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Chemosensitivity in Mealworms and Darkling Beetles (*Tenebrio molitor*) across Oxygen and Carbon Dioxide Gradients

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Chemosensitivity in mealworms and Darkling beetles (*Tenebrio molitor*) across oxygen and carbon dioxide gradients

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Andrew Patterson ENTITLED Chemosensitivity in mealworms and Darkling beetles (*Tenebrio molitor*) across oxygen and carbon dioxide gradients BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

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ABSTRACT

Patterson, Andrew. M.S., Department of Biological Sciences, Wright State University, 2016. Chemosensitivity in mealworms and Darkling beetles (*Tenebrio molitor*) across oxygen and carbon dioxide gradients.

Breathing in most insects is controlled through a negative feedback loop consisting of signals (O_2, CO_2, pH), sensors (chemoreceptors), integrators (neural ganglia), and effectors (spiracles over tracheae). I hypothesized that mealworms and their adult counterparts Darkling beetles, *Tenebrio molitor,* can sense anoxic and hyperoxic environments and preferentially avoid these environments. I also hypothesize that mealworms are attracted to hypercarbia while Darkling beetles avoid hypercarbia. I constructed a test arena to create an O_2 or CO_2 gradient. Velocity, total distance traveled, and time spent in each area of the O_2 or CO_2 gradients were compared for 0%, 21% and 100% O_2, and 0.04%, 1% and 5% CO_2. Air flow alone decreased velocity and distance traveled by Darkling beetles compared to the no air flow protocol ($p<0.05$, one-way ANOVA). Darkling beetles spent more time in 21% O_2 than in 100% O_2 than in 0% O_2 ($p<0.05$, one-way ANOVA). There was no evidence that the Darkling beetles preferred any portion of the CO_2 gradient over another. I infer from my data that Darkling beetles prefer to avoid anoxic and to a lesser extent hyperoxic environments. Mealworms spent more time in anoxia than normoxia ($p<0.05$, one-way ANOVA). Mealworms spent more time in 5% CO_2 than 1% CO_2 and 0.04% CO_2 ($p<0.05$, one-way ANOVA). I infer from my data that mealworms prefer anoxia over normoxia and are attracted to hypercarbia.

Darkling beetles and mealworms are able to sense their gaseous environments and appear to avoid environments that may be insalubrious to them.

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LIST OF ABBREVIATIONS

Central Pattern Generator:	CPG
Discontinuous Gas Exchange:	DGE
Partial Pressure of Oxygen:	pO_2
Partial Pressure of Carbon Dioxide:	pCO_2

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I. INTRODUCTION

Environment

Darkling beetles, *Tenebrio molitor*, and their larvae mealworms are burrowing insects and can be found all over the world (Cotton, 1941). They are considered pests because they tend to flourish where grain is plentiful such as farm silos. The genus *Tenebrio* means darkness in Latin which is appropriate due to their nocturnal habits, and they are usually found in damp places in accumulations of grain in neglected corners of mills (Cotton, 1941). *T. molitor* are scavengers and prefer to feed on decaying grain or milled cereals that are in poor conditions (Cotton, 1941). Mealworms will usually stay hidden in their burrows of grain to protect themselves; they do not have the hard elytra that they develop when they become adult beetles. The adult beetle can be found both inside and outside the burrow.

Metamorphosis

T. molitor are holometabolous with four distinct stages of life. They begin as eggs and hatch into larvae. The larvae go through several molts depending on environmental conditions (Loudon, 1987). Larvae reared in a 10.5% oxygen environment undergo more molts than larvae reared at 21% oxygen; they also have a greater mortality rate and an increased chance of developmental abnormalities (Loudon, 1987). *T. molitor* raised in 15% oxygen developed larger in cross-sectional tracheal area on average than larvae raised at 21% oxygen (Loudon, 1989).

After the larval stage *T. molitor* will move on to the pupal stage; during this time no nutrition is gathered. Finally, the pupa will metamorphose into an adult darkling beetle.

Insect respiratory system

The insect tracheae are gas-filled structures that invaginate the insect's body down to the tissues (Quinlan & Gibbs, 2006). The insect respiratory system relies mainly upon diffusion of gases through the tracheae and so requires an oxygen partial pressure gradient for movement of oxygen to the tissues. Oxygen moves down its partial pressure gradient to reach these tissues. There is very little diffusion of gases through the hemolymph (Loudon, 1989). The portals of the insect respiratory system are pores on the lateral body surface called spiracles. Spiracles can open to allow gas exchange or close for extended periods of time to prevent gas exchange. Spiracles are holes in insects connected to tracheae that are covered by valves that are operated by an opener and a closer muscle or by a closer muscle that acts against cuticular elasticity (Quinlan & Gibbs, 2006). Since gas exchange in an insect is regulated by opening and closing spiracles, this is known as "diffusion control" (Wigglesworth, 1935 citing Hazelhoff, 1926). Regulation of diffusion in insects has been a topic of debate: one hypothesis is to prevent water loss in insects (Mellanby, 1934) and another to prevent oxygen toxicity (Hetz & Bradley, 2005). Spiracular opening is thought to be controlled through a negative feedback loop consisting of signals (oxygen, carbon dioxide, pH), sensors (chemoreceptors), integrators (neural ganglia), and effectors (tracheae) (Heinrich & Bradley, 2014; Wigglesworth, 1935).

Many insects do not breathe at a constant rate but rather exhibit an irregular respiratory pattern known as discontinuous gas exchange (DGE). During DGE the spiracles can remain closed for periods as long as several hours or several days and periodically open for a few moments to release accumulated carbon dioxide (Burmester, 2005). Wigglesworth (1935) found that by decreasing his fleas' atmospheric oxygen spiracles can open and close rapidly and are difficult to record. Ventilation can change from discontinuous to continuous due to different types of locomotion; insects that fly have a higher metabolic rate during flight and a higher resting metabolic rate than those insects that have less challenging types of locomotion (Woodman *et al.*, 2007; Reinhold, 1999). Some competing theories for DGE include prevention of water loss through the spiracular system to prevent desiccation (Mellanby, 1934), adaptation to underground habitats where CO_2 levels are high and O_2 levels are low (Lighton, 1996), and prevention of oxygen toxicity which can cause oxidative damage to tissues even at low levels (Hetz & Bradley, 2005).

Each DGE cycle is composed of three different phases: the closed phase, the flutter phase, and the open phase. During the closed phase O_2 consumption by the tissues lowers the endotracheal partial pressure of O_2 (pO_2), (Hetz *et al.*, 1993). When endotracheal pO_2 levels fall and partial pressure of CO_2 (pCO_2) increases (Hetz & Bradley, 2005), the spiracular valves begin to flutter. During the flutter phase convective flow of air will occur due to negative endotracheal pressure from consumption of O_2 (Wobschall & Hetz, 2004; Quinlan & Gibbs, 2006). Later in the fluttering period opening duration and frequency increases leading to the open phase where O_2 and CO_2 move by diffusion (Wobschall & Hetz, 2004; Hetz & Bradley, 2005) and accumulated

CO_2 is released during the open phase. After the CO_2 is released the spiracles will close and the cycle will repeat.

Why insects developed DGE and how it is coordinated is poorly understood (Chown *et al.*, 2006). A central pattern generator (CPG) controls the motor activity that coordinates insect ventilation (Marder & Bucher, 2001). A CPG in one body segment can impose respiratory rhythm in another segment through interneurons (Lewis *et al.*, 1973; Burrows 1975, 1981). In locusts these interneurons innervate muscles for abdominal pumping and spiracular control (Woodman *et al.*, 2007).

Importance of environmental chemosensation in insects

Chemosensation is important for other facets in insect life in addition to regulation of breathing. Studies have shown there is a significant role CO_2 plays between insects and plants and between insects and mammals in their natural environment (Nicolas & Sillans, 1989). For example, blood-feeding female mosquitoes sense CO_2 that is emitted in the breath of animal with their olfactory network (Mboera & Takken, 1999). CO_2, along with host body odor, will stimulate blood feeding mosquitoes to produce "host-seeking behaviors" (Mboera & Takken, 1999). The hawk moth, *Manduca sexta*, assesses the quality of *Datura wrightii* flowers by sensing an increased amount of CO_2 that flowers produce with its labial-palp pit organs; newly blossomed flowers emit more CO_2 and offer more nectar (Thom *et al.*, 2004). *T. molitor* lives and feeds on stored grain in silos that can have elevated levels of CO_2. Members of the Tenebrionidae family have chemoreceptors in head tissues (Abdel-latief, 2007). Perhaps these chemoreceptors

are able to sense environmental levels of CO_2 and this may be how *T. molitor* detects its food source.

In this study I constructed a test arena where I was able to create oxygen and carbon dioxide gradients and measure time, distance and velocity in three different areas of my arena to test my hypothesis that mealworms and their adult form Darkling beetles (*Tenebrio molitor*) can sense anoxic, hyperoxic and hypercarbic environments and preferentially avoid an environment that is insalubrious for them. *T. molitor* are stored grain pests that live in environments where hypoxic and hypercarbic environments can exist (Cotton, 1941). Tiger beetle larvae (*Cicindela togata*) have been observed surviving in anoxic environments for up to 6 days (Hoback *et al.*, 1998). I predict that mealworms and beetles will spend the least amount of time in an anoxic environment because there is no oxygen to feed metabolic demand. I predict that mealworms and beetles will spend less time in a hyperoxic environment than a normoxic environment because hyperoxia is rare in nature (Greenberg & Ar, 1996) and may cause oxidative damage to tissues (Hetz & Bradley, 2005). I predict that mealworms will be attracted to and spend the most time in hypercarbic environments because stored grain can have elevated CO_2 which is where my study species can be found (Cotton, 1941). Based on my observations of my mealworms in my storage container, I predict that they will spend the least amount of time in a normocarbic environment because they only surfaced to feed on the fruits and vegetables that I put in the container. *Cryptolestes ferrugineus* have been observed moving towards environments of elevated CO_2 (Parde *et al.*, 2004). I predict the opposite for my beetles, I predict they would spend more time in a normocarbic environment followed by a hypercarbic environment based my observations

of my beetles in my storage containers where they could be found on the surface regardless of the nutrition I supplied. It has been observed by Groner & Ayal (2001) that Darkling beetles use plant cover to avoid predation that is on the surface and is a normocarbic environment. I would expect if my animals enter an environment that is insalubrious they would travel the farthest and the fastest to leave that environment.

