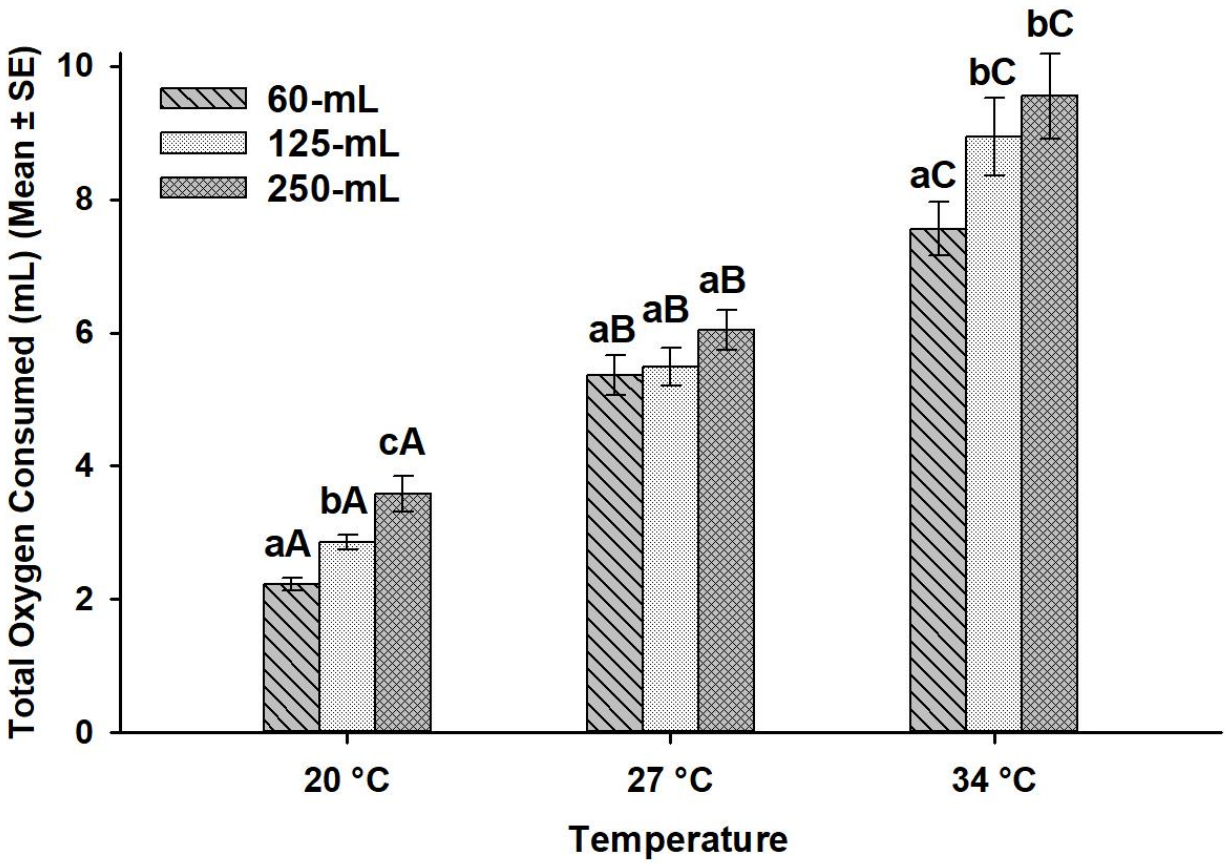

Figure 2.3. Average total oxygen consumption by *T. castaneum* adult over 21 d held inside different sized sealed containers at different temperatures. Means among temperature levels (upper case letters) and container size (lower case letters) with the same letter are not significantly different based on adjusted Tukey (alpha = 0.05).

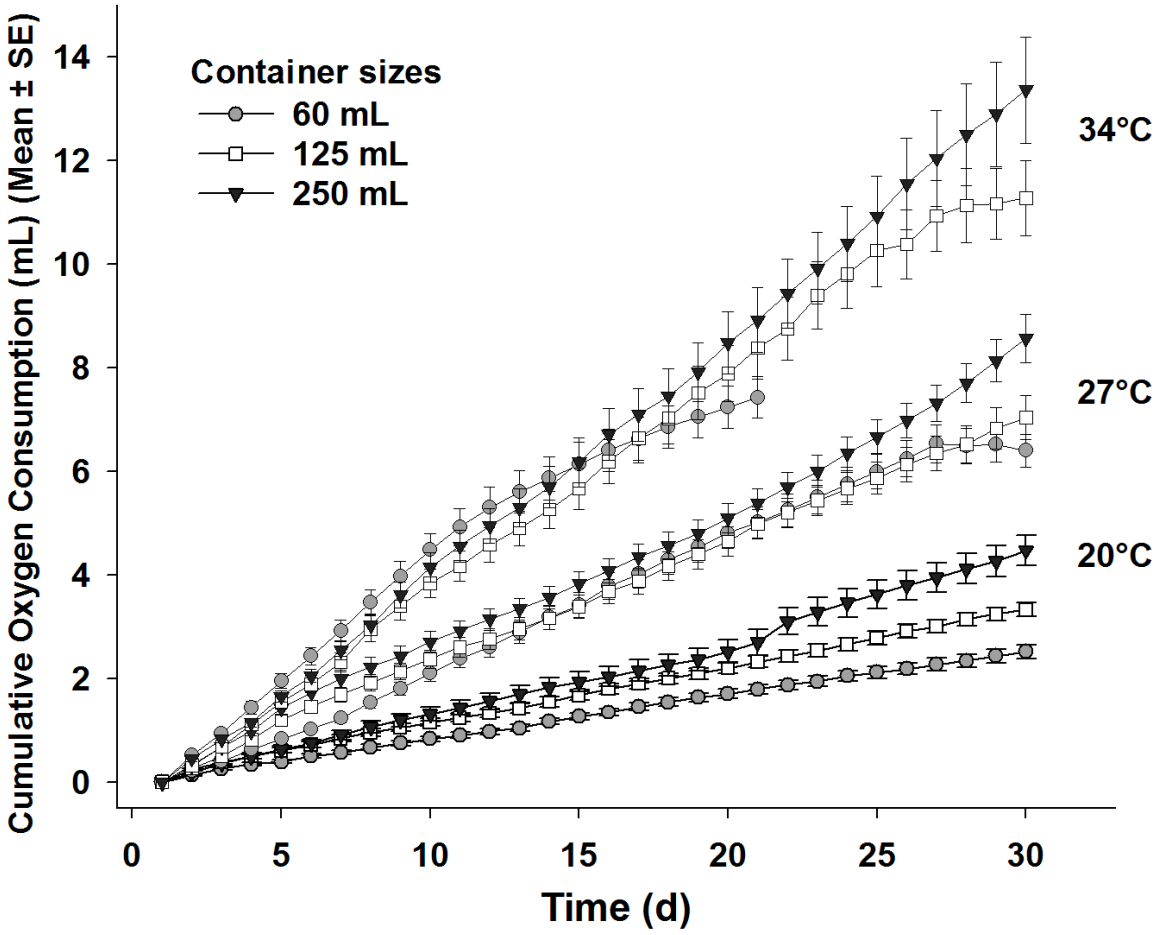

Figure 2.4. Cumulative oxygen consumption by *T. castaneum* adults held inside different sized sealed containers at different temperatures.

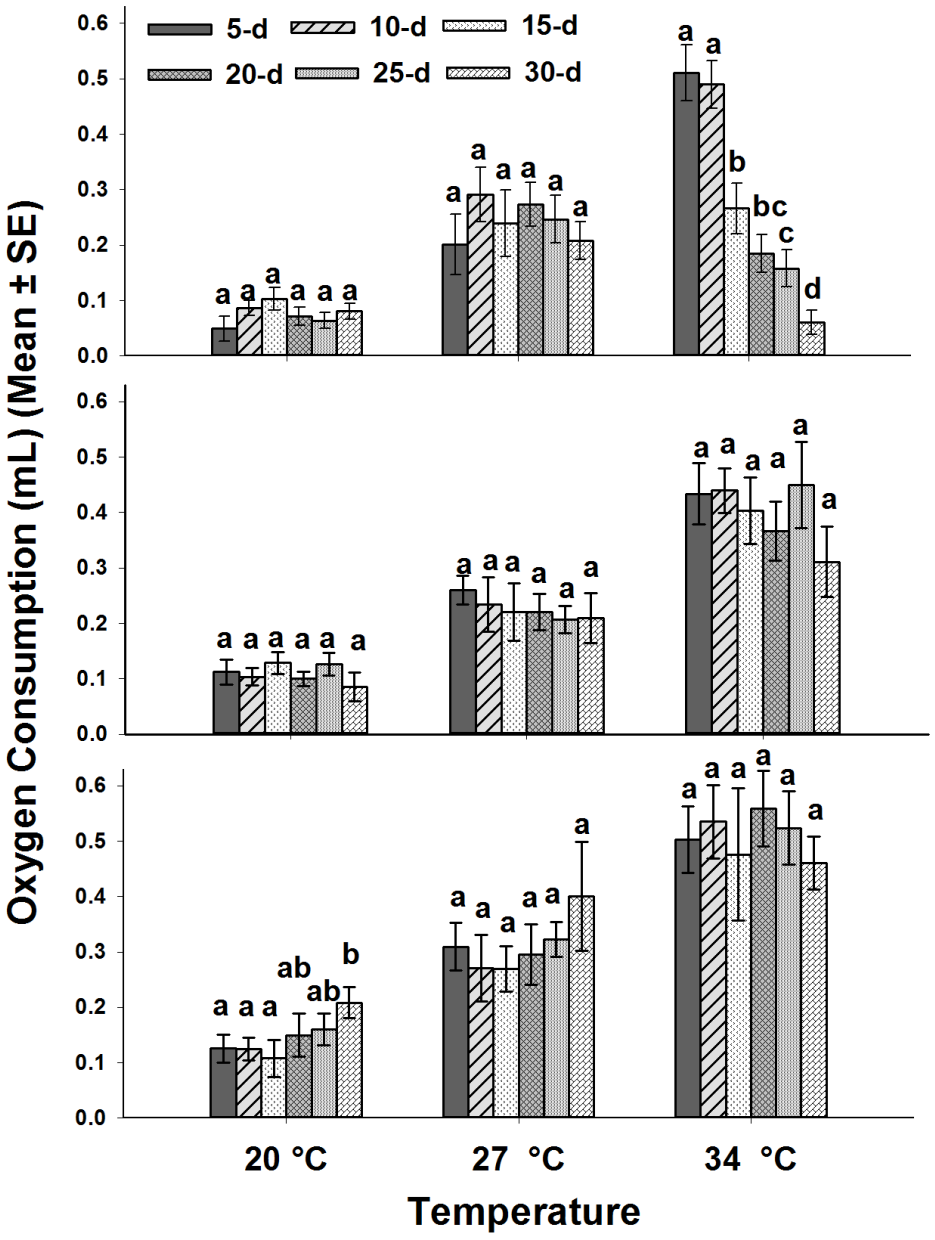

Figure 2.5. Average daily oxygen consumption by *T. castaneum* adults at 5, 10, 15, 20, 25, and 30 days (d) held inside different sized sealed containers at different temperatures; Top: 60 mL, middle: 125 mL, bottom 250 mL. Means among days (d) within temperature levels with the same letter are not significantly different based on adjusted Tukey (alpha = 0.05)

CHAPTER 3. EFFECTS OF HYPOXIA AND EXPOSURE TIME ON *TRIBOLIUM CASTANEUM* (HERBST)

3.1 Abstract

Current management of stored grain insects relies heavily on grain fumigants and insecticidal dust. Exposing grain to toxic chemicals is a concern due to potential chemical residues and insect resistance. Hermetic grain storage technology offers a viable chemical-free approach to control storage insects. However, there is limited understanding of how hypoxic conditions in hermetic containers affect insect development. We exposed *Tribolium castaneum* (Herbst) eggs (2 d), young larvae (7 d), old larvae (21 d) pupae (28 d), and adults (2 d after emergence) to 2, 4, and 8% oxygen levels as well as to ambient oxygen (20.9%) for 1, 3, 5, 10, and 15 d and assessed subsequent mortality. Eggs and young larvae were the most susceptible stage, experiencing complete mortality when exposed to 2% oxygen level for three or more days. Old larvae and pupae experienced 100% percent mortality when exposed to 2% oxygen level for ten days or more. Adults required the longest (15 d) hypoxic conditions to achieve complete mortality at 2% oxygen. Similarly, 4% oxygen levels required 5 d to produce complete mortality of eggs and more than 15 d to kill other life stages of *T. castaneum.* By contrast, exposure to 8% oxygen for 15 d produced less than 20% mortality both in adults and immatures, while eggs experienced higher mortality (>90%). Even so, exposure to 8% oxygen levels even for a relatively short time (1-3 d) caused significant developmental delays in immatures. Nonetheless, our study shows potential utility of hermetic technology for control of *T. castaneum*, but the internal oxygen should be maintained below 2% levels for more than 15 d. If those conditions are not achievable, the crop storage duration should be extended to a minimum of 3-6 month as previously shown by other studies.

Keywords: Hermetic storage, *T. castaneum*, life-stages, hypoxia treatment, developmental delay

3.2 Introduction

Tribolium castaneum (Herbst) (Coleoptera, Tenebrionidae), the red flour beetle, is an important pest of stored cereal grains, pulses, and oilseeds. It has one of the highest population growth potentials for any stored-product insects (White, 1988). *T. castaneum* causes damage by feeding on the germ and endosperm of grain kernels and by contaminating the grain with body parts and feces. When the infestation level is high, *T. castaneum* secretes benzoquinones, which imparts a pungent odor to the commodity, rendering it unfit for consumption (Campbell and Runnion 2003). Pest control tactics for *T. castaneum* in stored grain rely heavily on synthetic insecticides, including slow-release grain fumigants such as phostoxin and essential oils. The grain may be treated with insecticidal dusts, residual sprays, or with other materials before storage (i.e., diatomaceous earth, Malathion, Chlorpyrifos-methyl) (Zettler and Cuperus 1990, Lee et al. 2002, Epidi and Odili 2009). Extensive use of chemicals has led *T. castaneum* to be resistant to many conventional insecticides (Jagadeesan et al. 2012). Furthermore, usage of insecticides on stored food commodities is a concern due to human and environmental health hazards associated with exposure, including danger to applicators using improper application practices.

Hermetic storage technology, a chemical-free approach, is a promising alternative for management of insect pests of stored grains and pulses. The technology reduces the oxygen supply of these pests by fostering the development of a hypoxic environment inside the storage system. Suboptimal levels of oxygen suppress insect population growth and survival. Hypoxic conditions are achieved mainly due to (i) respiration of insects and other organisms present including the grain itself, and (ii) making the storage system airtight to greatly restrict the movement of air from outside (Navarro et al. 1994). Studies have shown that hermetic

bagging systems such as Purdue Improved Crop Storage (PICS) bags can maintain oxygen levels at 2-15% for extended periods of time, typically for 3-6 months, which limits insect population growth (Murdock et al. 2012, Baoua et al. 2014, Kharel et al. 2018). Oxygen levels below 5% are effective in controlling various storage insects including *Callosobruchus maculatus*, *Cryptolestes ferrugineus*, *Rhyzopertha dominica*, *Oryzaephilus surinamensis*, *Sitophilus oryzae*, *S. zeamais* and *Trogoderma granarium* (Bailey 1965, Baributsa et al. 2010, Murdock et al. 2012, Baoua et al. 2014, Martin et al. 2015, Yan et al. 2016, Kharel et al. 2018). In the past, hermetic storage technology was mainly used by small-scale farmers to store their surplus crops for subsequent consumption or marketing. Thanks to its proven effectiveness against a broad range of storage pests, hermetic technology has begun to attract the attention of a wider circle of users such as large-scale farmers, grain traders, and organic food distributors.

Little is known about how the different *T. castaneum* life stages are affected by exposure to hypoxic conditions. The immature stages of most stored product insects spend significant amounts of their developmental time inside grain kernels or buried beneath the flour surface as in the case of *T. castaneum* (Mason et al. 2012). In such locations, oxygen may be limited because oxygen inflow is retarded by the compactly filled stored commodities and the grain itself presents a barrier to oxygen movement (Williams et al. 2016). We hypothesized that the different developmental stages of insects might exhibit varying degrees of susceptibility to hypoxia. To evaluate this hypothesis, we designed life stage-specific studies in an attempt to understand in more detail the effects of hypoxia. Better understanding of these effects could lead to improving the effectiveness of hermetic storage for the control of *T. castaneum* and, by implication, other insects. Here we report the results of a laboratory study on the response of *T. castaneum* life stages exposed for different times to artificially-created hypoxic conditions in hermetic containers.

3.3 Methods

3.3.1 Insects

T. castaneum were obtained from colonies maintained on a mixture of whole wheat flour and brewer's yeast (95:5) used by the Stored Product Insect Rearing Facility at the Department of Entomology, Purdue University, West Lafayette, Indiana, USA. Colonies were reared in a CARON Insect Growth Chamber (CARON model 6025-1) (Caron Products & Services, Inc., Ohio, USA) at 27 ± 1°C, 60% R.H. and at 16:8 light:dark photoperiod. The developmental life stages selected for the experiment were: egg (2 d), young larva (7 d), old larva (21 d), pupa (28 d), and freshly emerged unmated (selected and kept separately from the pupal stage), and mixed-sex adults (1-2 d after emergence).

3.3.2 Experimental preparation

Clear glass containers (30 ml) with plastic lids containing approximately 0.5 g of wheat flour added as food for the insects were used for each hypoxia treatment (Wheaton Glass Sample Bottle, CP Lab Safety, California, USA). Lids were perforated with several small holes to allow gas exchange. Each container received a single *T. castaneum* (either an egg, young larvae, old larvae, pupae, or adult). For each life stage, 40 glass containers were prepared. Thus, for each hypoxia condition (8, 4, or 2% oxygen), there were 40 containers for each of the five life stages. There were also 40 containers in which the insects were held in normoxia (20.9% oxygen) as controls.

3.3.3 Hypoxia treatment

Three chambers consisting of clear polycarbonate vacuum chambers 16.5 x 13.6 x 15 cubic inches, 35 L capacity, were obtained from Bel-Art - SP Scienceware, New Jersey, USA, and used to expose insects to different levels of hypoxia. The experiment was

conducted at room temperature (27 ± 2°C). Each chamber received ten glass containers for each of the life stages of *T. castaneum* (3 x 10 = 30 containers), and the remaining ten containers for each life stage were kept at ambient oxygen levels (control- 20.9%). Three of the polycarbonate chambers were randomly chosen for given levels of hypoxia (2, 4, or 8% oxygen levels) using a random sequence generator (Haahr, 2002). The individual hypoxia levels in the chambers were created by flushing the air out of chambers while replacing it with nitrogen gas from a gas cylinder until the target concentration of oxygen in the chamber was attained. Oxygen content of the chambers was subsequently monitored every three to four hours during the day and every six hours during the night. An Oxysense 5250i® oxygen reader device was used in conjunction with fluorescent yellow Oxydots attached to the inner surfaces of the chamber (Oxysense, Dallas, TX). The Oxysense device is a non-invasive method for monitoring the oxygen content of a sealed translucent container. Oxygen content was maintained within ± 0.25% of the target oxygen levels. If the oxygen levels elevated beyond ± 0.25%, nitrogen gas was pumped into the hypoxia chamber to restore the target level, and if the oxygen level declined below the target, the inlets in the chambers were opened for a short period to admit ambient air.

3.3.4 Exposure duration and experimental run

The duration of exposure to each hypoxia condition was 1, 3, 5, 10, and 15 days. The hypoxia treatment for the different exposure days was conducted separately, i.e., when the individuals were taken out of the hypoxia chamber after exposing for a given number of days, the treatment for another exposure day was initiated. The whole experiment was repeated three times (true replicates). This was done because the glass containers (n = 10) for each treatment combination were held inside the same hypoxia chamber and were considered as pseudoreplicates.

3.3.5 Data collection

3.3.5.1 Adult survivorship

At the end of each hypoxia treatment, the adults were removed from the hypoxia chamber and examined. They were classified as live if they were capable of reflex movement and dead if they did not move when prodded with a probe. Data were recorded as a binary response. If an adult was alive, it was given the value 1, and a value of 0 if dead. Controls were classified in the same manner. After recording the initial survivorship, all adults for each treatment groups were collected and placed in vials with food (n = 10). They were held in growth chambers (27 ±1°C, 60% R.H. and 16:8 light:dark photoperiod). After ten days, post-exposure survivorship was determined using the same assessment as above.

3.3.5.2 Immature stage experiment

Exposed eggs, young larvae, old larvae, and pupae were held for a maximum of 90 d post-treatment in the incubator (27°C, 65% RH) until any adult emergence had occurred. Adult emergence from exposed immatures of each treatment was recorded as a binary response, as above. Thus, if an adult emerged from an immature stage that had been exposed to hypoxia, it was recorded as 1. Similarly, if the immature stage failed to develop as an adult within 90 d, it was assigned a value of 0. In addition, the immature developmental time for *T. castaneum* was determined for the control and hypoxia-exposed young larvae. Only young larvae and no eggs were used for the developmental time studies because there was a high level of mortality in eggs exposed to hypoxia for even a short time. Young larvae exposed to 4 and 8% oxygen levels for 1 and 3 d had at least 80% hypoxia survival. Therefore, these two groups were chosen for further developmental time observations. The young larvae were then allowed to develop at ambient oxygen for 30 d post-treatment, after that they were checked every 3d to record the time (d) of adult emergence.

3.3.6 Statistical analysis

Wald's test was used to determine if the covariance estimate of the random effect from three experimental runs was significantly different from 0 or not. If the experimental run showed no significant differences, data were pooled over the variable experimental run for further analysis. For the adult stage, logistic regressions were constructed to model the percentage adult survival using SAS® PROC GLIMMIX Maximum Likelihood Estimation based on Laplace Approximation with the fixed effects of oxygen levels and exposure duration. For the post-exposure survivorship, the number of live adults in each treatment combination was determined and converted to percentage values and then transformed to angular values (Zar 2010). After that, the data were subjected to repeated measure ANOVA to determine the effects of oxygen levels, exposure duration, and post-exposure days on adult survivorship. However, the data presented in the text are all untransformed mean percentage of live adults from three experimental runs.

Similarly, for the immature stages, logistic regression was constructed to model the proportion of adult emergence from immature stages using SAS® PROC GLIMMIX Maximum Likelihood Estimation based on Laplace Approximation with the main effects of oxygen levels, exposure duration, and life stages. Data for the developmental period were analyzed using PROC GLM. Treatment means were separated using adjusted Tukey at $P \leq 0.05$.

3.4 Results

3.4.1 Adult experiment

The test of covariance parameters based on the likelihood ratio test showed that random effects from the experimental run were not significant ($\chi^2 = 0.67$, $P = 0.21$). Accordingly, the data were pooled together and analyzed for the effects of oxygen levels and

exposure time. In the post-treatment assessment begun immediately after returning them to normoxia, 100% of the adults in control treatments survived as did those exposed at all levels of hypoxia for 1 and 3 d. The data for 1 and 3 d and the controls were excluded from further analysis since there was no variation in the responses. We only used the hypoxia treatments of 2, 4, and 8% oxygen levels and for 5, 10 and 15 d to examine if there were posttreatment effects on adult survival. The GLIMMIX test of fixed effects showed that adult survival was significantly affected by (1) the oxygen levels ($F = 18.51$; $df = 2, 259$; $P < 0.01$) (2) exposure time ($F = 3.39$; $df = 2, 259$; $P = 0.03$) and (3) the interactions of oxygen levels x survival days ($F = 32.29$; $df = 2, 259$; $P < 0.01$). The probability of adult survival decreased as the oxygen levels decreased from 8 to 2% and likewise decreased as the length of exposure increased from 5 to 15 d. When exposed to 2% oxygen for 15 d, more than 99% of the exposed adults died. By contrast, at 8% oxygen levels, over 90% of the insects survived even when exposed for 15 d (Fig 1).

Post-exposure adult survival was affected by the level of hypoxia ($F= 261.29$; $df = 2, 58$; $P < 0.01$), duration of exposure to hypoxia ($F= 446.50$; $df = 4, 58$; $P < 0.01$), and observation time after hypoxia treatment (d) ($F= 43.70$; $df = 1, 58$; $P < 0.01$) (Table 3.1). About 20- 45% of the *T. castaneum* adults that initially survived the hypoxia treatment at 2% and 4% oxygen levels for 5 to 15 d were found dead 10-days post-treatment. However, at 8% oxygen levels, less than 4% mortality occurred at 10 d after hypoxia treatment compared to immediately after exposure (Table 3.1).

3.4.2 Immature stages

The test of covariance parameters based on the likelihood ratio test showed that random effects from the experimental run were not significant ($\chi^2 = 0.53$, $P = 0.23$). The test of fixed effects revealed that adult emergence from exposed immatures was significantly affected by (1) the insect developmental life stage exposed to hypoxia ($F = 769.12$; $df = 3$,

2318; P <0.01), (2) oxygen levels (F= 810.17; df = 3, 2318; P <0.01), (3) exposure time (F= 348.22; df = 4, 2318; P <0.01), and (4) the interactions of life stages x oxygen levels x exposure time (F= 142.69; df = 3, 2318; P <0.01). Eggs were the most susceptible immature life stage to hypoxia treatment (Table 3.2). Oxygen levels of 4% or less for 3 d or more killed all eggs. A hypoxia level of 4% oxygen or less for at least 10 d killed over 75% of the young larvae, older larvae, and pupae. About 80% of the young larvae, old larvae, and pupae exposed to the 8% oxygen levels survived regardless of exposure duration (Table 3.2).

Furthermore, the two-way ANOVA to determine the effects of different levels of oxygen and exposure periods on the adult emergence time from the exposed young larvae showed that developmental time was significantly affected by the oxygen levels, exposure days, and their interactions. The young larvae-to-adulthood developmental time increased by 14 d at 4% and by 8 d at 8% oxygen level compared to control when young larvae were exposed to hypoxia for 1 d. Similarly, when exposed to hypoxia for 3 d, the developmental time increased by 18 d at 4% oxygen and by 15 d at 8% compared to the control (Figure 3.2).

3.5 Discussion

3.5.1 Hypoxia exposure of adults

Exposing *T. castaneum* adults to 2% oxygen for 10 d killed 90% of them. Increasing the exposure period to 15 d resulted in greater than 99% mortality. However, to reach 90% mortality at 4% oxygen, adults required exposure for 15 d. On the other hand, 8% oxygen and 15 d of exposure period produced less than 5% mortality. Our findings are consistent with those of previous researchers who reported that most stored product insects are sensitive to the oxygen levels <5%, (Donahaye 1990, Viebrantz et al. 2016, Yan et al. 2016, Njoroge et al. 2017).