II. MATERIALS AND METHODS

Study species

I purchased mealworms (*Tenebrio molitor*) from Jack's Aquarium® (Beavercreek, OH) and raised them in plastic containers (15x30.5x6cm) from larvae. I cut two holes in the top of the containers to allow for aeration. The floor of the container contained oatmeal to provide a burrowing substrate and food source for my mealworms and beetles. Once a week I provided fruit and vegetables such as banana peels, apple cores, peppers, carrots, celery, broccoli, cauliflower, cucumbers, and processed orange cubes which served as a source of water and extra nutrition.

Test arena

I constructed a test area with dimensions 36x31x0.9cm (figure 1) from a wooden frame with a glass floor and a removable glass sheet on top to create a closed chamber. I placed a white piece of poster board on top of the glass floor to provide a surface for my animals to walk on and create a color contrast between my animals and the poster board. I coated the perimeter of the poster board approximately 1.5cm from the chamber frame in a thin layer of high vacuum grease (Dow Corning®, Midland, Michigan) to prevent my beetles and mealworms from climbing out of the test area. I drilled two sets of three evenly spaced holes. I made the inlet holes 6mm in diameter and the outlet holes 8mm in diameter. The inlet and outlet holes were on opposite sides of the wooden frame with each aligned so air would flow straight through the test arena (figure 1). I connected

clear plastic tubes 4mm in diameter to the holes on one side. These tubes were stabilized in place with clay to reduce gas leakage around tubing and used as air inlets (figure 2). The holes on the opposite side of the inlets were used as air outlets for air to leave the test arena. The movements of evaporative mist from dry ice pellets confirmed air was entering each area at the inlet with minimal mixing on the borders of each area and exiting through the outlet (figure 3). The test arena was broken down into three areas. Flow rate through each area was $0.2L \cdot min^{-1}$.

Experimental design

I developed four separate protocols: control (no air flow), sham (room air flow ($0.2L \cdot min^{-1}$) in all areas of test arena), an oxygen gradient (0% O_2 – 100% O_2; $0.2L \cdot min^{-1}$), and a carbon dioxide gradient (0.04% CO_2 – 5% CO_2; $0.2L \cdot min^{-1}$) as illustrated in table 1. The range for the oxygen gradient was chosen to maximize the likelihood that I would elicit a hyperoxic response from my animals. Hyperoxic niches are rare in nature (Greenberg & Amos, 1996) and behavior in a hyperoxic environment is understudied. The range for the carbon dioxide gradient was chosen because 5% CO_2 is what I would expect to see in a burrow or grain silo, and excessive CO_2 can anesthetize my animals.

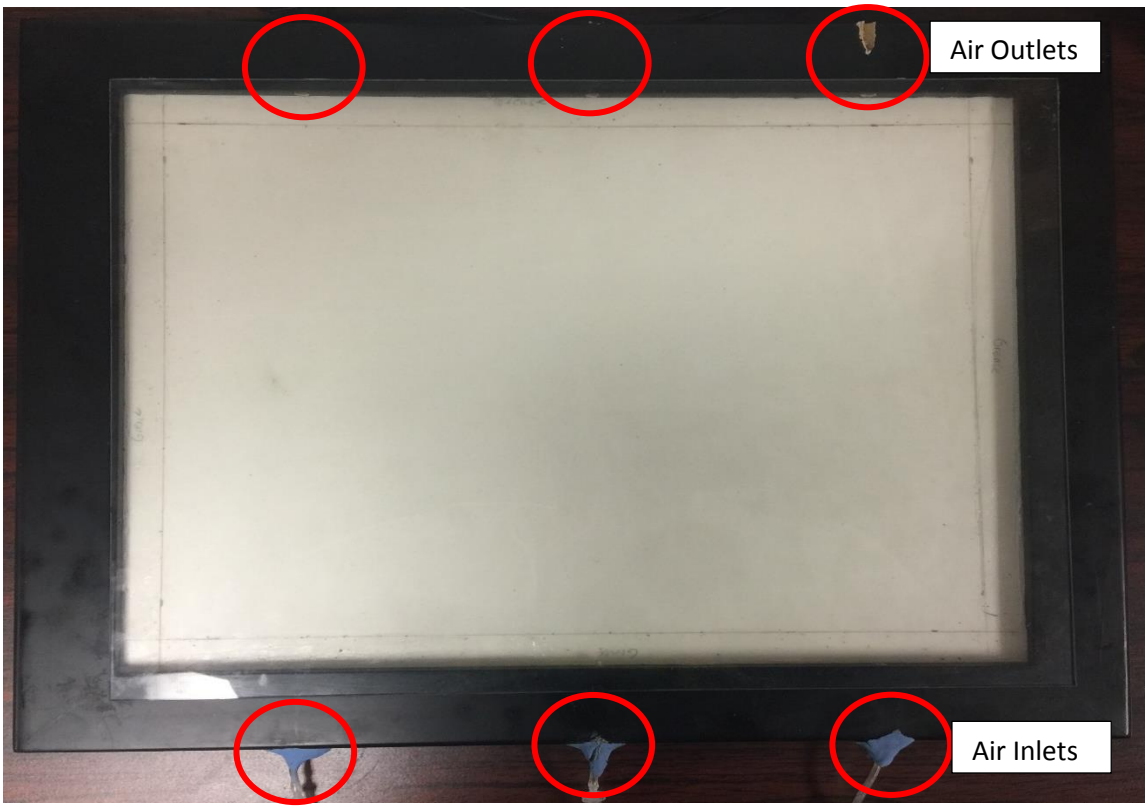


Figure 1. A top view of the test arena showing the three evenly spaced parallel inlet and outlet air holes circled in red. Air would flow through each area in the arena with minimal mixing at 0.2L·min^{-1}. 0.2L·min^{-1} was the maximal air flow before there would be turbulent flow through the arena. Inlet holes were 6mm in diameter while outlet holes were 8mm in diameter to help reduce resistance to airflow.

Figure 2. Inlet holes were stabilized with clay wrapped around the plastic tube to reduce air leakage around air inlets.

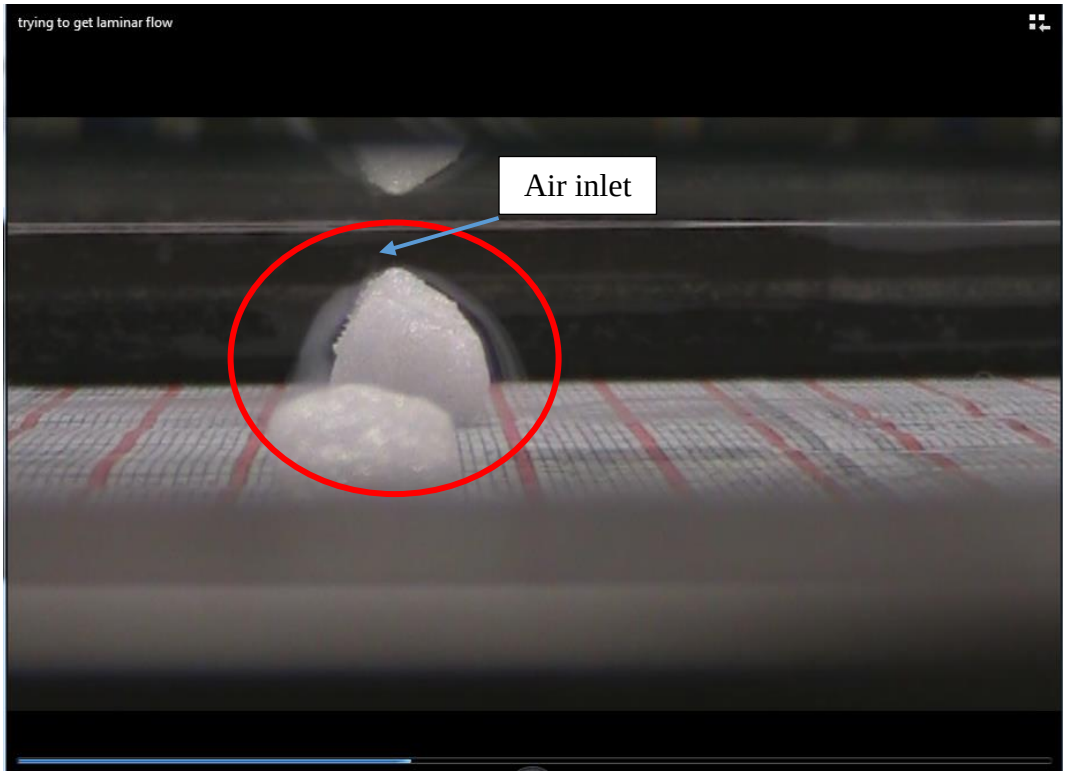


Figure 3. This picture of the test arena was taken at the level of the air inlet to show the air flowing over dry ice. Inside the circle the movements of evaporative mist from dry ice confirmed air was coming in the inlets and flowing through the test arena with minimal mixing on the edges exiting through the outlet (outlet not pictured).

Table 1. Four protocols were designed for various gas concentrations to be streamed through each area of the test arena. This table shows the remaining gases for the 1% slightly hypercarbic and 5% moderately hypercarbic areas of the test arena. "No air flow" means air was not streamed through any of the three areas, but movement of gases was by diffusion alone.

Behavioral Protocols	Area 1	Area 2	Area 3
No air flow (Control)	No air flow	No air flow	No air flow
Room air flow (Sham)	Room air flow	Room air flow	Room air flow
O_2 gradient	100% N_2	Room air flow	100% O_2
CO_2 gradient	Room air flow	20.95% O_2, 1% CO_2, 78.05% N_2	20.95% O_2, 5% CO_2, 74.05% N_2

For each trial the animal's movement was recorded by a camera (U Eye, National Instruments Corporation, Austin, TX) for 1800 ± 90 seconds (30 ± 1.5 minutes) and ShuttleSoft software (Loligo Systems, Tjele, Denmark) would calculate distance and velocity. Distance and velocity for beetles and mealworms were captured in cm/s. Mealworms distance and velocity were converted to mm/s because they did not travel as far or as fast as beetles. The U Eye camera reads distance and velocity in pixels. In order for me to convert my data to centimeters I had to calibrate the camera before each test by setting a 25cm ruler in the test arena and telling the software that X amount of pixels' equals 25cm. Data were then transferred to a Microsoft Excel® spreadsheet for analysis. The average velocity and average distance were calculated for each of the four protocols. Distance and velocity in each area of the test arena were calculated for all four protocols as well.

The U Eye camera tracks each animal in the test arena (figure 4) by a contrast between the white poster board and the dark color of the subject. Mealworm color can vary from brown to a light tan color; this made it difficult for the U Eye to track them. Avoiding their spiracles, I painted the first two or three sections of the mealworms' back with acrylic black Palmer Paint® products so the U Eye could then pick up the contrast. I only used mealworms in their late instars because movement between a 1st instar and late instar would be completely different due to their size difference.