Depletion of oxygen levels in hermetic containers is a gradual, biologically-driven process. Depending on the initial insect infestation level, it can take several days or even weeks to reach <5% levels. It would enhance the value of hermetic storage if there were a mechanism to accelerate oxygen depletion in the hermetic container. On the other hand, in many cases, oxygen levels within hermetic storage systems are depleted rapidly reaching <5% days within a few days. This happens when the infestation level is relatively high and when the ambient temperature is warmer. These conditions promote a more rapid rate of insect metabolism and in some cases, respiration of the grain (Williams et al. 2016, Kharel et al. 2018). Additionally, it is useful to remember that hypoxia may affect other aspects of the insect life cycle besides survivorship. Hypoxia has been shown to reduce oviposition, progeny development, body mass, and longevity of the insects (Spratt 1979, Cheng et al. 2012, Yan et al. 2016), all of which slow overall population growth. Further evidence of the sublethal effects of hypoxia is our present observations of additional mortality of *T. castaneum* adults at 10 d post-treatment. This suggests that many of the adults that responded positively to the test probing (i.e., survive) immediately after hypoxia treatments of low oxygen levels such as 2 or 4% were, in fact, moribund and died by 10 d post-treatment. Further investigations of insect behavior and physiology of the surviving adults would shed valuable light on the still largely unknown sublethal effects of hypoxia on insects.

3.5.2 Hypoxia effects on immature stages

Our study shows that the different life stages of *T. castaneum* respond differently to hypoxia. The egg was the most susceptible stage, with 100% mortality to 2% oxygen when exposed for at least 3 d. Yan et al. (2016) found that exposing eggs of *C. maculatus* to 2% oxygen for two days suppressed adult emergence by over 80%, and increasing the exposure time to three days, reduced adult emergence by > 98% compared to eggs exposed to normal ambient oxygen levels. For other juvenile stages, exposure to hypoxia of 2%

oxygen for at least fifteen days was critical to arrest adult emergence of exposed individuals. According to Zrubek and Wood (2005), adult and juvenile insects possess efficient systems to regulate oxygen flux and water balance, but eggs lack sensory systems to monitor hypoxia, thereby making the egg stage the most sensitive to hypoxic environments. Interestingly, many studies in the past have shown *T. castaneum* eggs to be the most tolerant stages to conventional pest control methods such as grain fumigants used in crop bagging systems (Bell et al. 1998, Walse et al. 2009). Hence, storing grains and processed food commodities in hermetic bags in food facilities could contribute to control population development of *T. castaneum*. The pupal stage was the least sensitive of the immature stages to hypoxia. This may be due to depressed respiratory metabolism during pupation (Navarro 2006). Further studies are needed to understand the mechanisms underlying the difference in the susceptibility to hypoxia among the different developmental stages of *T. castaneum*. Nevertheless, when exposed to 2% or 4% oxygen levels for 15 d, over 93% of the individuals of all developmental stages succumbed. As with the adult stage, immature stages were less susceptible to 8% oxygen compared to lower levels, as over 75% of individuals of all developmental stages survived 15 days of exposure to this level of hypoxia. Even so, even a short exposure (<3 d) to 8% oxygen levels caused marked developmental delays in immature *T. castaneum*. Previously Loudon (1988) reported that the larvae of *Tenebrio molitor* L. exposed to 10% oxygen levels molted 12.3 times compared to 5.8 at normoxia, thereby supporting the assumptions of hypoxia-related developmental delay in insects. This delay will have the effect of retarding population growth, thereby helping make the hermetic system more effective in controlling the numbers of insect present.

In conclusion, *T. castaneum* eggs, the most tolerant stage to the conventional fumigants, can be controlled if the hermetic storage maintains an oxygen level of 2% for at least 3 d. Other juvenile stages *T. castaneum* can be controlled when exposed to oxygen

levels of 2% for 10 d. However, adults of *T. castaneum* require exposure period of 15 d at 2% oxygen level to produce complete mortality (statistically, it is possible to obtain complete mortality of *T. castaneum* adults in 10 d at 2% oxygen levels). However, the hermetic containers which can maintain oxygen levels around 4% require at least 15 d to kills 100% eggs and over 93% of the other life stages. Higher levels of oxygen (such as 8% oxygen), may not directly contribute to the death of adults and immature stages of *T. castaneum*, that level of hypoxia, even for a short period (1 or 3 d) can increase the developmental time of the immature stages, thereby slowing the rate of population expansion inside hermetic containers. Therefore, the oxygen levels inside hermetic containers should be maintained below 2% for at least 15 d for the control of *T. castaneum* life stages. The oxygen levels of 4% for at least 15 d can completely suppress the eggs and larvae and kill over 90% of the pupae and adults of *T. castaneum*, thus contributing greatly to the control of the population development of *T. castaneum* inside hermetic storage. Nevertheless, the non-lethal oxygen levels such as 8% can help to reduce *T. castaneum* population growth by increasing the developmental time of the immature stages.

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Table 3.1. Percent *T. castaneum* adult survival when exposed to 2, 4, and 8% oxygen levels for 1, 3, 5, 10, and 15 days when assessed immediately after exposure and 10 days (d) post-exposure.

Exposure periods (days)	Percent adult survival (Mean ± SE)					
	8% Oxygen		4% Oxygen		2% Oxygen	
	Immediately after exposure	10 d after	Immediately after exposure	10 d after	Immediately after exposure	10 d after
1	100a	100a	100a	96.7 ± 3.3b	100a	100a
3	100a	96.7 ± 3.3b	100a	90b	100a	70 ± 5.8
5	100a	96.7 ± 3.3b	80 ± 5.7a	66.7 ± 3.3b	23.3 ± 6.7a	13.3 ± 6.7b
10	100a	100a	36.7 ± 6.6a	26.7 ± 3.3b	10 ± 10a	6.7 ± 3.3b
15	96.7 ± 3.3a	96.7 ± 3.3a	10 ± 5.7a	6.6 ± 3.3a	0a	0a

Initially, ten *T. castaneum* adults were exposed to each treatment, and the experiment was repeated three times. The values presented in the table are the means of percent adult survival from three experimental runs. Means among columns (post-exposure observation days) within a hypoxia level and an exposure day with the same letter are not significantly different ($P \geq 0.05$, n=3, adjusted Tukey).

Table 3.2. Percent live adult emergence from the immature life stages of *T. castaneum* exposed to 2, 4, 8 and 20.9% levels of oxygen for 1, 3, 5, 10, and 15 days

Oxygen levels	Exposure periods (days)	Percent live adult emergence (Mean ± SE)			
		Egg	Young larvae	Old larvae	Pupae
2%	1	50 ± 9.3Ab	39.9 ± 9.1Ab	90 ± 5.5Aa	93.4 ± 4.6Aa
	3	0 ± 0Bc	0 ± 0Bc	16.6 ± 6.8Bb	66.7 ± 8.7Ba
	5	0 ± 0Bb	3.3 ± 3.3Bab	6.7 ± 4.6Ba	3.3 ± 3.2Cab
	10	0 ± 0Ba	0 ± 0Ba	0 ± 0Ca	0 ± 0Ca
	15	0 ± 0Ba	0 ± 0Ba	0 ± 0Ca	0 ± 0Ca
4%	1	56.7 ± 9.2Ab	80.1 ± 7.4Aa	93.4 ± 4.6Aa	86.7 ± 6.2Ba
	3	4 ± 1.1Bc	90.1 ± 5.5Aa	73.4 ± 8.2Bb	100 ± 0Aa
	5	3.3 ± 3.3BCd	29.9 ± 8.5Bc	56.7 ± 9.3Ba	70.1 ± 8.5Ca
	10	3.3 ± 3.2BCb	23.3 ± 7.9Ba	9.9 ± 5.5Ca	19.9 ± 7.4Da
	15	0 ± 0Cb	6.6 ± 4.8Ca	0 ± 0Db	6.7 ± 4.6Ea
8%	1	73.4 ± 8.2Ac	90.1 ± 5.5Ab	96.7 ± 3.3Aab	100 ± 0Aa
	3	36.6 ± 8.9BCb	96.7 ± 3.2Aa	100 ± 0Aa	100 ± 0Aa
	5	53.3 ± 9.3Bc	96.6 ± 3.3Aa	83.4 ± 6.8Bb	100 ± 0Aa
	10	23.3 ± 7.8Cb	96.7 ± 3.2Aa	90.1 ± 5.5ABa	93.4 ± 4.6Ba
	15	6.6 ± 4.5Db	73.4 ± 8.2Ba	83.4 ± 6Ba	80.1 ± 7.4Ca

Eggs (2 d), young larvae (7 d), old larvae (21 d), and pupae (28 d) were exposed to 2, 4, 8, 20.9% oxygen levels for 1, 3, 5, 10, and 15 d. After hypoxia treatments, the immature stages were held for a maximum of 90 d at normoxia to observe any adult emergence had occurred. All immature stages kept at 20.9% (control) survived (data not presented). Means among exposure days (upper case letters) and immature life stages (lower case letters) within given oxygen levels with the same letter are not significantly different ($P \geq 0.05$, n=30, adjusted Tukey).

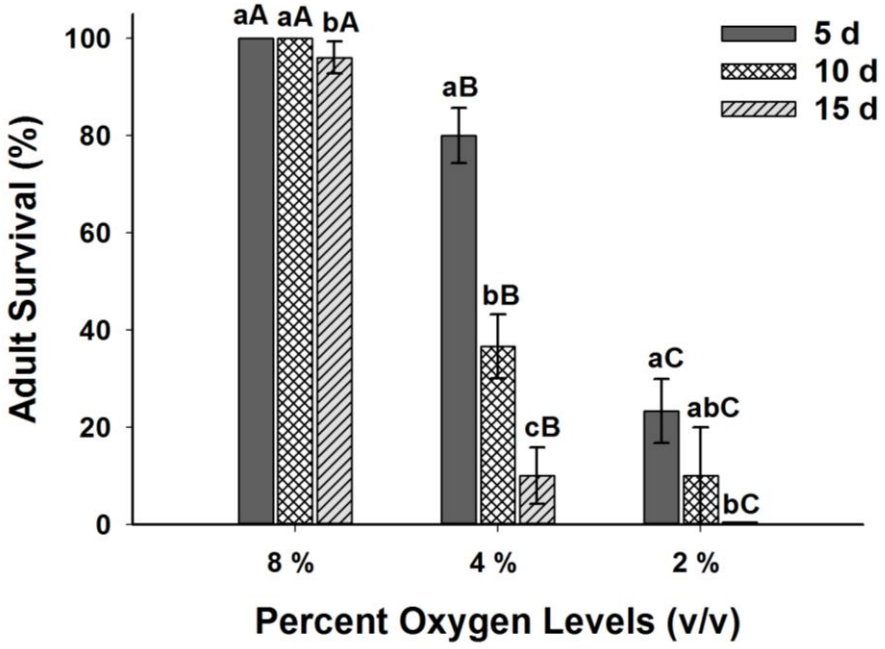

Figure 3.1. Percent *T. castaneum* adult survival after exposure to 2, 4, 8% hypoxia for 5,10 and 15 days. The assessment was done immediately after returning the hypoxia-exposed adults to normoxia. Means among hypoxia levels (upper case letters) and exposure periods (lower case letters) with the same letter are not significantly different ($P \geq 0.05$, n=30, adjusted Tukey)

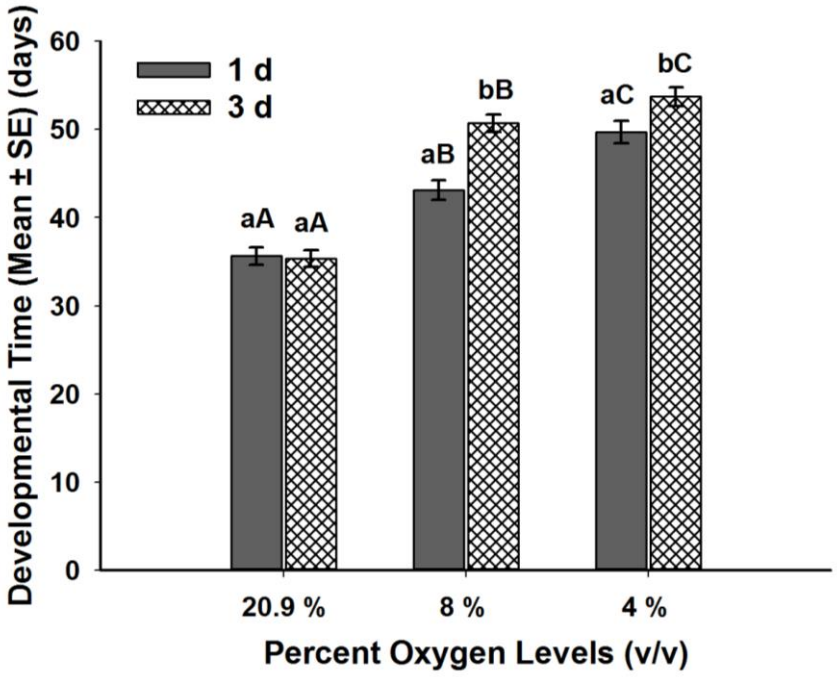

Figure 3.2. Total time required by young larvae of *T. castaneum* (7 d old) to reach adulthood when exposed to 4%, 8%, and atmospheric levels of oxygen (20.9%) for 1 and 3 days (d). Means among hypoxia levels (upper case letters) and exposure periods (lower case letters) with the same letter are not significantly different ($P \geq 0.05$, n=30, adjusted Tukey).

CHAPTER 4. EFFECTS OF HYPOXIA ON THE BEHAVIORAL ACTIVITY AND CELLULAR ENERGY IN *TRIBOLIUM CASTANEUM* (HERBST)

4.1 Abstract

Modified atmosphere (MA) techniques such as hermetic systems are viable and chemical-free pest control methods for postharvest crop storage. However, information about how insects respond to the hypoxic conditions during hermetic storage is limited. We utilized ultrasonic monitoring to characterize the individual activity of adults and immature stages of *Tribolium castaneum* (Herbst) at 2, 4, 8 and 20.9% oxygen levels. We observed that the activities of both adults and larvae were reduced with decreasing oxygen levels, and the effects were apparent within the first 30 minutes of hypoxia exposure. The average activity for adults and immature stages at 2, 4, and 8% oxygen levels were reduced to at least 7.1, 16.1, and 23.5%, respectively, when compared to the activity at the normoxia. We also determined the adenylate energy charge (AEC) in adults of *T. castaneum* exposed to 2, 4, 8, and 20% oxygen levels for 48 h as well as <1% oxygen levels for 96 h by measuring ATP, ADP, and AMP utilizing high-performance liquid chromatography (HPLC). The AEC index of *T. castaneum* adults at normoxia was 0.70 ± 0.0, while the AEC at <1%, 2, 4, 8% oxygen levels were 0.18 ± 0.0, 0.19 ± 0.01, 0.25 ± 0.02, and 0.58 ±0.01, respectively. Complete mortality of *T. castaneum* adults was achieved at hypoxia treatment of <1% oxygen for 96 h. Taken together, our results suggest that the MA technology that maintains oxygen levels ≥2% for 48 h does not completely eliminate the *T. castaneum* population. However, lower levels of hypoxia can markedly reduce available cellular energy in *T. castaneum* adults, and thus lowering their overall activity including feeding and movement. This, in turn, suppresses population development and grain damage potential. Keywords: Hermetic storage, *T. castaneum*, insect activity, ultrasonic signal, cellular energy

4.2 Introduction

Modified atmosphere (MAs) techniques are widely used for the control of postharvest insect pests in stored grain commodities. Hermetic systems are a type of MA method that can naturally create a hypoxic environment inside a container due to respiration of living organisms present in the grain. Oxygen levels inside hermetic grain storage bags can be depleted within a few days to levels which are unsuitable for insect development (Baoua et al. 2012, Murdock et al. 2012). Hermetic bags are particularly popular among small-scale farmers in developing countries as a viable and chemical-free pest control measure to protect their stored crop commodities. Nevertheless, information as to how insect behavior and physiological functioning are affected by the hypoxic environment inside the hermetic storage is presently unavailable.

Previous researchers have shown that hypoxia affects various aspects of insect population development including feeding, reproduction, developmental time, and survival. Murdock et al. (2012) utilized an ultrasonic device called the Purdue Insect Feeding Monitor (PIFM) to detect and record the feeding behavior of *Callosobruchus maculatus* (F.) exposed to different levels of oxygen. They reported that *C. maculatus* stops feeding when the oxygen levels reach below 5%. Furthermore, Njoroge et al. (2017) used a piezoelectric sensor-preamplifier module to observe the activity of *Sitophilus oryzae* L. inside hermetic containers and reported that the activity of *S. oryzae* decreases at oxygen levels below 5% and completely ceases when oxygen levels are below 2%. However, there are no reports on hypoxia and the activity of external feeders like *T. castaneum* within hermetic containers. Feeding behavior and overall activity of insects over time inside hermetic storages are of interest because they are directly related to the degree of feeding damage to grains and the efficacy of hermetic storage.

Hypoxic can affect various important biochemical pathways in insects. One of the most important pathways affected is carbohydrate catabolism. This involves the conversion of glucose molecules in the presence of oxygen into carbon dioxide, water, and energy. Energy produced through the oxidation of carbohydrates is used for regulating various biochemical pathways such as maintaining homeostasis, muscle activity, and biosynthesis. The primary function of oxidative metabolic pathways is to regenerate adenosine triphosphate (ATP) from adenosine diphosphate (ADP), which puts energy into the adenylate system, while the biosynthesis in the cells converts ATP to ADP, thereby removing the energy from the adenylate system and using it to do work (Baker et al. 2010). The phosphotransferase enzyme called adenylate kinase catalyzes the interconversion of adenine nucleotides (ATP, ADP, and adenosine monophosphate (AMP) in cells. Regulation of these pathways is critical to meet the metabolic needs of the cell (Raffin and Thebault 1996). Previous studies have extensively used the quantification of ATP to determine the effects of hypoxia in insects (Friedlander and Navarro 1979, Voorhies 2009). However, the cellular metabolism is regulated by the proportion of the adenylate nucleotides (ATP, ADP, AMP) in the adenylate system rather than the absolute concentrations of individual adenylates (Swedes et al. 1975).

The ratio of the adenylates in living cells or the energy status of the adenylate system can be measured with the index called adenylate energy charge (AEC), a concept developed by Atkinson (1968). The AEC index has been used to determine the environmental stresses such as nutritional stress, oxygen depletion, desiccation, heat and chemical stresses in an organism through the status of the metabolic energy (Ivanovici and Wiebe 1981, Hochachka et al. 1996, Milusheva et al. 1996). Exposure to hypoxic conditions can lead to the reduction of AEC levels in the cell of insects (Hoffmann and Parsons 1989; Mitcham et al. 2006). However, more studies

are required to understand the status of the cellular energy in stored product insects exposed to hypoxia.

The red flour beetle, Tr*ibolium castaneum* (Herbst) is one of the most important pests of grain storage and food processing industries. It is also an important model insect for many physiological and molecular studies. In the present study, we utilized PIFM to characterize the temporal activity of individual *T. castaneum* adults and larvae exposed to different oxygen levels for 48 hours (h) in the presence or absence of food; and also determined the AEC levels in *T. castaneum* adults exposed to different levels of hypoxia for 48 and 96 h.

4.3 Methods

4.3.1 Experiment 1: Monitoring the activity of *T. castaneum* adults and immature stages

The experiments were carried out in Purdue University's insect quarantine room at ambient room temperatures (27 ± 1°C) during the fall of 2017. The room was isolated and offered a quiet environment for this experiment.

4.3.1.1 Insects

T. castaneum were obtained from colonies maintained on a mixture of whole wheat flour and brewer's yeast (95:5) by the Stored Product Insect Rearing Facilities at the Department of Entomology, Purdue University, West Lafayette, IN, USA. The colonies were reared in a CARON Insect Growth Chamber (CARON model 6025-1) (Caron Products & Services, Inc., Ohio, USA) at 27 ± 1°C, 60% R.H. and a 16:8 light:dark photoperiod. Young larvae (1-week-old), old larvae (3-weeks-old) and freshly emerged mixed-sex adults (1-2 d after emergence) were selected for the study.

4.3.1.2 Purdue Insect Feeding Monitor (PIFM)

The PIFM device can detect, amplify, and record the signals generated by the insects by means of piezoelectric transducers adjacent to the insects. Data is collected by means of LabVIEW computer software (Shade et al. 1989). This device has been used previously by many researchers to study the feeding behavior of internally feeding insects such as *C. maculatus* and *Sitophilus oryzae* (Shade et al. 1990, Pittendrigh et al. 1997, Salzman et al. 2003). It was used for monitoring the effects of hypoxia on the feeding behavior of *C. maculatus* by Murdock et al. (2012). This is the first time the PIFM has been used to record the activity of the external feeder, *T. castaneum*.

An 8-channel PIFM device consisting of eight transducers was used for the experiments. Small wells were prepared using cell-culture plates to hold a single insect. The wells were modeled to fit inside the transducers.

4.3.1.3 Treatments

Two sets of wells were prepared with a single individual insect, either a young larva, an old larva, an adult, or no insect (control). Approximately 0.1 g of wheat flour was added to one set of wells whereas the other set had no food/flour. The opening of the wells was covered using filter paper to prevent the insects from escaping. Eight wells were used for placing each insect stage plus control w/ and w/o flour randomly in the eight transducers of the PIFM. Cylindrical wooden blocks were positioned above the wells to ensure good contact between the wells and the metal surface of the transducer to increase signal detection (Murdock et al. 2012). Subsequently, the PIFM device was placed inside the hypoxia treatment chambers (Pittendrigh et al. 1997) and maintained at 2, 4, 8% oxygen levels or 20.9% oxygen levels (room condition). Target hypoxia

levels inside the chambers were obtained by flushing air out of chambers while pumping in nitrogen gas from a compressed gas cylinder. Oxygen content of the chambers was monitored every three to four hours (h) using an Oxysense 5250i® oxygen reader device and fluorescent yellow Oxydots attached to the inside surface of the chamber (Oxysense, Dallas, TX). The Oxysense device is a non-invasive method to record the oxygen content of a sealed container. The oxygen content was maintained within ± 0.25% of the target oxygen levels. If the oxygen levels in the chamber began to be elevated, nitrogen gas was pumped into the chamber to return the level to the target values. If the oxygen level in the chamber began to decline below target, the inlets in the chambers were opened for a short period to let ambient air flow inside until the target value was reattained.

4.3.1.4 Monitoring activities

Physical movements of *T. castaneum* adults or larvae in contact with the surface of the transducers generated electrical signals that reflected their degree of physical activities. Individual insect activities were monitored for sequential 30 min intervals for 48 h. Data was collected using a Microsoft Windows 8 operating computer running LabView II software (National Instruments Corp., 1990). Any background noise observed in wells with insects were removed by subtracting activity recorded on control channel transducers (empty wells w/ or w/o flour) from the activity recorded from each of the treatment channels. The experiment was replicated four times (four experimental runs).

4.3.1.5 Statistical analysis

The activity data throughout every three-hour period were summed to produce 16 data points to reflect the overall pattern of activity at different oxygen levels for 48 h. Similarly, data

was segregated to examine activity at 30 min intervals for the first five hours. Activity data were log transformed to normalize skewness. Statistical analyses were conducted with the Statistical Analysis System (SAS Institute Inc. 2013, SAS Institute, Cary, NC) (SAS, 2013). The log-transformed insect activity data were subjected to a mixed model repeated measure analysis of variance (ANOVA) to determine the significance of the random effects from the four-experimental run on the insect activity in the presence of the fixed effects from oxygen levels, the presence of flour, and observation time (h). If the random effects of the four-experimental run (repetition of the experiment) were not significant, data were pooled over experimental run for further analysis. Then, the activity data were subjected to repeated measure ANOVA to observe the effects of hypoxia treatment on average activity during the first 5 and 48 h. However, the raw data were used in the text for 5 and 48 h average activity rather than log-transformed data for better visualization. Treatment means were separated using adjusted Tukey at $P \leq 0.05$.

4.3.2. Experiment 2: Assessment of Adenylate Energy Charge (AEC) in *T. castaneum* adults

This experiment was carried out during the spring of 2018 at the Department of Entomology, Purdue University.

4.3.2.1 Hypoxia treatment of insects

Each hypoxia chambers received glass containers (n = 6) containing 120 adults of *T. castaneum* mixed-sex adults (1-2 d after emergence) (125 mL capacity glass containers, The Lab Depot Inc., Georgia, USA). Similarly, six containers with *T. castaneum* adults were kept at the ambient oxygen condition (control - 20.9% oxygen levels). The target hypoxia levels (<1, 2, 4, and 8% oxygen) inside the polycarbonate vacuum chambers (Bel-Art - SP Science ware, New Jersey, USA) were obtained by flushing air out while pumping the nitrogen gas in from a

compressed gas cylinder. Oxygen content kept within ±0.25% of the target value was monitored using an Oxysense 5250i® oxygen reader device (Oxysense, Dallas, TX). Following insect exposure for 48 h, insects in three glass containers were assessed for the number of live or dead individuals in each treatment (n =3), while insects in other three containers were quickly flash frozen in liquid nitrogen and kept at -80°C until analysis (n =3). Insects exposed to <1% oxygen levels were exposed to hypoxia for an additional 48 h (96 h total) until they were all dead. This was done to observe and compare the status AEC in dead vs. hypoxia treated adults of *T. castaneum*. The whole experiment was performed in triplicate.

4.3.2.2 Sample preparation

Approximately 0.3 g of insects were homogenized using Precellys24 homogenizer (Bertin Corp, Maryland, USA) at 5000 rpm for two cycles of 20 seconds. The homogenate was extracted using 500 µl of 8% perchloric acid in an ice bath for 10 min. The extracts were vortexed for 1 min and centrifuged at 15,000 rpm for 10 min, 4°C (Eppendorf™ 5424R Microcentrifuges). The supernatant (300 µL) solution was diluted with 1 mL of 1 M phosphate buffer, and the final pH of the insect sample was maintained at 6.5 to 7.

4.3.2.3. Instrumental method

Target analytes were analyzed using high-performance liquid chromatography (HPLC) in tandem with an ultraviolet (UV) detector (Agilent 1100 HPLC System). Analytes were separated using an Agilent Zorbax SB C18 column (4.6 i.d. x 250 mm x 5 µm). Mobile phase A consisted of 0.06 mol/L dipotassium hydrogen phosphate and 0.04 mol/L potassium dihydrogen phosphate in nanopure water at pH ~7.0, and the mobile phase B was 100% acetonitrile (Yang et al. 2002, Liu et al. 2006). The mobile phase flow rate was 1 mL/min at the solvent gradient of 100% A (0-

9 min), 25% A (9-13 min), 25% A (13-15 min), and 100% A (15-29 min). The diode array detector was used at 254 nm with the bandwidth of 8 nm.

4.3.2.4 Quality assurance and quality controls (QAQC)

A method blank consisting of 500 µL perchloric acid and 1 mL of 1M phosphate buffer was included to evaluate methodological contamination. The calibration curve was prepared in each batch of sample analysis. Method blank sample was also run prior to the analysis of insect samples to minimize potential carryover of analytes from the standard solutions. Chromatographic peaks of ATP, ADP, AMP (referred to as ATPX hereafter) were identified based on the retention time of analytes in standard solutions while analytes were quantified using an external method of calibrations. A mixture of ATPX standards stock solution was prepared at 2 mg/mL in nanopure water. Stock solutions were diluted with nanopure water to prepare 0.025, 0.05, 0.1, 0.2, 0.4 mg/mL levels. All standard solutions and insect samples were stored at -20°C.