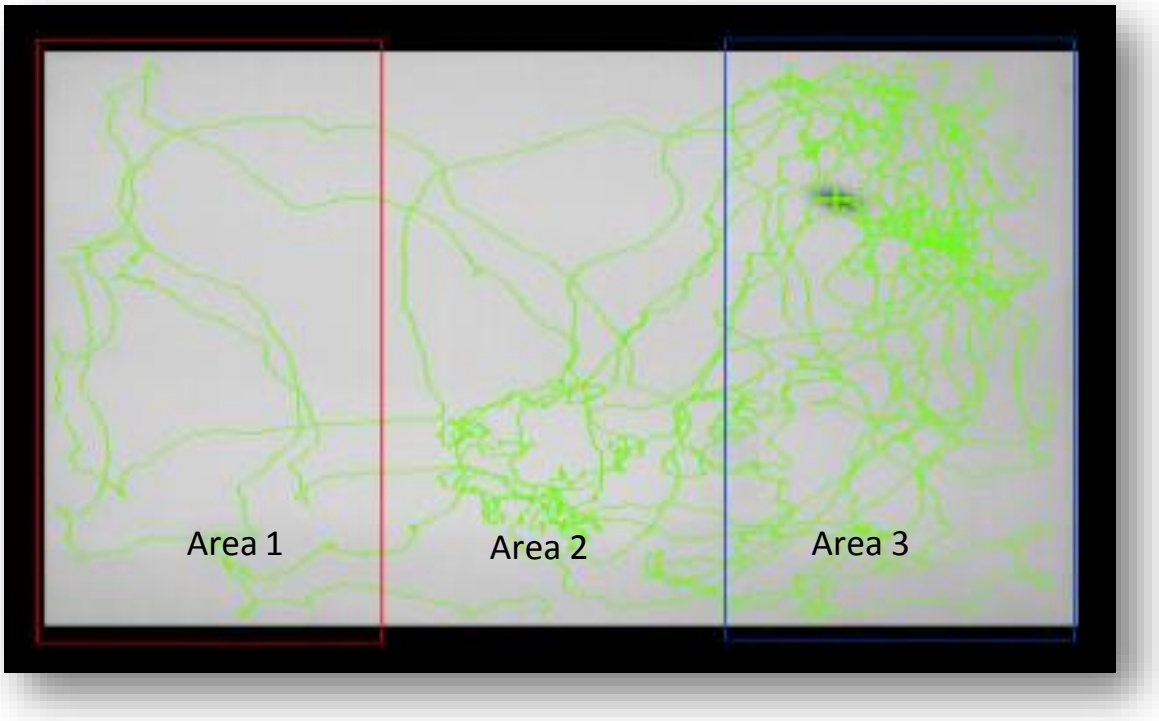


Figure 4. A screen shot of a recording looking down on the test arena taken during a 30-minute experiment. The trace line represents the path the beetle created. The dark spot in area 3 is the beetle. The crosshair over the beetle tracks the dark/light contrast movement. This image shows how the software divided the areas of the test arena. The red straight line after "Area 1" indicates the end of area 1 and the beginning of area 2. The blue straight line after "Area 2" indicates the end of area 2 and the beginning of area 3.

Each trial the glass cover was removed from the test arena, the animal was placed on the arena floor and the glass cover was replaced. The animal was given 5 minutes to adapt to the test arena with no air or gas mixtures streamed through to make sure they would explore the test arena. Every trial for all protocols were conducted with the lights on in the laboratory and between consistent temperatures of 23ºC and 25ºC. All trails were conducted between 10am and midnight. The number of times an animal started in each area was recorded and reported in table 2. After recording the animals' movement for thirty minutes it was removed from the test arena and placed in a separate container not to be used again.

The control protocol designated as "no air" did not have air streamed through the test arena. I used a one-way ANOVA to confirm that the animals did not spend more time, travel farther, or move faster in any one area over another. This protocol was used to eliminate the possibility that there was any other variable attracting the animals to any area in the arena other than the oxygen and carbon dioxide gas mixtures.

A 0.2L·min^{-1} (TSI model 4140 D flowmeter, TSI, Inc., Shoreview, MN) stream of room air (sham protocol) was moved through each area of the test arena (figure 5) to identify if airflow alone disrupted the locomotion of the animals. Airflow can have an effect on the behavior of the animals so it was important to document that even with streaming room air through the arena my animals were not attracted to any one area of the test arena. A one-way ANOVA was used to confirm lack of preference due to the presence of airflow alone.

Table 2. Number of times an animal started in each area for all four protocols when the 30-minute trial began. Animals were intentionally not started in the same area. Animals were given 5 minutes to explore the test arena before the trial began and starting location recorded.

Beetles	**Area 1**	**Area 2**	**Area 3**
Control (no air)	7	4	3
Sham (room air)	4	2	5
O_2 Gradient	2	4	4
CO_2 Gradient	2	4	4
Mealworms	**Area 1**	**Area 2**	**Area 3**
Control (no air)	4	2	4
Sham (room air)	3	4	7
O_2 Gradient	2	0	8
CO_2 Gradient	4	1	5

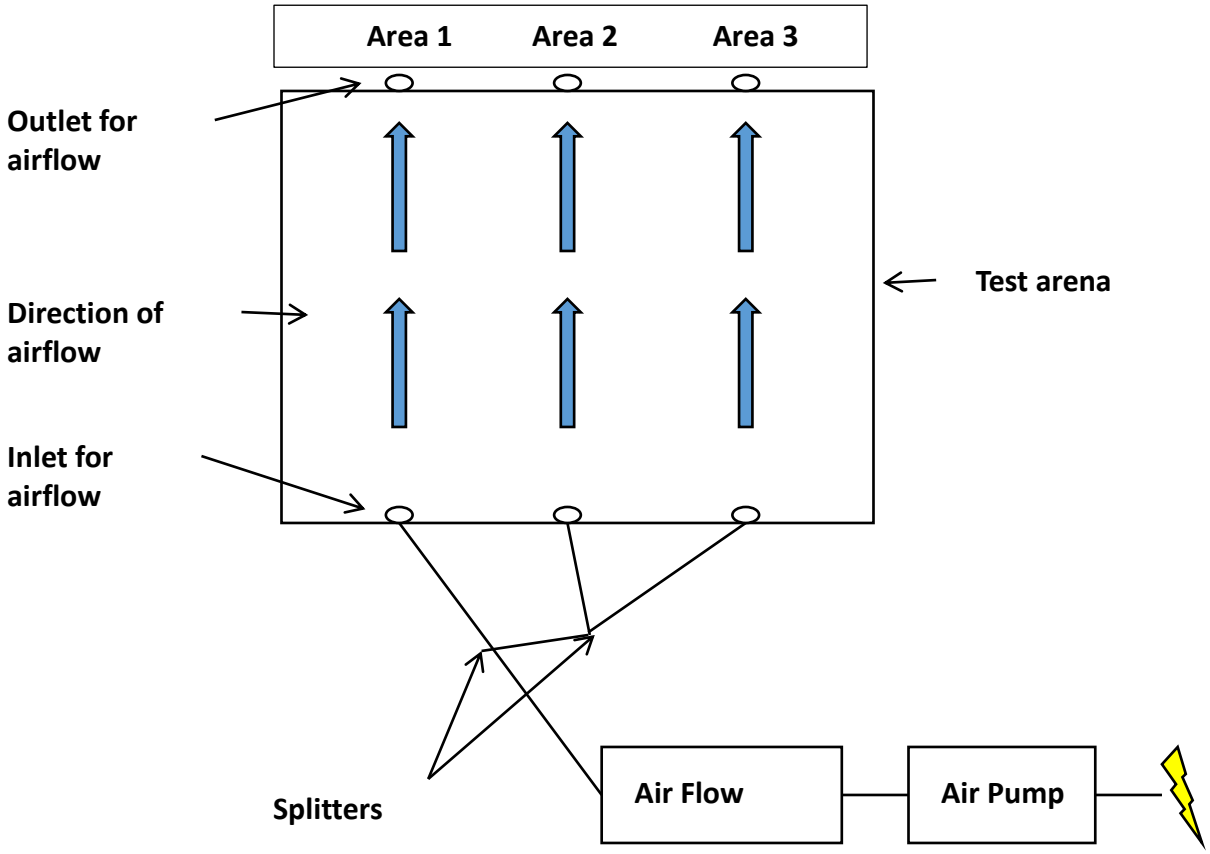


Figure 5. Diagram of the experimental set up for room air (sham) protocol. Room air was streamed in the direction of the blue arrows through all three areas in this protocol. The room airflow experiments were used as a secondary control (sham) to determine if the mealworm and beetle movements were affected by airflow and to ensure they would not prefer any one area over another in the absence of oxygen and carbon dioxide gas mixtures.

To create an oxygen gradient, I streamed 100% N_2 (0% O_2) through area 1 of the test arena, I streamed room air (20.95% O_2, 0.04% CO_2, 79.01% N_2) through area 2, area 3 received a stream of 100% O_2. Each stream was 0.2L min^{-1} (figure 6).

To create a carbon dioxide gradient, I streamed room air (20.95% O_2, 0.04% CO_2, 79.01% N_2) through area 1, I streamed 1% CO_2 (remaining gases were 20.95% O_2 and 78.05% N_2) through area 2, and area 3 received a stream of 5% CO_2 (remaining gases were 20.95% O_2, 74.05% N_2). Each stream was 0.2L·min^{-1} (figure 7). Flow rate for all protocols requiring air was determined by using the evaporative mist of dry ice and decreasing the flow of air 1L·min^{-1} until I saw pooling of gases by the outlets. The lowest flow rate achieved without seeing pooling by the outlets was 0.2L·min^{-1}.