Extraction efficiency was evaluated by spiking 20 µL of randomly selected treatment samples (all three replicates of the treatment in each batch) with a 100 µL mixture of ATPX at 0.1 mg/mL level. An aliquot of each sample (20 µL) was injected for the HPLC analysis. The corresponding detector response (area) for insect sample was subtracted from the spiked samples to determine the recovery of analytes as below:

$$\%\ Recovery = \frac{(area\ of\ spiked\ sample) - (area\ of\ insect\ sample)}{area\ of\ standard} \times 100$$

4.3.2.5 Analytes quantifications

ATP, ADP, and AMP were quantified using the linear regression equations from five-point external standard calibration curves for individual analytes. The adenylate energy charge (AEC) index was determined for each treatment samples using the following equation.

$$AEC = \frac{ATP + \frac{1}{2}\,ADP}{ATP + ADP + AMP}$$

4.3.2.6 Statistical analysis

Statistical analyses were conducted with SAS 2013. The quantitative data from the HPLC analysis (AEC index) were subjected to a mixed model using PROC MIXED to determine the significance of the random effects of the experimental run on the AEC levels in the presence of the fixed effects of oxygen levels. AEC index for treatments was expressed as means of replicate determinations. Treatment means were separated using adjusted Tukey at $P \leq 0.05$.

4.4 Results

4.4.1 Feeding monitor test

The mixed model procedure showed that the estimated random effects from the experimental run were not significantly different for the adult stage (Z = 1, P = 0.15), old larval stages (Z = 0.35, P = 0.36), and young larval stages (Z = 0.53, P = 0.29). Similarly, the repeated measure ANOVA showed that the effects on the activity due to the presence of flour were not significant for adults ($F_{1, 381}$ = 0.33, P =0.56), old larvae ($F_{1, 381}$ = 0.04, P =0.85), and young larvae ($F_{1, 381}$ = 3.27, P =0.07). Accordingly, the data were pooled and analyzed for the effects of oxygen levels and exposure time for adult and immature stages. The subsequent repeated ANOVA showed that adult activity was significantly affected by oxygen levels, exposure time,

and the interaction of oxygen levels x exposure time ($F_{3, 445}$ = 127.58, P <0.01, $F_{15, 445}$ = 5.08, P <0.01, $F_{45, 445}$ = 2.06, P <0.01, respectively). For old larvae, the repeated measure ANOVA showed that activity was significantly different due to oxygen levels and exposure time, but the interaction of oxygen levels x exposure time was not significant ($F_{3, 445}$ = 32.53, P <0.01, $F_{15, 445}$ = 2.43, P <0.01, $F_{45, 445}$ = 1.37, P =0.06, respectively). Similarly, for young larvae, the repeated measure ANOVA showed that the activity was significantly different due to oxygen levels and exposure time but not due to the interaction of oxygen levels x exposure time ($F_{3, 445}$ = 69.92, P <0.01, $F_{15, 445}$ = 3.42, P <0.01, $F_{45, 445}$ = 1.37, P =0.06, respectively). For both adults and larvae, activity was reduced with decreasing oxygen levels right from the beginning of the treatments (Figure 4.1). Therefore, we focused on trend for the first five hours using the data taken at every 30 min intervals (Figure 4.2).

For the first five-hours observation (i.e., data monitored every 30 min intervals), the mixed model procedure showed that the estimated random effects from the experimental run were not significantly different for the adults (Z = 0.53, P = 0.29), old larvae (Z = 1.07, P = 0.141), and young larvae (Z = 0.78, P = 0.21). Similarly, the repeated measure ANOVA showed that the effects on activity due to the presence of flour were not significant for adults ($F_{1, 237}$ = 1.72, P =0.190) old larvae ($F_{1, 237}$ = 0.08, P =0.776), and young larvae ($F_{1, 237}$ = 2.19, P =0.140). Accordingly, the data for adults were pooled as well and with larvae; and analyzed separately for each insect stage to assess the effects of oxygen levels and exposure time. The repeated measure ANOVA showed that the adult activity was significantly affected due to oxygen levels and exposure time ($F_{3, 277}$ = 27.53, P <0.01, and $F_{9, 277}$ = 6.33, P <0.01, respectively) but not interaction of oxygen levels x exposure time ($F_{27, 277}$ = 1.34, P =0.1262). For the old larvae, the repeated measure ANOVA showed that the activity was significantly affected by oxygen levels

and exposure time and there was an interaction of oxygen levels x exposure time ($F_{3, 277}$ = 32.0, P <0.01, $F_{9, 277}$ = 4.03, P <0.01, and $F_{27, 277}$ = 1.62, P =0.03, respectively). Similarly, for young larvae, the repeated measure ANOVA showed that the activity was significantly affected by oxygen levels and exposure time ($F_{3, 277}$ = 17.40, P <0.01, and $F_{9, 277}$ = 9.35, P <0.01, but not by the interaction of oxygen levels and exposure time ($F_{27, 277}$ = 1.08, P =0.3684). Activities for both adults and immatures were reduced with decreasing oxygen levels. Interestingly, hypoxic effects on behavior were observed in the insects within the first 30 min of treatments (Figure 4.2).

Average insect activity after 5 and 48 h hypoxia exposure showed significant differences due to oxygen levels for adults ($F_{3, 313}$ = 21.56, P <0.01 and $F_{3, 3057}$ = 382.08, P <0.01, respectively), old larvae ($F_{3, 313}$ = 36.60, P <0.01 and $F_{3, 3057}$ = 450.11, P <0.01, respectively), and young larvae ($F_{3, 313}$ = 21.56, P <0.01 and $F_{3, 3057}$ = 232.81, P <0.01, respectively). The average activity for both 5 and 48 h also decreased with decreasing oxygen levels (Table 4.1). At the 2, 4, and 8% oxygen level, the average activity for adults and immature stages were reduced to at least 7.1, 16.1, and 23.5% of that obtained in normoxia (Table 4.1).

4.4.2 Determination of AEC through HPLC tests

4.4.2.1 QAQC

No peaks were detected at the retention time of three analytes in a method blank, thus no blank correction was required. A prepared calibration curve was included in each batch of samples. The linear regression fits resulted in the coefficient of determination (R^2) ≥0.995. A solvent blank analyzed prior to the insect samples did not show carryover of analytes from the standard solutions. The average recoveries of spiked analytes at the middle point of calibration levels were above 96% for all three analytes tested (Figure 4.3).

4.4.2.2 Analytes quantifications

One set of *T. castaneum* adults exposed to different levels of oxygen levels for 48 or 96 h was used for assessing mortality, while another set of treatments was used for the analysis of the adenylates in the insects. The bioassay showed that *T. castaneum* adults exposed to 2, 4, 8, and 20.9% oxygen levels were 100% alive while the *T. castaneum* adults exposed to <1% oxygen levels for 96 h were 100% dead (data not shown).

For the AEC index data, a mixed model using PROC MIXED showed that random effects from the experimental run were not significant. ($Z = 0.66$, $P = 0.254$). Subsequent, one-way ANOVA to determine the effects of hypoxia on cellular energy levels show that the AEC index was significantly affected by the oxygen level ($F_{5, 46} = 364.37$, $P < 0.01$). The AEC value decreased sharply with the decreasing levels of oxygen, with one exception. The AEC level was not significantly different for the insects exposed to 2% oxygen levels for 48 h and <1% oxygen levels for 96 h (Figure 4.4).

4.5 Discussion

4.5.1 Effects on insect activity

Results showed that the average activity of both adults and larvae decreased to at least 7.1, 16.1 and 23.5%, at 2,4 and 8% oxygen levels, respectivelycompared to normoxia, and the decrease was apparent beginning after the first 30 min of hypoxia treatment. Comparable to our findings, Murdock et al. (2012) reported that the feeding activity of the larvae of *C. maculatus* dropped to about 20% and 5% of control at 6 and 3% oxygen levels. In the present study, we did not find any difference in the insect-generated signal due to the insect being exposed with or without food for both adult and larvae of *T. castaneum*. This suggests that the ultrasonic sounds

produced by the *T. castaneum* adults and larvae were not due to feeding alone but a combined detection of feeding and movement. Accordingly, we refer to the signal produced by the individual insect in the transducer as overall insect activity rather than feeding and locomotory activity.

Recently, Njoroge et al. (2017) used an acoustic device to observe the activity of *S. oryzae*. They reported a sharp drop in acoustic signals for 1-2 d after sealing hermetic containers, after which the signal continued to drop slowly. The activity of *S. oryzae* ceased entirely when oxygen levels went below 2% level over 3-14 d of sealing the hermetic containers, suggesting the death of *S. oryzae* population inside the hermetic containers (Njoroge et al. 2017). Insect activity data were collected twice a day by Njoroge et al. (2017). In the present study, we collected data every 30 min for 48 h, which allowed us to better observe the pattern over time. The effect of hypoxia treatment on insect activity (feeding and movement) can further suggest the effect on their population development and grain damage potential, which can happen within the first 30 min of hypoxia treatment.

In real field scenarios such as in sealed PICS hermetic bags, the oxygen levels can fall below 2% levels in a few days to several weeks after closure, depending upon various physical and biological factors including initial infestation levels, headspace, and temperature (Varnava et al. 1995, Tubbs et al. 2016, Williams et al. 2016, Kharel et al. 2018). Generally, storage insects are killed when the oxygen in a strictly airtight hermetic container falls to approximately 2% levels (Oxley and Gloria 1963, Njoroge et al. 2017). However, our study was conducted in a controlled environment, and no dead larvae and adults of *T. castaneum* were found even at 2% oxygen levels when assessed after 48 h of exposure. There could be several plausible reasons for this observation. Inside hermetic containers, insects are exposed to a suboptimal level of oxygen

for a long time before reaching 2% oxygen levels. At suboptimal oxygen levels, insects are unable to balance their metabolic water (Murdock et al. 2012, Navarro and Navarro 2014), and finally, die of desiccation (Murdock et al. 2012). Additionally, the size of the insect population inside the hermetic containers can influence how fast the available oxygen is consumed (Oxley and Gloria 1963, Kharel et al. 2018). In our study, we exposed six *T. castaneum* larvae and adults per treatment inside hermetic treatment chambers. By contrast, Njoroge et al. (2017) had 25, 50, or 100 *S. oryzae* inside airtight 500 and 1000 mL mason jar suggesting that oxygen was highly limiting for each individual insect.

Furthermore, we observed that the signals produced by the adults were higher on the transducers than the larvae. This may be due to the heavier body size of the adults that produced more impact on the transducers. Alternatively, the softer bodies of insects may have less capacity to produce a detectable signal on the transducers. Our findings corroborate those of Shade et al. (1990) who reported that the feeding activity increased from the smaller first instar to larger last instars of *C. maculatus*; however, it does not imply that younger instars fed less than the old instars as with Shade et al. (1990). Further, we observed a wide range in the activity for individual adults and larvae. A similar finding has been reported by Pittendrigh et al. (1997) in rice weevils who reported a wide range in the total number of feeding events per insect. The wide range in the activity of the insect may be due to the variation in their natural feeding behavior (Shade et al. 1990), an insect being close to the transducer while the data are being recorded (Pittendrigh et al. 1997), or it may be due to the variation in sensitivity among the individuals in the same group to the treatment being used.

Nonetheless, our study shows that the feeding and movement of *T. castaneum* are decreased substantially at oxygen levels of 8% or below, beginning 30 min of hypoxia exposure.

The reduction of activity may be a strategy to conserve energy during hypoxic stresses. Further studies are warranted to shed light on the hypoxia tolerance mechanisms. Additionally, the reduction of insect activity can occur as early as 30 min of the beginning of hypoxia treatment. Hence, the sublethal effects of hypoxia and consequently retardation in insect population development and their grain damage potential inside the hermetic storage can began much earlier than previously thought.

4.5.2 Effects on insect cellular energy

Our result shows that the AEC index declines with a decrease in oxygen level. When *T. castaneum* adults were held at normoxia (20.9%) for 48 h, the AEC index was 0.7 ± 0.0. Exposure to 8% oxygen levels for 48 h, depressed the AEC levels (0.58 ±0.01) by 16.8%. However, the AEC levels in the insects exposed to 8% oxygen levels for 48 h were not different from the insects held at normoxia for 96 h (0.59 ±0.01). This may be because the feeding activity of *T. castaneum* is significantly decreased below 8% levels as suggested by the ultrasonic bio monitor test where the combined activity of feeding and movement was reduced to 23.7% of normoxia activity. Additionally, the AEC level was reduced in *T. castaneum* adults exposed to normoxia for 96 h compared to 48 h which might be due to insects at 96 h exposure normoxia experienced a higher amount of stress from the absence of food and crowding compared to insects exposed to normoxia for 48 h. For, e.g., Chapman et al. (1971) demonstrated the AEC levels in *Escherichia coli* depressed from 0.85 in a healthy individual to 0.5 or less due to 20 h of starvation.

The AEC index dropped sharply (0.25 ±0.02) when the insects were exposed to 4% oxygen levels for 48h. This is very important from the practical pest management point of view because any AEC level below 0.5 is considered to have irreversible viability losses to the

organisms even after returning them to normal conditions (Ivanovici and Wiebe 1981). Ridge (1972) working with rat-cerebral cells showed it was not possible to obtain a steady state at the AEC level less than 0.5 and any reduction below that level was lethal. However, invertebrates can survive at lower AEC (0.3–0.4) than vertebrates (0.5–0.6) (Raffin and Thebault 1996). In our study, we found that *T. castaneum* adults can survive an AEC level of 0.19 ± 0.01.