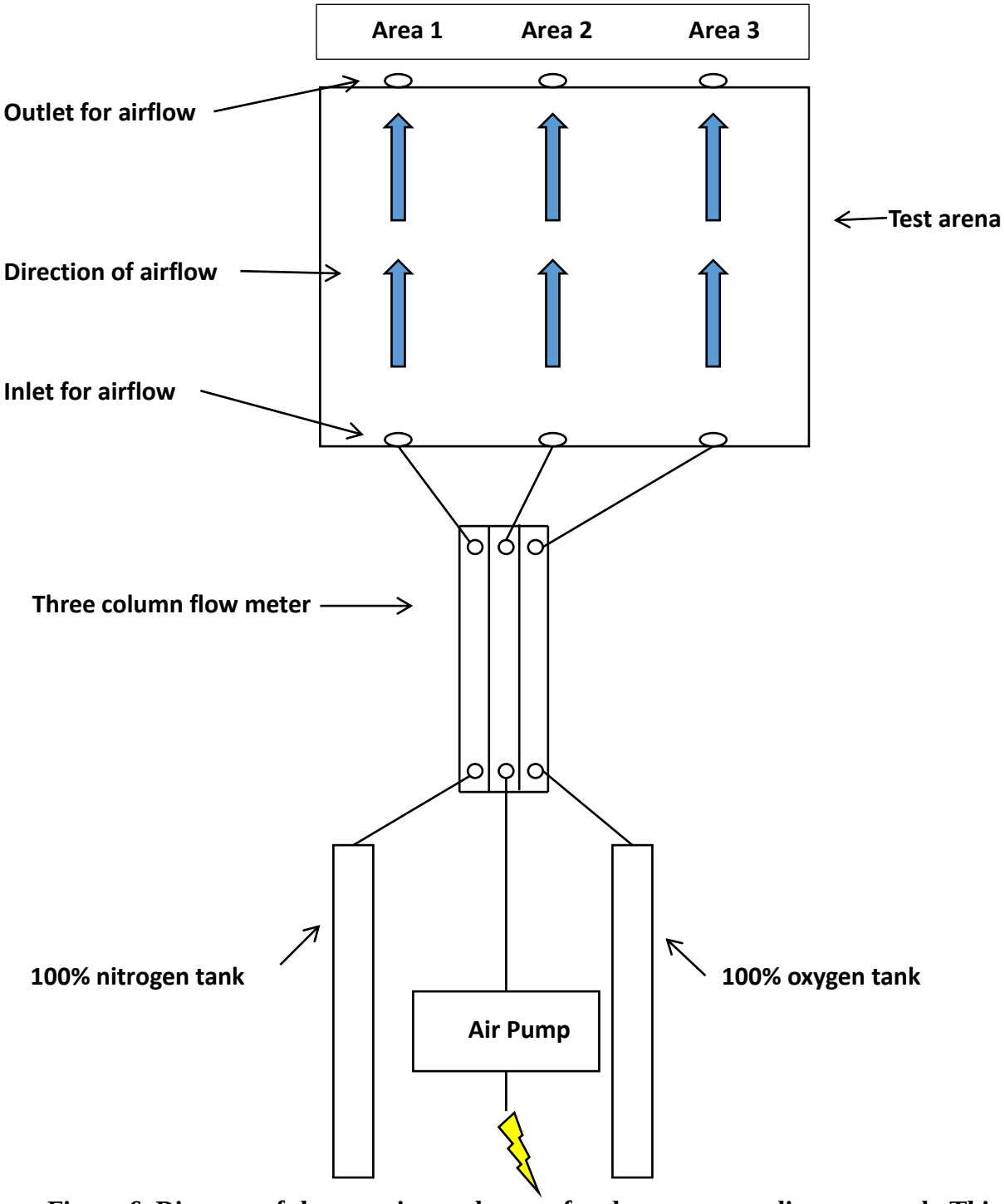


Figure 6. Diagram of the experimental set up for the oxygen gradient protocol. This gradient was used to test my hypothesis that my animals would avoid anoxia and hyperoxia. Flow through each area was 0.2L·min^{-1}.

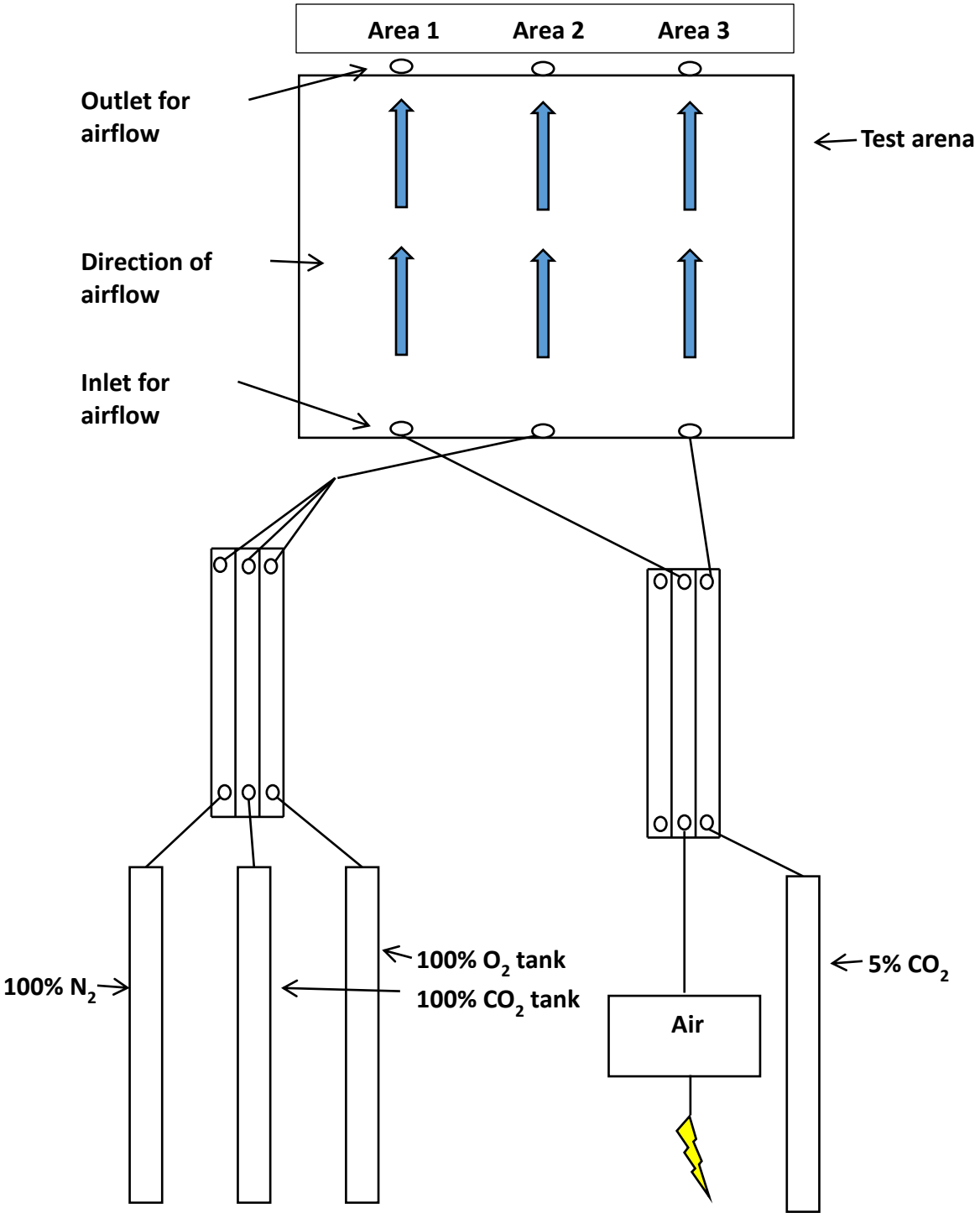


Figure 7. Diagram of the experimental set up for the carbon dioxide gradient protocol. This gradient was used to test my hypothesis that mealworms are attracted to hypercarbia and beetles would be attracted to normocarbia. Flow through each area was 0.2L·min^{-1}.

Statistics and comparisons

Upon completion of all protocols for mealworms and beetles, I analyzed and compared the data using one-way ANOVA and Student-Newman-Keuls Post-Hoc test using the statistics program GraphPad Prism Version 6.01 by GraphPad Software, Inc. (La Jolla, California). I ran Pearson Correlations on the amount of times an animal started in an area *versus* the time spent in that area, distance traveled in that area, or velocity in that area (table 8). My Pearson Correlations were calculated using GraphPad Prism Version 6.01 and significant differences were accepted where $p<0.05$. The "r value" represents the strength of the relationship between the number of times my animals started in an area and the time spent in that area, distance traveled in that area or velocity in that area for the duration of the trial. The "p value" is the significance level and "n" is my sample size. R^2 is the coefficient of determination that is the percentage of my data that can be explained by the relationship *versus* the percentage of data that cannot be explained.

I compared the average distance and average velocity among all four protocols. I also compared average time spent in each area, average distance in each area, and average velocity in each area for each protocol separately. Sample sizes for each protocol were between 10 and 14. Data are presented as means ± standard deviation of means. Significant differences were accepted where $p<0.05$.

III. RESULTS

Effects of air flow on average distance and velocity for control protocols

Both beetles and mealworms average distance traveled and average velocity for all trials in each protocol were calculated. Average distance traveled in each area, and average velocity in each area were calculated for all four protocols (control, sham, oxygen gradient, carbon dioxide gradient). Air flow alone was enough to cause a significant difference between the no air flow protocol (control) and the three remaining protocols requiring air flow, for both average distance traveled ($F(3,41) = 15.61$ $p < 0.0001$) and average velocity ($F(3,41) = 16.30$ $p < 0.0001$) for the beetles.

Time spent in each area for all four protocols

Analyses of time spent in each area for the control and sham protocols are illustrated in Table 3. Table 3 shows that both beetles and mealworms had no preference for any one area over another in the test arena.

Table 3. No significant differences were found between the time beetles spent in the control (F (2,39) = 2.230 p = 0.1211) or sham (F (2,30) = 2.973 p = 0.0664) protocols between the three areas (mean ± SD). No significant differences were found in the time mealworms spent in the control (F (2,27) = 2.751 p = 0.0818) or sham (F (2.39) = 1.987 p = 0.1508) protocols between the three areas (mean ± SD). If a significant difference had been found, that could mean there was another variable attracting beetles and mealworms to the area other than the O_2 and CO_2 gas mixtures.

Beetles	**Area 1**	**Area 2**	**Area 3**
No Airflow (control) n=14	11.6 ± 4.3min	9.2 ± 2.9min	8.7 ± 4.3min
Room Air (sham) n=11	13.5 ± 5.6min	8.3 ± 4.6min	8.1 ± 7.1min
Mealworms	**Area 1**	**Area 2**	**Area 3**
No Air (control) n=10	11.1 ± 4.2min	7.5 ± 2.35min	11.4 ± 5.6min
Room Air (sham) n=14	11.6 ± 6.51min	8 ± 3.81min	10.5 ± 4.1min

Figure 8 represents the average time beetles and mealworms spent in each area of the oxygen gradient. Figure 8A shows beetles spent more time in 20.95% O_2 area than the 0% O_2 area, and they spent more time in the 100% O_2 area of the gradient over the 0% O_2 area ($F(2,27) = 17.68$ $p < 0.0001$). Figure 8B shows mealworms spent more time in 0% O_2 area of the gradient over the 20.95% O_2 area ($F(2,27) = 4.694$ $p = 0.0178$).

Figure 9 represents the average time beetles and mealworms spent in each area of the carbon dioxide gradient. Figure 9A shows beetles had no preference across the CO_2 gradient ($F(2,27) = 0.06267$ $p = 0.9394$). Figure 9B shows mealworms spent more time in the 5% CO_2 area of the gradient over the 1% CO_2 and 0.04% CO_2 areas ($F(2,27) = 7.499$ $p = 0.0026$).

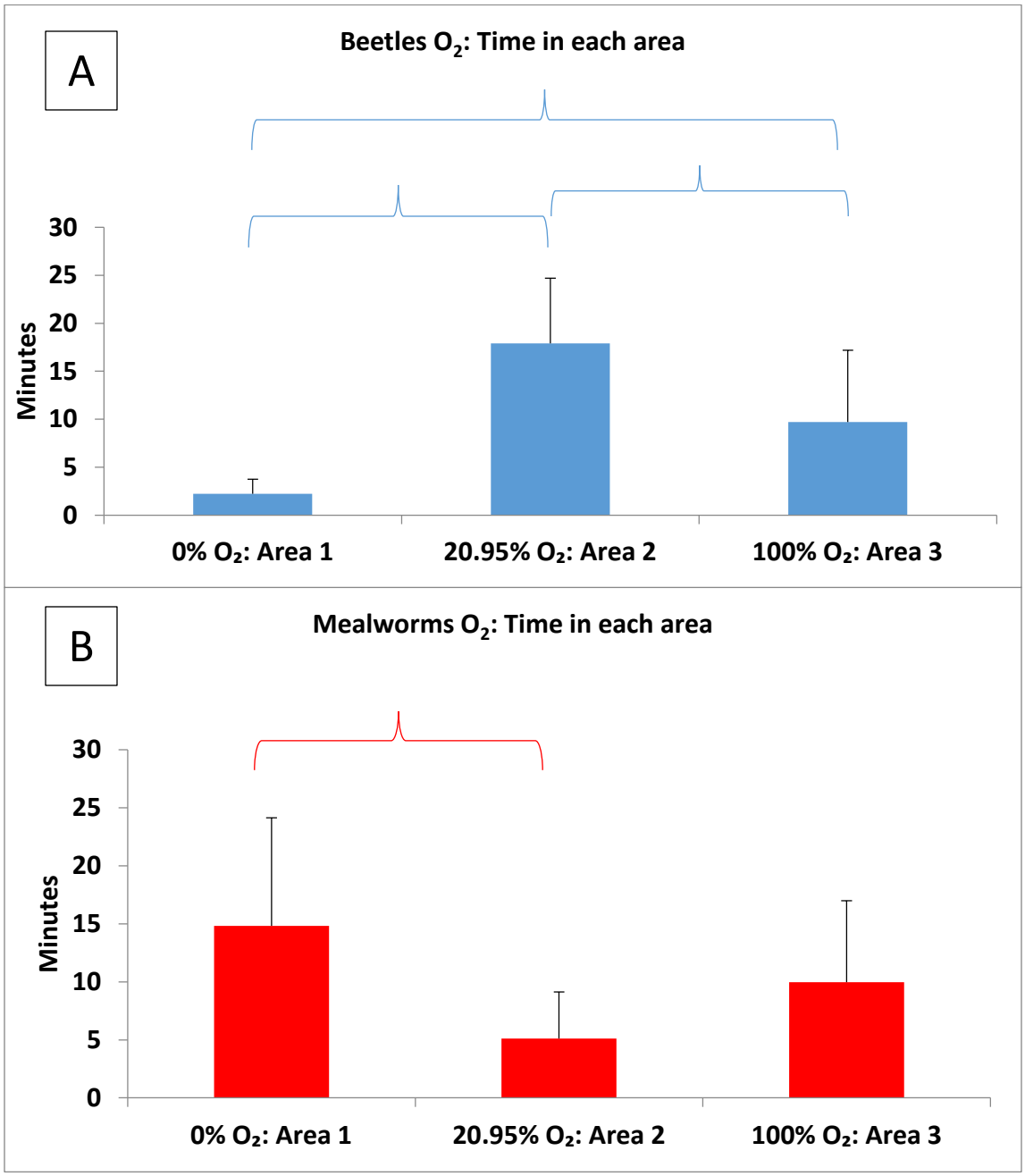
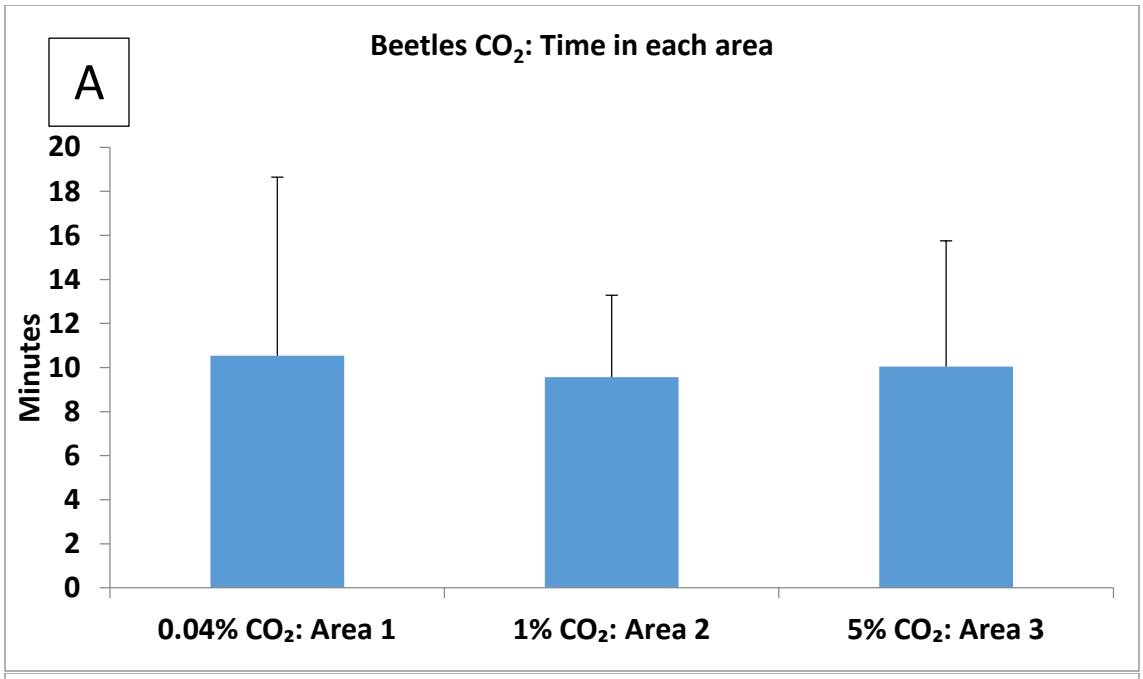


Figure 8. Darkling beetles (A) spent more time in 20.95% O_2 > 100% O_2 > 0% O_2 ($F_{(2,27)} = 17.68$ $p < 0.0001$). The mealworms (B) spent more time in the 0% O_2 area of the gradient than the 20.95% O_2 area; no significant differences were found between 0% and 100% O_2 and 20.95% O_2 and 100% O_2 ($F_{(2,27)} = 4.694$ $p = 0.0178$). The brackets represent significant differences between means ± SD.



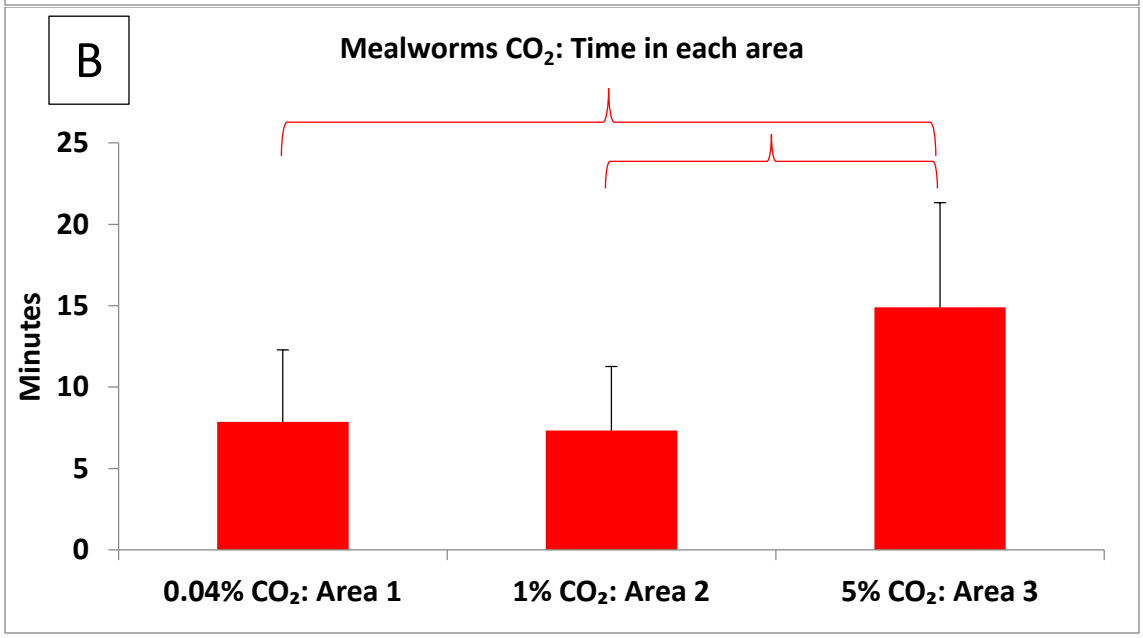


Figure 9. Beetles (A) did not spend more time in any one area than another in the CO_2 gradient (F (2,27) = 0.06267 p = 0.9394). Mealworms (B) spent more time in the 5% CO_2 than the 1% CO_2 area of the gradient and also spent more time in the 5% CO_2 over 0.04% CO_2 area of the gradient (F (2,27) = 7.499 p = 0.0026). The brackets represent significant differences between means ± SD.

Distance traveled in each area for all four protocols

Table 4 depicts the distance beetles and mealworms traveled in each area for the control and sham protocols. Beetles did not travel more in any one area of the test arena over another in both the control ($F(2,39) = 1.064$ $p = 0.3549$) and sham ($F(2,30) = 2.632$ $p = 0.0885$) protocols. Mealworms did not travel more in any one area of the test arena over another in both the control ($F(2,27) = 0.3446$ $p = 0.7115$) and sham ($F(2.39) = 0.07589$ $p = 0.9271$) protocols.