Our results showed that the AEC level in the insects exposed to 2% oxygen for 48 h was not significantly different from the individuals exposed to <1% for 96 h. However, the survival rate at 2 and <1% treatments was 100 and 0% alive, respectively. There might be several plausible reasons behind this observation. The slow decline of the AEC levels near mortality might be due to the inhibition of their consumption leading to the accumulation of energetic nucleotides (primarily the ATP and ADP) as seen in *Diaphorina citri* infected with *Candidatus Liberibacter asiaticus* (CLas) (Killiny et al. 2017). Previously, Marazza et al. (1996) showed that the AEC of shrimp, *Palaemonetes varians,* was slightly increased upon exposure to a near-lethal levels dose of ammonia. Pullin and Bale (1988) who studied the effects on AEC levels due to freezing injury in nettle aphid *Microlophium carnosum*, also reported that the energy charge declined relatively slowly at low temperature than at higher temperatures. They interpreted their results of slow decrease of AEC levels at low temperature due to insusceptibility of catabolic respiratory processes to chill injury. We assumed that below 2% oxygen levels, the adenylate molecules could not be generally processed due to disruption in mitochondrial metabolism to maintain membrane integrity (Neven, 2000).

Our findings are in agreement with (Mitcham et al. 2001) who suggested that the oxygen level should be lower than 1% for the rapid kill of storage pests. However, low oxygen levels below

4% for extended periods of time (>15 d) can be detrimental for the survival of storage insects as shown in chapter 2. Limited oxygen supply in the cells can affect various oxygen-requiring pathways of cellular respiration including Krebs' cycle and electron transport chain (ETC) (Dean and English 2013). The effects on those two critical steps of cellular respiration will further affect the process of ATP synthesis, thereby impacting the overall energy balance of the adenylate system (Ildefonso et al. 2014). The decreased energy in the cell has been linked to disruption of the mitochondrial mechanisms to regulate structural dynamics (mitochondria continuously undergo structural changes under normal conditions) causing cell injury (Khacho et al. 2014). Further studies on mitochondrial dysfunctioning would shed light on the mode of action of hypoxia. Additionally, decreased cellular energy might affect various energy-requiring biosynthesis of macromolecules at the subcellular level (Baker et al. 2010). At the organismal level, hypoxia has been shown to reduce oviposition, progeny development, body mass, and longevity of the insects (Spratt 1979, Cheng et al. 2012, Yan et al. 2016), all of which contribute to pest control.

In conclusion, oxygen levels <1% levels for at least 96 h can completely control *T. castaneum* adult. Considering that all *T. castaneum* adult and larvae remained alive at hypoxia treatments of 2-8% oxygen levels for 48 h, the exposure time should be extended for such hermetic containers to increase their efficacy. However, the movement and feeding (overall activity) of *T. castaneum*, as well their cellular energy is substantially reduced when oxygen levels fall below 8%, the effects being more prominent below 4% levels. The negative effects on the behavior as well as the cellular energy can negatively affect *T. castaneum* population development as well as their damage to stored crop commodities inside hermetic storages.

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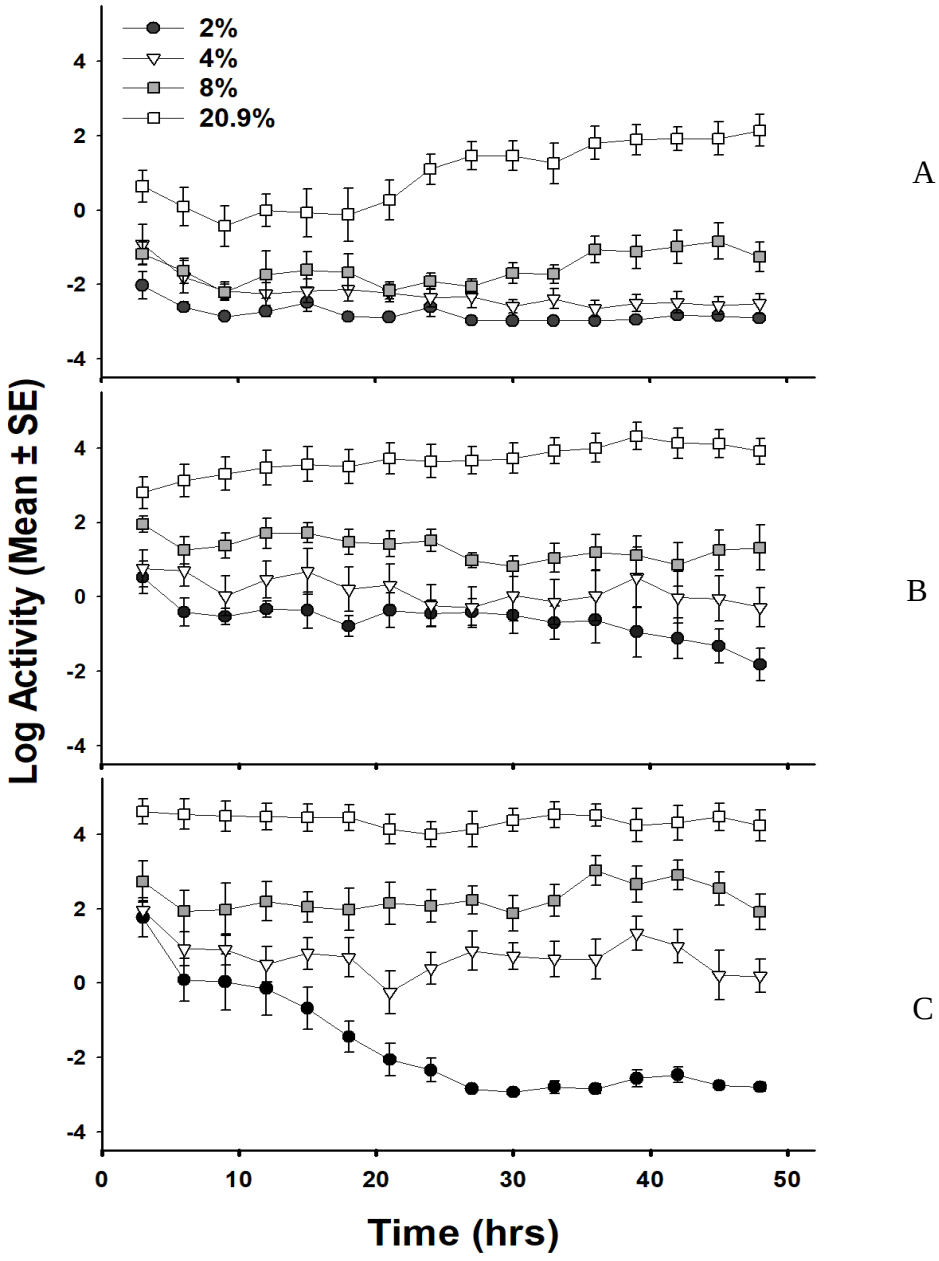

Figure 4.1. Temporal pattern of *T. castaneum* activity at different oxygen levels; A: young larvae (1-week-old), B: old larvae (3-weeks-old) C: adult (1-2 d after emergence). The activity of individual insect was recorded every 30 min for 48 h. The data were then summed for every three hours to create 16-point time points.

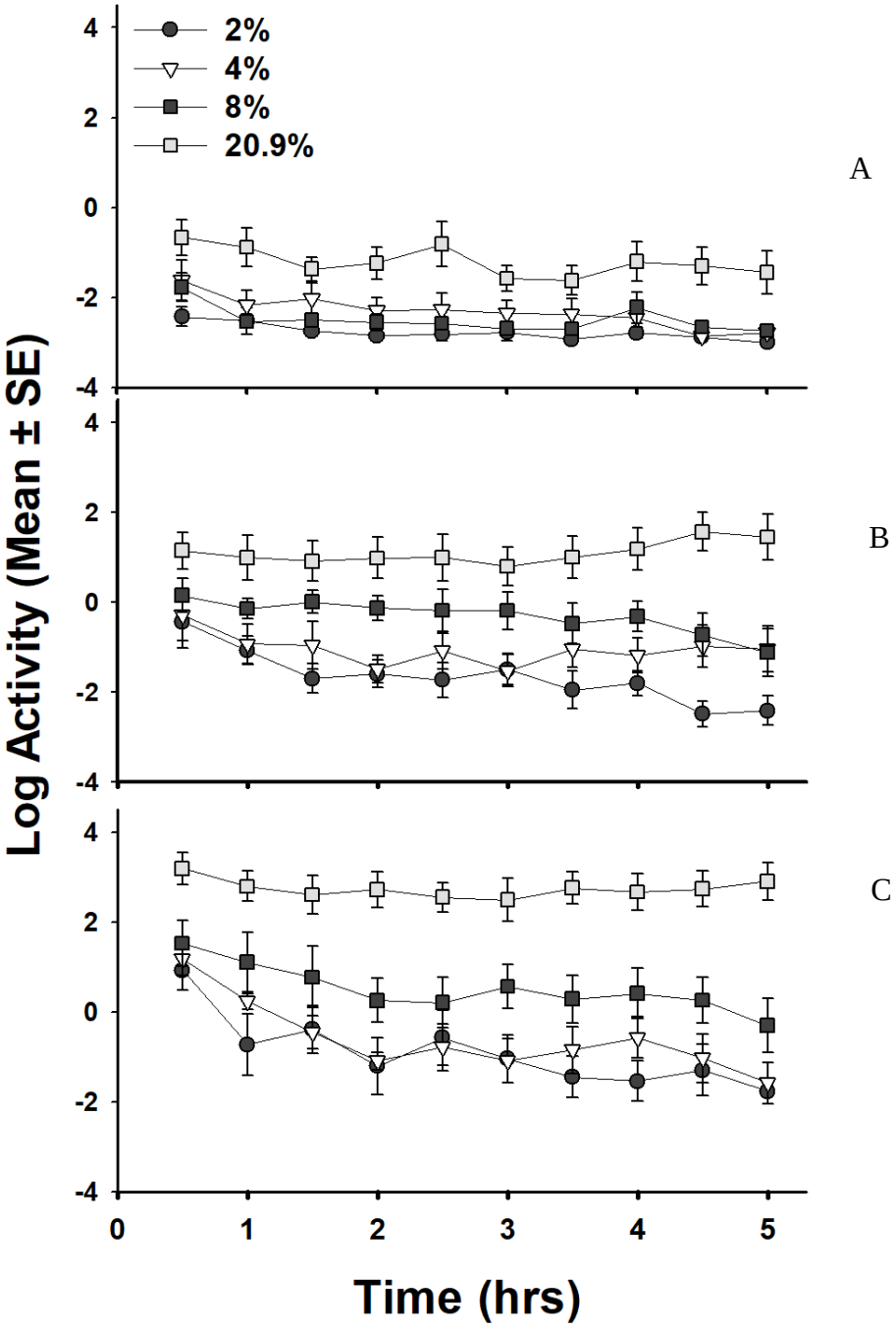

Figure 4.2. Temporal pattern of *T. castaneum* activity recorded at every 30 min intervals at different oxygen levels during the first five hours of hypoxia treatments; A: young larvae (1-week-old), B: old larvae (3-weeks-old) C: adult (1-2 d after emergence).

Table 4.1. The average activity of different stages of *T. castaneum* for the first 5 and 48 h after hypoxia exposure

Oxygen levels	Number of events (bites/0.5 h/individual) (Mean ± SE)					
	Adults		Old Larvae		Young Larvae	
	5 h	48 h	5 h	48 h	5h	48 h
20%	2371.8 ± 199.5a	1986.4 ± 58.2a	522.6 ± 53.1a	1228.9 ± 54.6a	50.0 ± 8.3a	108.3 ± 5.7a
8%	474.5 ± 80.2b	378.4 ± 21.3b	122.6 ± 14.9b	102.27 ± 6.1b	5.8 ± 1.6c	8.3 ± 1.1b
4%	133.4 ± 24.5c	72.6 ± 4.8c	84.1 ± 19.2c	59.9 ± 4.8c	11.1 ± 2.5b	2.4 ± 0.3c
2%	142.0 ± 31.1c	29.0 ± 4.4d	36.8 ± 7.8d	17.5 ± 1.4d	2.2 ± 0.7d	0.4 ± 0.1d

Means within a column with the same letter are not significantly different ($P \geq 0.05$, n=80 (5 h), n=768 (48 h) adjusted Tukey). The activity data for 5 h is the average activity for 5 h at 30 min intervals for eight insects (4 replications, 2 insects per oxygen levels): n= 88. The activity data for 48 h is the average activity for 48 h at 30 min intervals for eight insects (4 replications, 2 insects per oxygen levels): n= 768.

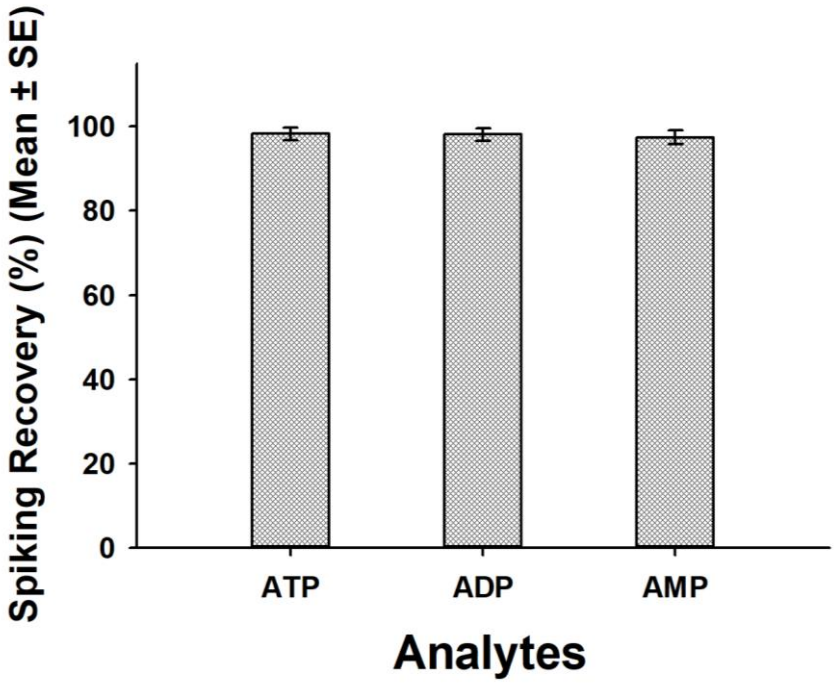

Figure 4.3. Percent recovery of spiked analytes at the middle point of a calibration level (0.1 mg/ml of ATP, ADP, and AMP standard solutions).