Table 4. No significant differences were found for beetles' distance traveled in the control (F (2,39) = 1.064 p = 0.3549) or sham (F (2,30) = 2.632 p = 0.0885) protocols between the three areas (mean ± SD). No significant differences were found for mealworms distance traveled in the control (F (2,27) = 0.3446 p = 0.7115) or sham (F (2.39) = 0.07589 p = 0.9271) protocols between the three areas (mean ± SD). If a significant difference had beenfound, then that could mean there was another variable attracting beetles and mealworms to the area other than the O_2 and CO_2 gas gradients.

Beetles	**Area 1**	**Area 2**	**Area 3**
No Air (control) n=14	387 ± 202cm	388 ± 325cm	273 ± 162cm
Room Air (sham) n=11	198 ± 135cm	141 ± 116cm	95 ± 42cm
Mealworms	**Area 1**	**Area 2**	**Area 3**
No Air (control) n=10	774 ± 552mm	746 ± 552mm	590 ± 495mm
Room Air (sham) n=14	386 ± 311mm	430 ± 313mm	400 ± 288mm

Figure 10 depicts the distance beetles and mealworms traveled in each area of the oxygen gradient. Figure 10A shows that the beetles traveled more in the 20.95% O_2 area of the oxygen gradient than the 0% O_2 area ($F(2,27) = 4.912$ $p = 0.0152$). There was no evidence that mealworms traveled farther in any area of the O_2 gradient over another seen in figure 10B ($F(2,27) = 0.4216$ $p = 0.6602$).

Figure 11 depicts the distance beetles and mealworms traveled in each area of the carbon dioxide gradient. There were no statistically significant differences ($F(2,27) = 0.7383$ $p = 0.4873$) found in distance across the CO_2 gradient for the beetles (figure 11A). Mealworms traveled further in the 1% CO_2 area than 0.04% CO_2 area (figure 11B) of the gradient ($F(2,27) = 4.387$ $p = 0.0224$).

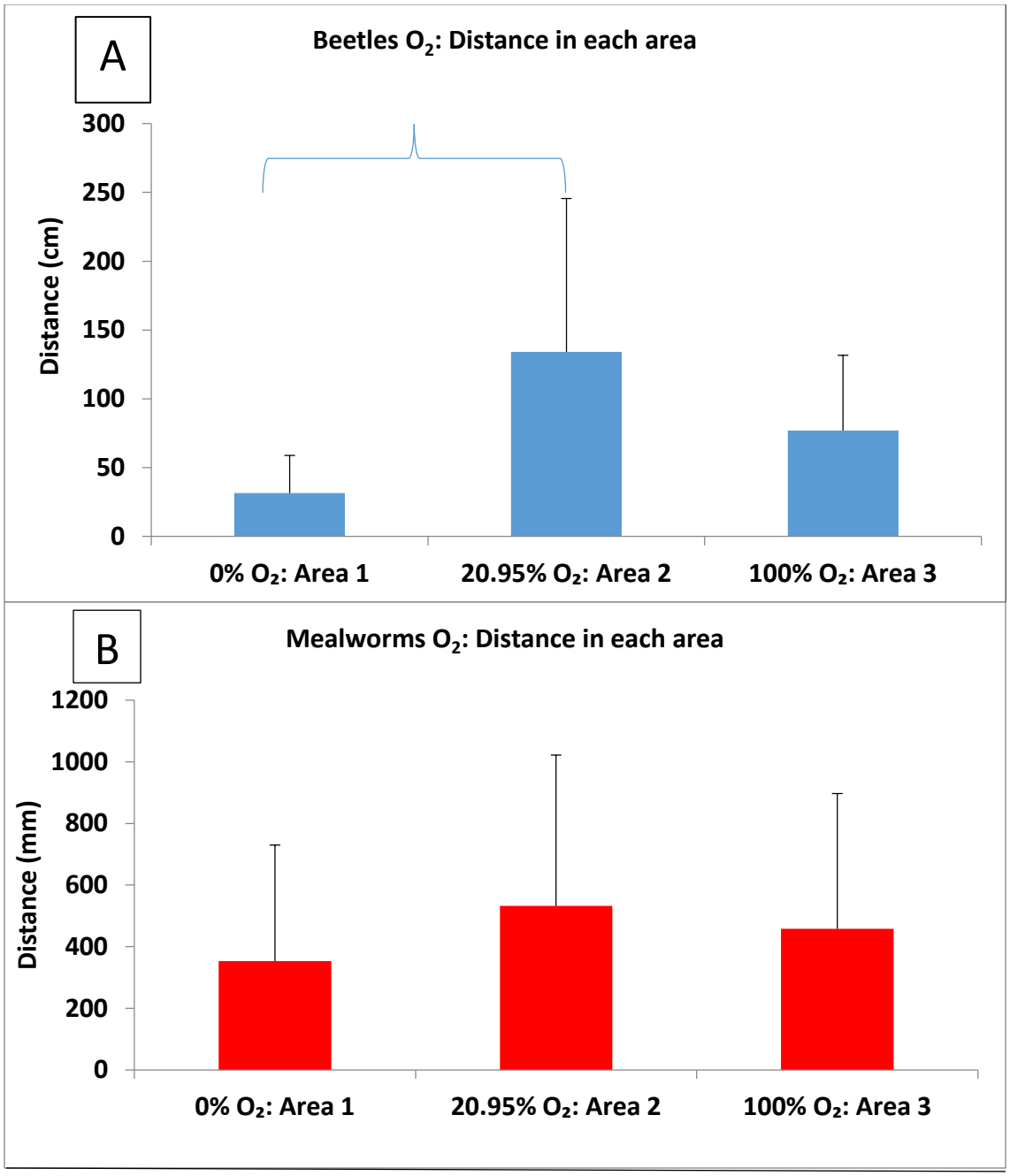


Figure 10. Beetles (A) traveled farther in the 20.95% O_2 area than the 0% O_2 area (F (2,27) = 4.912 p = 0.0152). No significant differences were found among the mealworm (B) trials (F (2,27) = 0.4216 p = 0.6602). The brackets represent significant differences between means ± SD.

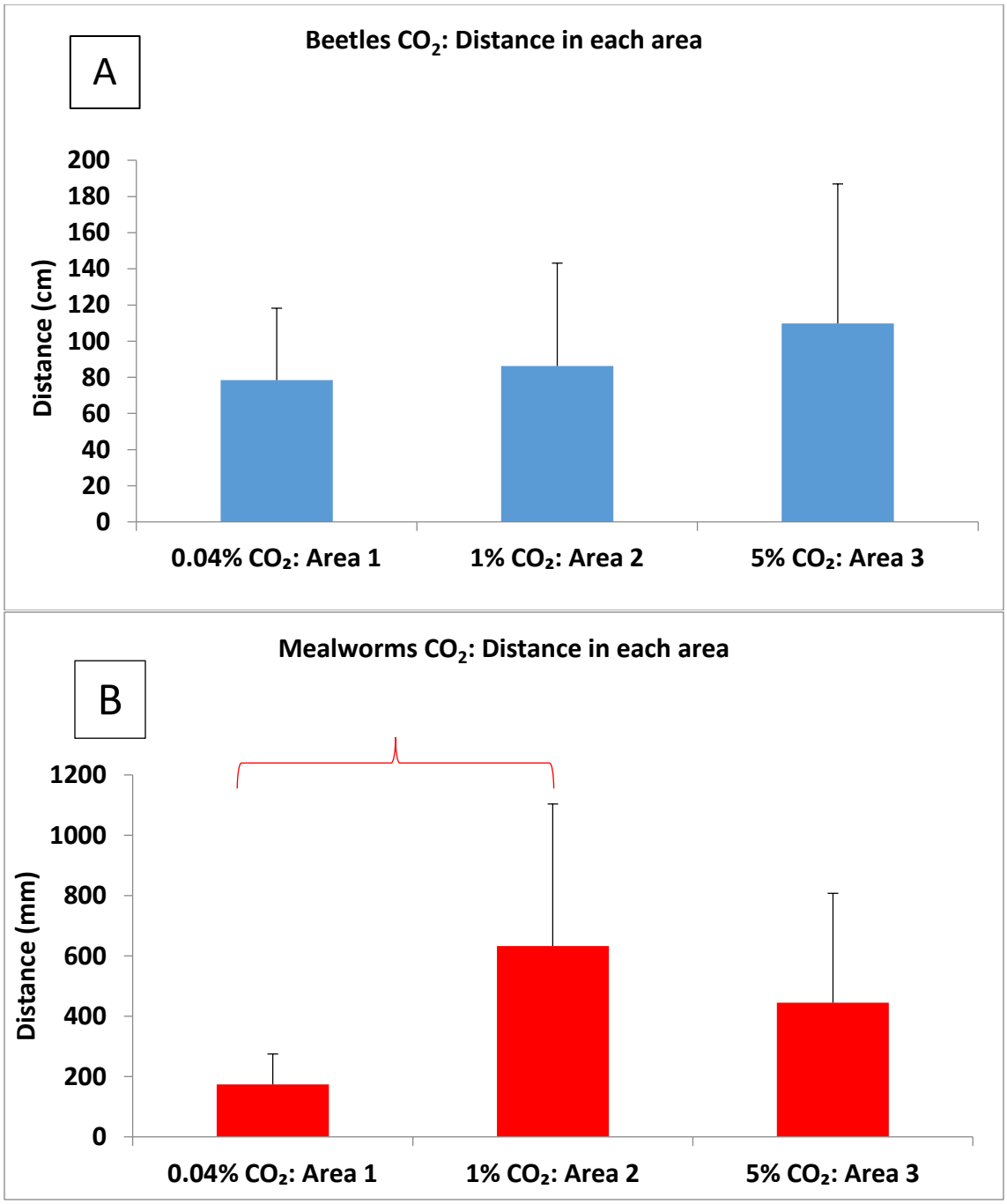


Figure 11. Beetles (A) showed no preference for any area of the CO_2 gradient (F (2,27) = 0.7383 p = 0.4873). Mealworms traveled farther in the 1% CO_2 area of the gradient then the 0.04% CO_2 area (F (2,27) = 4.387 p = 0.0224). The brackets represent significant differences between means ± SD.

Average velocity in each area for all four protocols

Table 5 shows the average velocities for beetles and mealworm for the control and sham protocols. Beetles did not travel faster in any one area over another for the control ($F(2,39) = 0.09913$ $p = 0.9059$) or sham ($F(2,30) = 0.4229$ $p = 0.6590$) protocols**.** Mealworms did not travel faster in any one area over another for the control ($F(2,27) = 1.665$ $p = 0.2080$) or sham ($F(2.39) = 1.308$ $p = 0.2819$) protocols.