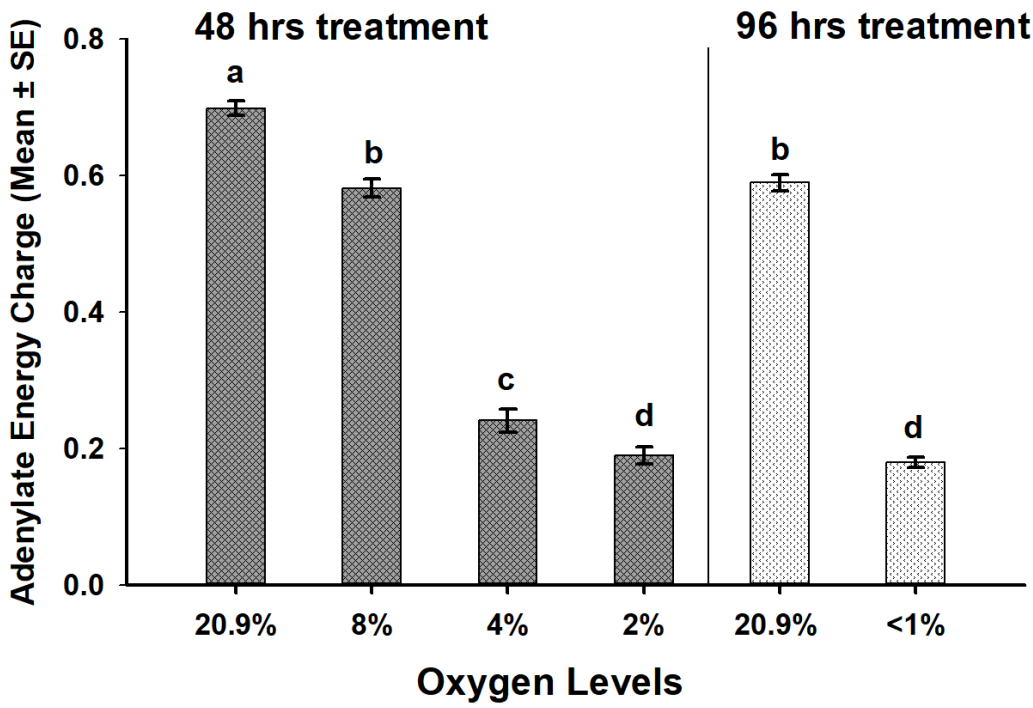

Figure 4.4. Adenylate energy charge (AEC) in *T. castaneum* adults exposed to different oxygen levels for 48 or 96 h. Means among hypoxia levels with the same letter are not significantly different ($P \geq 0.05$, n=30, adjusted Tukey).

CHAPTER 5. SUMMARY OF OBJECTIVES AND CONCLUSIONS

5.1 Review of Main Objectives

Current management of stored grain insects relies heavily on fumigants and insecticidal dusts. Hermetic grain storage technology offers a viable, chemical-free approach to control storage insects. Even so, there is limited understanding of how hypoxic conditions in hermetic containers affect insect development. Many portable hermetic grain storage bags such as PICS, GrainPro, GrainSafe, IRRI Super Bag or higher capacity storage system such as cocoons are commercially available. Hermetic technology has already been adopted by millions of smallholder farmers in Asia and Africa. This proven technology is likely to be adopted by a much wider circle of stakeholders. Given this scenario, we need a better understanding the mode of action of the hermetic system. The overall goal of this dissertation was to understand the response of *T. castaneum* exposed to different levels of hypoxia inside hermetic storage bags.

The sub-objectives included:

1. Investigating the daily and cumulative oxygen utilization of *T. castaneum* immature (egg-to-adulthood) and adult stages at the different availability of oxygen and temperature levels.
2. Understanding the life stage-specific response of *T. castaneum* exposed to artificially-created hypoxic conditions similar to that seen in hermetic containers (2, 4, 8% oxygen levels) as well as at ambient oxygen (20.9%) for different times (1, 3, 5, 10, and 15 days).
3. Assessing the effects of hypoxia on the immature developmental time.
4. Characterizing individual activity (feeding and movement) of adults and immatures of *T. castaneum* (Herbst) exposed to different levels of hypoxia (2, 4, 8% oxygen level) as well as at normoxia control (20.9%).

5. Determining the adenylate energy charge (AEC) in adult of *T. castaneum* exposed to hypoxia through the estimation of ATP, ADP, and AMP utilizing the high-performance liquid chromatography (HPLC) technique.

5.2 Summary and Conclusions

Our result showed that total oxygen consumption, as well as the daily rate of consumption, increase with increasing temperature and in the bigger size containers. *T. castaneum* consumed 6.72 ± 0.53 mL of oxygen during the egg-to-emerging adult stage, while adult *T. castaneum* consumed 5.37 ± 0.30 mL of oxygen over a 21-d period at 27°C. Understanding the total and daily oxygen consumption of immature and adult stages of *T. castaneum* may be helpful in predicting population development of *T. castaneum* under various oxygen levels attained in hermetic storage systems. Additionally, our findings support that the hermetic storage may perform better in the tropical and subtropical regions due to increased oxygen consumption at an elevated temperature.

Further, we exposed *T. castaneum* eggs (2 d), young larvae (7 d), old larvae (21 d) pupae (28 d), and adults (2 d after emergence) to 2, 4, 8 and 20.9% oxygen levels for 1, 3, 5, 10, and 15 d and assessed subsequent mortality. Our result showed (i) eggs, and young larvae were the most susceptible stage, experiencing complete mortality when exposed to 2% oxygen level for three or more days, (ii) old larvae and pupae required at least ten days for mortality to be observed, and (iii) adults required more than 15 d. Exposure time to achieve complete mortality increased with the increasing oxygen levels, for example at 4% oxygen levels, eggs required 5 d to produce total mortality and more than 15 d to kill other immature stages. The 8% oxygen levels were not lethal, but caused significant developmental delays in immatures. Hence, this

study has increased our knowledge regarding the response of *T. castaneum* adults and immature stages exposed to different hypoxia levels. A better understanding of these effects may lead to improving the effectiveness of hermetic storage for the control of *T. castaneum* and, by implication, other insects.

We used the Purdue Insect Feeding Monitor (PIFM) ultrasonic device to monitor the activity of the external feeding *T. castaneum* exposed to different levels of hypoxia. We observed that the activities of both adults and larvae were decreased at decreasing oxygen levels, and the decrease was apparent within the first 30 minutes of the hypoxia treatments. At the 2, 4, and 8% oxygen levels, the average activity for adults and larvae was reduced to at least 7.1, 16.1, and 23.5%, respectively, of the activity, observed at normoxia.

Lastly, we estimated ATP ADP, and AMP relative levels are utilizing the HPLC method for *T. castaneum* adults exposed to hypoxia and at normoxia control as a measure of available energy levels in the insects. The AEC level in the insects at normoxia was 0.70 ± 0, while the AEC at <1, 2, 4, 8% oxygen levels were 0.18 ± 0, 0.19 ± 0.01, 0.25 ± 0.02, and 0.58 ±0.01, respectively. Complete mortality of *T. castaneum* adults was achieved at hypoxia of <1% oxygen levels for 96 h. These results suggest the cellular energy in *T. castaneum* adults is markedly reduced at the oxygen levels of 8% and below, and suggest that affect their overall activity including feeding and movement. The decreased available energy probably affects various energy-requiring pathways at the cellular level. Therefore, the hypoxia toxicity is related to reduced cellular energy in the insects.

In conclusion, hermetic storage provides a practical solution for the control of *T. castaneum*, and the efficacy is related to the internal oxygen levels attained in the hermetic

containers and exposure time. Maintainance of the oxygen level below 2% can provide complete control of *T. castaneum* in 15 d. For more rapid kill of *T. castaneum* adults, the oxygen level should be decreased below <1% for at least for 4 d. Efficacy of hermetic storage may be greater in tropical and subtropical regions or when the hermetic storage is combined with temperature treatment due to increased rates of oxygen consumption due to the higher metabolic rate at a higher temperature. Exposure to 4% oxygen levels can provide complete control in 15 d against immature stages of *T. castaneum* but does not control adults. By contrast, 8% oxygen level is generally not lethal. Even so, the 4-8% oxygen levels can increase the developmental time, reduce feeding damage and mobility, and deplete cellular energy in *T. castaneum*; together these can suppress their population development in the hermetic containers.

APPENDIX A. TEMPERATURE (°C) AND RELATIVE HUMIDITY (RH%) DURING *T. CASTANEUM* EGG-TO-ADULT EMERGENCE EXPERIMENT

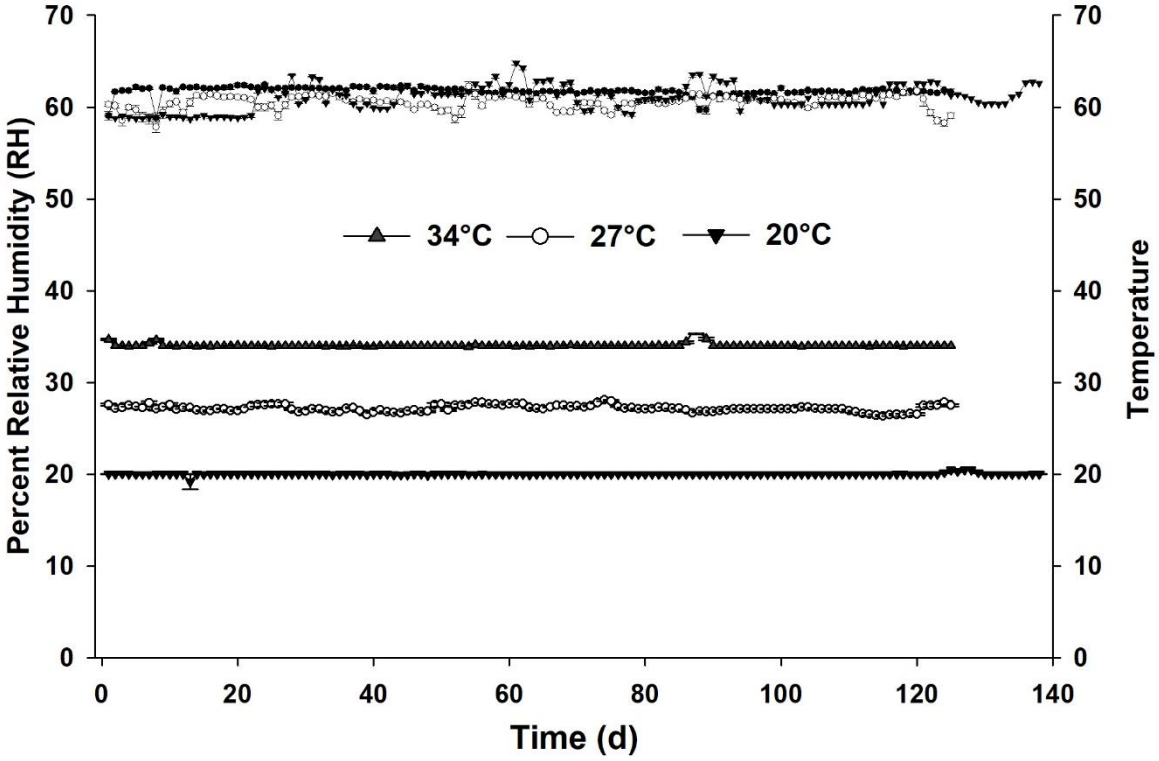

Figure A. Temperature and RH% were recorded using a USB data logger placed inside each incubator set with the target temperature and relative humidity during *T. castaneum* egg-to-adult emergence experiment. The temperature and RH% were recorded every hour and averaged for each day over the experimental period.

APPENDIX B. TEMPERATURE (°C) AND RELATIVE HUMIDITY (RH %) DURING T. CASTANEUM ADULTS' OXYGEN CONSUMPTION EXPERIMENT

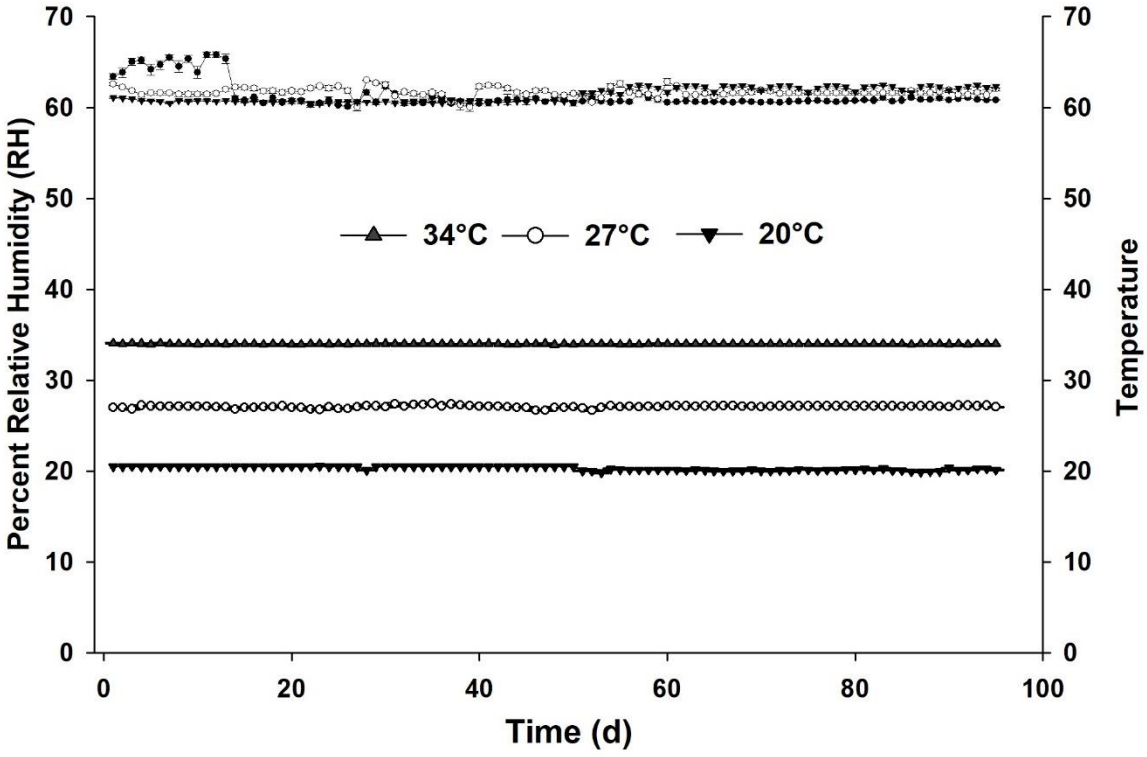

Figure B. Temperature and RH% were recorded using a USB data logger placed inside each incubator set with the target temperature and relative humidity during the *T. castaneum* adults oxygen consumption experiment. The temperature and RH% were recorded every hour and averaged for each day over the experimental period.