Table 5. No significant differences were found for beetles' velocity in the control (F (2,39) = 0.09913 p = 0.9059) or sham (F (2,30) = 0.4229 p = 0.6590) protocols between the three areas (mean ± SD). No significant differences were found for mealworms velocity in the control (F (2,27) = 1.665 p = 0.2080) or sham (F (2.39) = 1.308 p = 0.2819) protocols between the three areas (mean ± SD). If a significant difference had been found, then that could mean there was another variable attracting beetles and mealworms to the area other than the O_2 and CO_2 gas mixtures.

Beetles	**Area 1**	**Area 2**	**Area 3**
No Air (control) n=14	0.59 ± 0.29cm/s	0.66 ± 0.36cm/s	0.63 ± 0.51cm/s
Room Air (sham) n=11	0.24 ± 0.14cm/s	0.28 ± 0.16cm/s	0.29 ± 0.16cm/s
Mealworms	**Area 1**	**Area 2**	**Area 3**
No Air (control) n=10	1.06 ± 0.74mm/s	1.66 ± 1.07mm/s	0.99 ± 0.85mm/s
Room Air (sham) n=14	0.60 ± 0.47mm/s	0.92 ± 0.64mm/s	0.66 ± 0.54mm/s

Figure 12 depicts beetle and mealworm velocity over an oxygen gradient. There were no statistically significant differences ($F(2,27) = 1.181$ $p = 0.3222$) found over the oxygen gradient for the beetles (figure 12A). Mealworms traveled faster in the 20.95% O_2 area of the oxygen gradient (figure 12B) than the 0% O_2 and 100% O_2 areas ($F(2,27) = 3.944$ $p = 0.0314$).

Figure 13 represents beetle and mealworm velocity over a carbon dioxide gradient. No statistically significant differences ($F(2,27) = 0.1175$ $p = 0.8896$) were found across the carbon dioxide gradient for the beetles (figure 13A). Mealworms traveled faster in the 1% CO_2 area of the gradient (figure 13B) than in the 0.04% CO_2 and 5% CO_2 areas of the gradient ($F(2,27) = 8.132$ $p = 0.0017$).

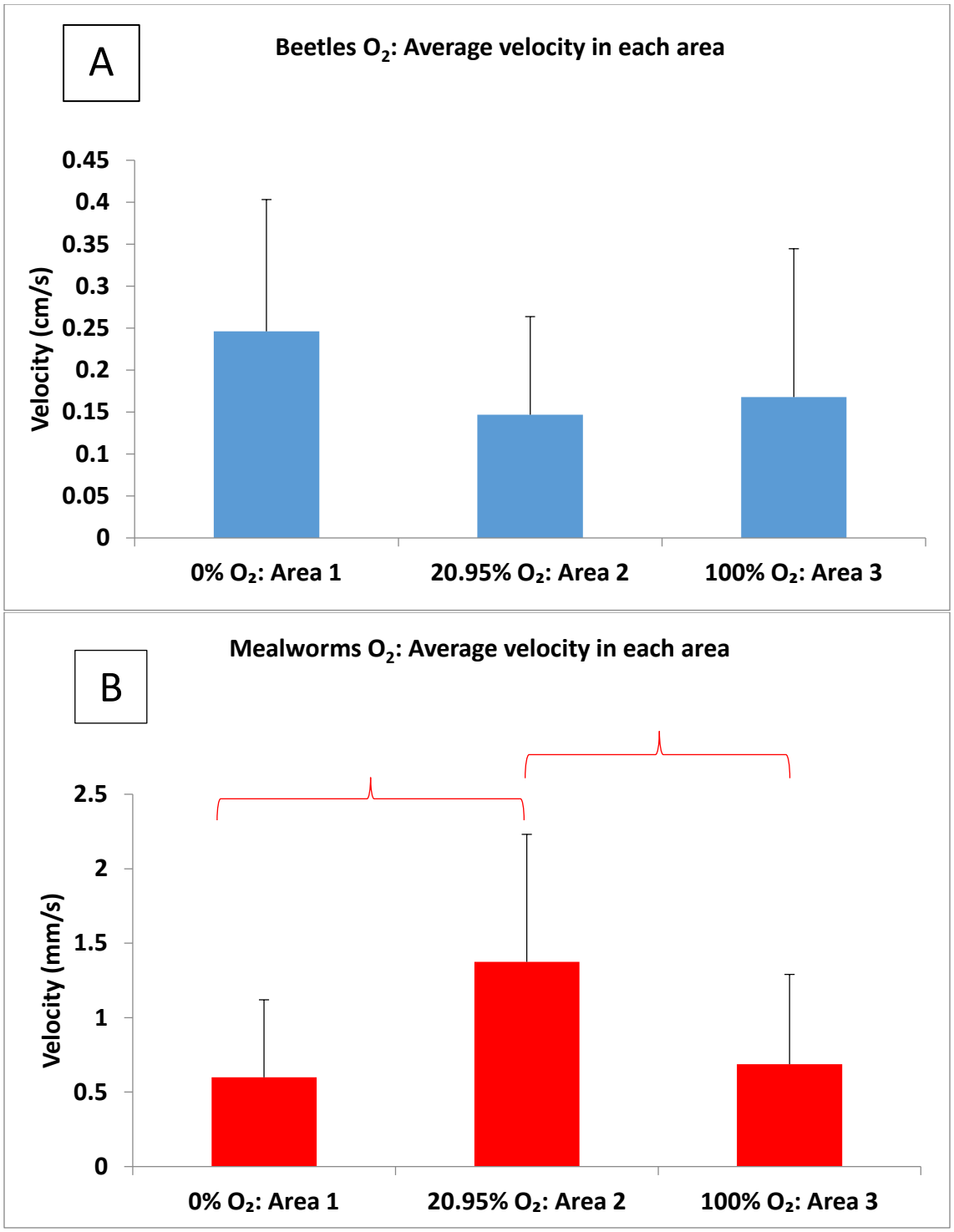


Figure 12. Beetles (A) did not travel faster in any one area over another in the O_2 gradient ($F_{(2,27)} = 1.181$ $p = 0.3222$). Mealworms traveled with increased velocity in the 20.95% O_2 which was significantly different from the remaining areas of the O_2 gradient ($F_{(2,27)} = 3.944$ $p = 0.0314$). The brackets represent significant differences between means ± SD.

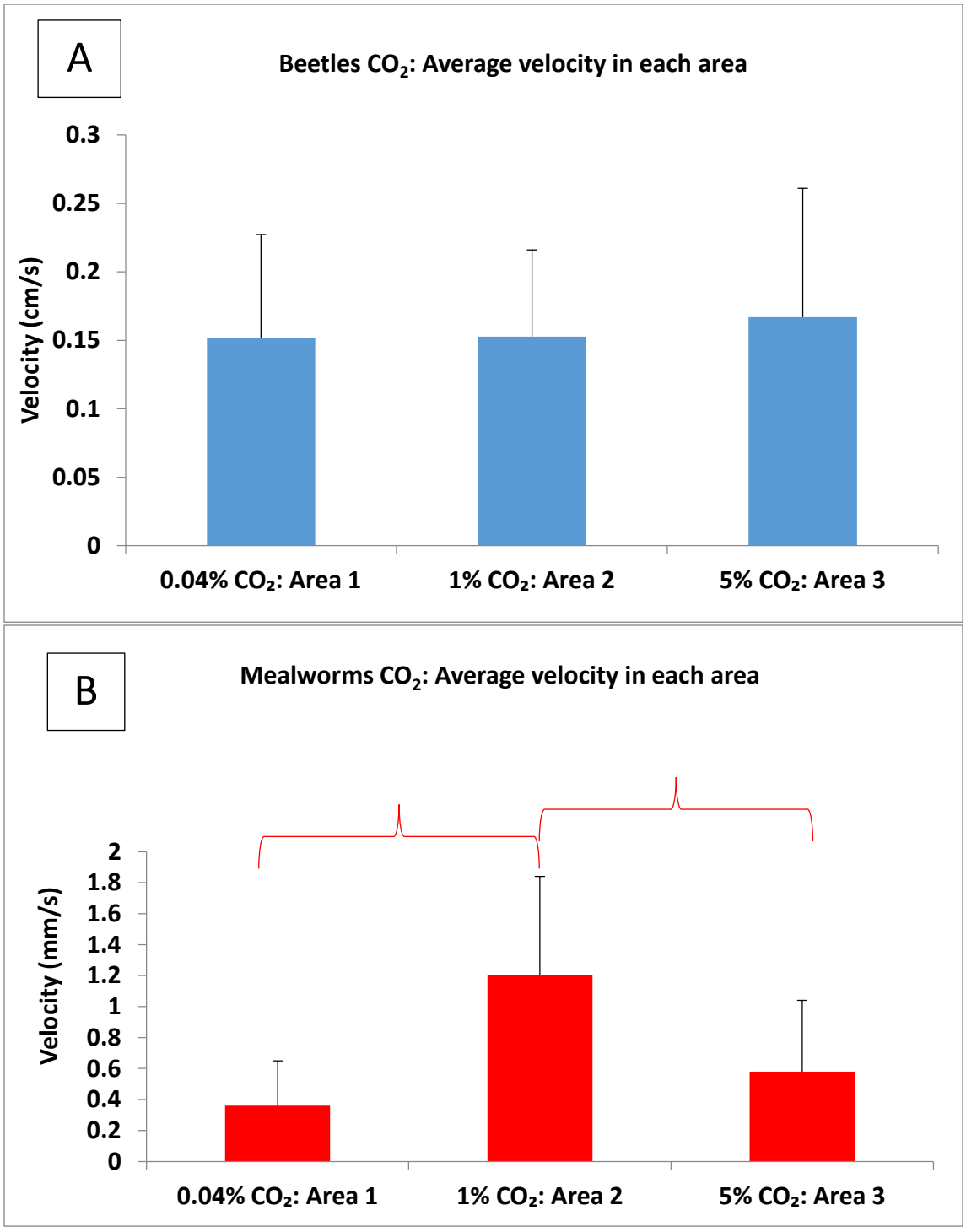


Figure 13. Beetles (A) did not travel faster in any one area over another in the CO_2 gradient (F (2,27) = 0.1175 p = 0.8896). Mealworms (B) traveled with an increased velocity in the 1% CO_2 area of the gradient (F (2,27) = 8.132 p = 0.0017). The brackets represent significant differences between means ± SD.

Time and distance in each area as a percentage

Table 6 compares the average time as a percentage that beetles and mealworms spent in each area of the oxygen and carbon dioxide gradient. This was done by taking the total average time the subjects spent in each area of the gradient and dividing by the total time of the protocol. Table 7 compares the average distance as a percentage that beetles and mealworms traveled in each area of the oxygen and carbon dioxide gradients. This was done by taking the total average distance the animals traveled in each area of the gradient and dividing by the total distance each animal traveled.