VITA

Kabita Kharel

E-mail: kkharel@purdue.edu; Phone: (765) 479-5418

Career Goal

- To join a research institute where I can apply and expand my knowledge and ability in Entomology, Insect Pest Management, and Toxicology through independent and collaborative research

Research Interests

- Studying behavioral and ecological aspects of insect pests and their natural enemies
- Evaluating and understanding the mode of actions of toxicants in both target and non-target organisms
- Determining the effects of environmental stresses such as hypoxia, high-temperature, and food availability on various physiological processes in insect pests
- Developing applied control methods for sustainable pest management

Academic Achievements

Ph.D., Entomology (Fall 2018), Purdue University, West Lafayette, IN

Co-Advisors: Prof. Linda J. Mason and Prof. Dieudonne Baribusta

Committee Members: Prof. Larry L. Murdock, Prof. Michael E. Scharf, and Prof. Lisa Mauer

Dissertation: Behavioral and physiological response of *Tribolium castaneum* (Herbst) exposed to hypoxia

M.S., Entomology (August 2013), Kansas State University, Manhattan, KS

Co-Advisors: Prof. Kun Yan Zhu and Prof. Frank H. Arthur

Committee Members: Prof. James F. Campbell and Prof. Subramanyam Bhadriraju

Thesis: Evaluation of pyrethrin aerosol insecticide as an alternative to methyl bromide for pest control in flour mills.

M.S., Conservation Ecology (November 2009), Tribhuvan University, Nepal

Advisor: Prof. Sant B. Gurung

Committee Members: Prof. Indira D. Bhattarai and Dr. Lal P. Amgain

Thesis: Biomonitoring the impact of ambient ozone on mungbean (*Vigna radiata* (L.) Wilczek) with the use of ethylenediurea (EDU) at Rampur, Chitwan.

Peer-Review Publications

1. Kharel, K., L.J. Mason, S.B. Williams, L.L. Murdock, I.B. Baoua, and D. Baributsa. 2018. A time-saving method for sealing Purdue Improved Crop Storage (PICS) bags. Journal of Stored Products Research. 77: 106-111.
2. Kharel, K., L.J. Mason, L.L. Murdock, M.E. Scharf, L.J. Mauer, and D. Baributsa. (In Preparation). Estimation of daily and total oxygen used by *Tribolium castaneum* to complete its life cycle.
3. Williams, S.B., L.L. Murdock, K. Kharel, and D. Baributsa. 2016. Grain size and grain depth restrict oxygen movement in leaky hermetic containers and contribute to the protective effect. Journal of Stored Products Research. 69: 65-71.
4. Kharel, K., F.H. Arthur, K.Y. Zhu, J.F. Campbell, and B. Subramanyam. 2015. Influence of temperature and artificially-created physical barriers on the efficacy of synergized pyrethrin aerosol. Journal of Stored Products Research. 60: 36-42.
5. Arthur, F.H., J.F. Campbell, K.Y. Zhu, and K. Kharel. 2015. Aerosol efficacy and direct and indirect exposure of flour beetles. *In*: Proceedings of the 11th International Working Conference on Stored Product Protection. November 2014, Chiang Mai, Thailand, pp 24-28.
6. Kharel, K., F.H. Arthur, K.Y. Zhu, J.F. Campbell, and B. Subramanyam. 2014. Susceptibility of different life stages of *Tribolium confusum* to pyrethrin aerosol: effects of flour source on insecticidal efficacy. Journal of Pest Science. 87: 295-300.
7. Kharel, K., F.H. Arthur, K.Y. Zhu, J.F. Campbell, and B. Subramanyam. 2014. Evaluation of synergized pyrethrin aerosol for control of *Tribolium castaneum* and *Tribolium confusum* (Coleoptera: Tenebrionidae). Journal of Economic Entomology. 107: 462-468.
8. Kharel, K., S.B. Gurung, I.D. Bhattarai, and L.P. Amgain. 2011. Effects of tropospheric ozone on crop yield and foliar injury at Rampur, Chitwan. Journal of Agricultural and Animal Science. 31: 225-230.
9. Kharel, K., and L.P. Amgain 2010. Assessing the impact of ambient ozone on growth and yield of crops at Rampur, Chitwan. Journal of Agriculture and Environment. 11: 40-45.

Miscellaneous Publications

– Kharel, K. 2009, June 15. A success story of a vermi-farmer. Nabayuba Monthly, 20, 7-9.
– Kharel, K. 2009, March 15. Ethnobotany: an indigenous Nepalese science. Nabayuba Monthly, 18, 7-9.

- Kharel, K. 2009, March 8. The significance of ethnobotanical study in today's context. Chitwan Post National Daily, p. 2.
- Kharel, K. 2008, December 11. Effect of climate change in the Himalayas. Chitwan Post National Daily, p. 2.
- Kharel, K. 2008. Ambient ozone: potential agricultural threat. In S. Gautam, A. Lamshal and K. Kharel (Eds.) Annual Agricultural Information Book, pp 80-81. Institute of Agriculture and Animals Sciences (IAAS), Post Graduate Students' Society, Rampur Chitwan.

Professional Presentations

- Kharel, K., L.J. Mason, and D. Baributsa. (March 2018). Estimation of daily and total oxygen used by *Tribolium castaneum* to complete its life cycle. (March 2018). Oral platform presentation. 73rd Annual Meeting of North Central Branch of Entomological Society of America (NCB ESA), Madison, WI.
- Kharel, K., D. Baributsa, S. Williams, and L.J. Mason. Influence of hypoxia on cowpea weevil egg-laying behavior and progeny development. (September 2016). XXV International Congress of Entomology. Orlando, FL.
- Kharel, K., D. Baributsa, S. Williams, L.L. Murdock, and L.J. Mason. (November 2016). Timely and effective sealing of Purdue Improved Crop Storage (PICS) bags. Oral platform presentation. 63rd Annual Meeting of the Entomological Society of America (ESA), Minneapolis, Minnesota.
- Kharel, K., and D. Baributsa. (May 2015). Evaluation of cowpea storage technologies used by traders in Burkina Faso and Niger. Poster presentation. 70th Annual Meeting of NCB ESA, Manhattan, KS.
- Kharel, K., Arthur, F.H., Zhu, K.Y., and Campbell, J.F. (November 2012). Sanitation increases the effectiveness of aerosol insecticides in milling facilities. Oral platform presentation. 60th Annual meeting of ESA, Knoxville, TN.
- Kharel, K., Arthur, F.H., Zhu, K.Y., and Campbell, J.F. (November 2012). Sanitation influences the efficacy of aerosol insecticides. Poster presentation. 16th Annual Research and the State: graduate student poster session, Kansas State University, Manhattan, KS.
- Kharel, K., Zhu, K.Y., Arthur, F.H., and Campbell, J.F. (June 2012). Presence of flour can influence the efficacy of pyrethrin aerosol spray against flour beetles. Oral platform presentation. 67th Annual meeting of NCB ESA, Lincoln, NE.

- Kharel, K., Zhu, K.Y., Arthur, F.H., and Campbell, J.F. (March 2012). Pyrethrin aerosol for pest control in food facilities. Poster presentation. 17th Annual Research and the State: graduate student poster session, Kansas State University, Manhattan, KS
- Kharel, K., Girase, J.R., Almas, L.K., and Colette, W.A. (October 2010). Optimizing soybean profitability and water use efficiency in the Texas Panhandle. Poster presentation. 8th Annual Pathways Student Research Symposium of the Texas A&M University System, Canyon, TX.

Leadership, Service, and Activities

- Founding executive board member. (Spring 2016- Present). US-Nepal Policy Research Center (UNPRC).
- Board member (Spring 2016-Present). Non-Residential Nepalese National Coordination Council of USA (NRN NCC USA), Education Task force.
- Reviewer. Journal of Stored Product Research
- Secretary. (Fall 2016/17). Society of Overseas Nepalese Entomologists (SONE).
- President (Fall 2015/16). Ohio Valley Entomological Association (OVEA).
- Ph.D. Session moderator. (Fall 2016). 29th Annual Forum of OVEA, Beck Agricultural Center, West Lafayette, IN.
- Secretary. (Fall 2015/16). Nepali Society at Purdue (NEPSAP), Purdue University.
- Student volunteer. (Fall 2014/15/16/17). Annual Pest Management Conference, Purdue University
- Poster Session Judge. (Spring 2016). 64th Annual Lafayette Regional Science and Engineering Fair, Purdue University and Science Education Foundation of Indiana.
- Student volunteer. (Fall 2016). 2016 XXV International Congress of Entomology.
- Student volunteer. (Spring 2015/2016). Bug Barn. Purdue University.
- Student volunteer. (Fall 2014/15/16). Insectaganza Program, Purdue University.
- Student Committee Member. (Spring 2011-2012). Department of Entomology Award Committee, Kansas State University.
- Student volunteer. (Fall 2011/12). Insect Zoo. Kansas State University.
- Treasurer. (Fall 2011-2012) Nepalese Student Association, Kansas State University.
- Editorial Board Member. (2008) Annual Agricultural Information Book, Post Graduate Students' Society, TU, Rampur, Nepal.

Honors and Awards

- ESA NCB Student Travel Scholarships. (Spring 2018). 73rd Annual Meeting of the Entomological Society of America, North Central Branch, Indianapolis, Indiana.
- Purdue Graduate Student Grant (PGSG) Travel Awards. (Spring 2018). Purdue University.
- Selected as 2017 U.S. Borlaug Fellow to participate in the U.S. Borlaug Summer Institute on Global Food Security (June 4-17). Center for Global Food Security, West Lafayette, IN.
- J. Edwin Sameth Memorial Scholarship. (2017). Department of Entomology, Purdue University.
- ESA NCB Student Travel Scholarships. (Spring 2017). 72nd Annual Meeting of NCB ESA, Indianapolis, Indiana.
- Selected to attend the International Student Leader Development Conference, James Madison University, Harrisonburg, VA (Spring 2016). Krach Leadership Center, Purdue University.
- PGSG Travel Awards. (Fall 2016). Purdue University.
- Indiana Pest Management Association Scholarship. (2016). Department of Entomology, Purdue University.
- PGSG Travel Awards. (Spring 2016). Purdue University.
- Megha Parajulee SONE Student Award for Academic Excellence (2015). Society of Overseas Nepalese Entomologists (SONE).
- Rhodes Family Scholarship. (2015). Department of Entomology, Purdue University.
- Ross Fellowship. (2014). Department of Entomology, Purdue University.
- Graduate Research Assistant. (2014-Present). Department of Entomology, Purdue University
- Research Assistant. (Spring 2013). Department of Entomology, Kansas State University.
- Floyd Holmes Scholarship. (2012). Department of Entomology, Kansas State University.
- First Place Student Platform Presentation Award. (2012). M.S. MUVE/PBT/SEB section. 67th Annual meeting of NCB ESA, Lincoln, NE.
- Graduate Student Council Travel Awards. (Fall 2012). Kansas State University.
- Abstract selected as Guide for How to Write an Abstract. (Fall 2012). Graduate Research Forum, Kansas State University.
- Selected as one of the Top Nine Presenters to represent K-State at the 10th Annual Capitol Graduate Research Summit (poster presentation). (Fall 2012). Graduate Research Forum, Kansas State University.

- Graduate Student Council Travel Awards. (Spring 2012), Kansas State University.
- Graduate Research Assistant. (2011-2012). Department of Entomology, Kansas State University.
- Nepalese Government Scholarship for M.S. degree. (2008-2009). Tribhuvan University (TU), Nepal.
- Nepalese Government Scholarship for B.S. degree. (2004-2007). TU, Nepal.
- Nepalese Government Scholarship for Proficiency Certificate Level. (2002-2003). TU, Nepal.

Grants Received

- The Conservation, Food & Health Foundation, Boston, MA, USA. (December 2017). "Assessing postharvest pest management practices and delivery of new storage innovations in Nepal" $14260.
- Global Future Institute (GFI), TX, USA. (July 2008). "Ambient ozone impact on crops at Rampur, Chitwan, Nepal" $600.

Professional Affiliations

- Entomological Society of America
- Society of Overseas Nepalese Entomologists
- Entomology Graduate Organization, Purdue University
- Pi Chi Omega: The National Pest Control Fraternity
- Ohio Valley Entomological Association
- Popenoe Entomology Club, Kansas State University