Tables 6 and 7 provide a brief overview of the percent differences between the areas in the test arena for beetles and mealworms for the different protocols. I did not compare beetles to mealworms because they are in different life stages. This was done to normalize these data due to their distance and velocity being presented in different units.

Animal starting points

The starting point for each trial was recored in table 2 and a Pearson Correlation was run to make sure that the amout of times the animals started in an area did not affect the results for time, distance or velocity. Had a correlation been found between the amount of time an animal spent in an area and the amount of times a trial was started while the animal was in that area, it is possible that the results could have been due to more animals starting in that area then the animals being attracted to that area. No correlations were found for any trials in any of the four protocols (table 8).

Summary of results

My results support my hypothesis that beetles can sense anoxic and hyperoxic environments, and I conclude that beetles will avoid anioxia and to a lesser extent hyperoxia. Beetles spent more time and traveled farther in a normoxic environment than an anoxic environment. I am unable to prove that beetles are attracted to normocarbia. My beetles did not seem to have a preference for any area in my CO_2 gradient.

Based on my data I can conclude mealworms can sense an anoxic and hyperoxic environment and prefer an anoxic environment. This goes against my hypothesis that mealworms would avoid anoxia. Mealworms spent the most time in anoxia, and, when they encountered a normoxic environment, they traveled the fastest to leave that environment. My mealworms can also sense a hypercarbic environment, and, based on my data, I think mealworms are attracted to hypercarbia over normocarbia supporting my hypothesis that mealworms are attracted to hypercarbia.

Table 6. Total time in each area is listed as a percentage of the total time in the expirment for both the oxygen and carbon dioxide gradient. Significant differences across gas gradients are represented by different letters when $p<0.05$.

	0% O_2	**20.95% O_2**	**100% O_2**
Beetle O_2	7% A	60% B	32% C
Mealworm O_2	49% A	17% B	33% AB
	0.04% CO_2	**1% CO_2**	**5% CO_2**
Beetle CO_2	34% A	31% A	33% A
Mealworm CO_2	26% A	24% A	49% B

Table 7. Total distance in each area is listed as a percentage of the total distance traveled in the experiment across both the oxygen and carbon dioxide gradients. These data were normailzed because beetles and mealworms distances were not recorded in the same units. Significant differences across gas gradients are represented by different letters when $p<0.05$.

	0% O_2	**20.95% O_2**	**100% O_2**
Beetle O_2	12% A	55% B	31% AB
Mealworm O_2	26% A	39% A	34% A
	0.04% CO_2	**1% CO_2**	**5% CO_2**
Beetle CO_2	28% A	31% A	39% A
Mealworm CO_2	13% A	50% B	35% A

Table 8. Pearson Correlations for all four protocols indicate that starting location had no effect on the time spent in each area, distance traveled in each area or velocity in each area. Significant differences were accepted at $p<0.05$. No significant differences were found.

Beetles Time	Control (no air)	Sham (room air)	O_2 Gradient	CO_2 Gradient
r value	-0.495	-0.305	0.248	-0.567
p value	0.071	0.360	0.489	0.086
n	14	11	10	10
R^2	0.245	0.093	0.061	0.322
Beetles Distance	Control (no air)	Sham (room air)	O_2 Gradient	CO_2 Gradient
r value	-0.245	-0.431	0.007	0.011
p value	0.396	0.185	0.984	0.976
n	14	11	10	10
R^2	0.061	0.186	5E-05	0.000
Beetles Velocity	Control (no air)	Sham (room air)	O_2 Gradient	CO_2 Gradient
r value	0.109	0.231	-0.623	0.397
p value	0.706	0.492	0.054	0.255
n	14	11	10	10
R^2	0.012	0.053	0.389	0.157
Mealworms Time	Control (no air)	Sham (room air)	O_2 Gradient	CO_2 Gradient
r value	-0.031	-0.326	-0.448	0.522
p value	0.931	0.255	0.194	0.121
n	10	14	10	10
R^2	0.001	0.106	0.201	0.273
Mealworms Distance	Control (no air)	Sham (room air)	O_2 Gradient	CO_2 Gradient
r value	-0.355	0.148	0.111	0.354
p value	0.313	0.613	0.758	0.314
n	10	14	10	10
R^2	0.126	0.022	0.013	0.126
Mealworms Velocity	Control (no air)	Sham (room air)	O_2 Gradient	CO_2 Gradient
r value	-0.083	0.048	0.081	0.018
p value	0.819	0.869	0.821	0.959
n	10	14	10	10
R^2	0.007	0.002	0.007	0.000

IV. DISCUSSION

The ability for an insect to sense environmental air composition is an important component of insect physiology. For example, an environment that is hypercarbic may anesthetize the insect. A mixture of 25% CO_2, 20% O_2 and 55% N_2 immobilized test subjects in Beadle & Beadle's (1949) experiments just as well as pure carbon dioxide. In contrast an environment that is hyperoxic may lead to oxygen toxicity in the insect and cause oxidative damage to tissues (Hetz & Bradley, 2005). In a normoxic environment when the spiracles are completely open during DGE, the tips of the tracheoles can reach 19 kPa which is an extremely high concentration of oxygen for tissues exposure (Hetz & Bradley, 2005). Vertebrates' pO_2 in the capillaries of inactive tissues can be about 5 kPa and in active tissues 0.5 kPa (Hetz & Bradley, 2005). An environment that is hyperoxic can increase the pO_2 in the tracheoles increasing the risk of oxidative damage to tissues. An attractive environment for Darkling beetles is between 15% and 21% oxygen. *T. molitor* larvae reared in 15% and 21% oxygen levels grew at the same rate; however, when reared at 10.5% oxygen, they grew more slowly and underwent a greater number of molts (Loudon, 1987).

I conducted a behavioral study. In my study I tested the hypothesis that *Tenebrio molitor* can sense anoxic and hyperoxic environments and avoid them. I also tested my hypothesis that mealworms are attracted to hypercarbia and beetles are attracted to normocarbia over hypercarbia. I measured the time, distance traveled, and velocity in

four different protocols. I did not investigate my animals for external chemosensors that may drive their behavior to select an area of hypercarbia, anoxia or hyperoxia.

Effects of Air Flow on Average Distance and Velocity for Control and Sham Protocols

A significant difference was found ($p<0.05$) between the no air flow protocol (control) and the three remaining protocols requiring air flow for average distance traveled and average velocity for beetles. Air flow alone was enough to cause this decrease in distance traveled and velocity. *Tenebrio molitor* have a number of antennal sensilla, smooth-surfaced pegs, hairs, and bristles that are normally associated with mechanoreception (Harbach & Larsen, 1977). The airflow could have activated these mechanoreceptors therefore making the beetles think they were above ground in an unsafe environment, or the lack of humidity in the gases streaming though could have caused this difference. Even with the decrease in distance traveled and velocity between the no air flow (control) protocol and the three remaining protocols having airflow, my animals did not spend more time, travel farther, or move faster in one area over another in the sham protocol. Had I found a significant difference between any areas in the sham protocol, that could mean there may have been some other variable attracting the animals to that area other than the O_2 and CO_2 mixtures I was streaming through the test arena.

In contrast to beetles, there was no significant difference found in distance traveled or velocity for mealworms between the control and three remaining protocols requiring air flow. I speculate this could indicate limited locomotion abilities or conservation of energy for metamorphosis.

Time Spent in Each Area for All Four Protocols

As Table 1 displays, beetles and mealworms did not spend more time in any one area of the test arena than another for the control and sham protocols. If there had been any significant differences between the areas, then there may have been some other variable besides the gas mixtures attracting my animals to that area of the arena.

Beetles spent more time in the normoxic area than the hyperoxic (100% O_2) area and, to a lesser extent, the anoxic area of the test arena (Figure 8A). I can conclude, based on my findings, that beetles can sense a normoxic environment and prefer that environment which supports my hypothesis that adult *T. molitor* will avoid anoxia and hyperoxia (100% O_2). My findings are similar to observations of tiger beetles (*Cicindela togata*) that are mobile and presumably able to remove themselves from flooded habitats to avoid anoxic environments (Hoback *et al.*, 1998). It would make sense to avoid an anoxic area. Increased metabolic demand (due to temperature, locomotion or consumption of food) decreases the closed phase during DGE and will continue to decrease it until there is a continuous pattern of gas exchange (Contreras & Bradley, 2008; Wigglesworth, 1935). In an environment lacking O_2 the exchange of respiratory gases may not occur.

Mealworms spent more time in anoxia than normoxia as seen in Figure 8B. Cotton (1941) observed *T. molitor* larvae living in stored grain where oxygen levels can be lower than in normal air. Tiger beetle (*C. togata*) larvae live in areas that can be flooded for extended periods of time; these submerged habitats become anoxic, and larvae can spend up to 6 days immersed in this anoxic environment before death (Hoback *et al.*, 1998; Hoback & Stanley, 2001). My findings are similar to the tiger beetle larvae

observations which does not support my hypothesis that mealworms would avoid anoxic environments. Greenberg & Ar (1996) found that growth rate slowed in hypoxia (10%) and increased the number of molts in *T. molitor* which delayed pupation if they survived. Based on the literature I can infer that mealworms would not be attracted to hypoxic or anoxic environments. Since Darkling beetles exhibit DGE, it is conceivable that they simply closed their spiracles and did not exchange gases.

I did not find a significant difference in time spent in each area of the CO_2 gradient for beetles (Figure 9A). Perhaps the gradient was not large enough for the beetles to recognize a difference between normocarbic, slightly hypercarbic (1% CO_2) and moderately hypercarbic (5% CO_2) environments. In a study done in stored grain cylinders, the adult rusty grain beetle (*Cryptolestes ferrugineus*) actually moved towards higher levels of CO_2 after 1-5 days (Parde *et al.*, 2004). In the flutter period during DGE tracheal pCO_2 gradually increases from roughly 3.6% to 6.4% CO_2 (Levy & Schneiderman, 1966). These concentrations are barely above my maximum gradient of 5% CO_2 and are, therefore, perhaps not enough to elicit a behavioral response. Parde *et al.* (2004) experiments had a gradient up to 10% CO_2, and 65% of their beetles moved towards the higher CO_2. The Parde *et al.* (2004) experiments lasted anywhere from 5 to 7 days while mine lasted only 30 minutes which may have been too short a time.

Mealworms spent more time in a moderately hypercarbic (5% CO_2) environment than in the normocarbic and slightly hypercarbic (1% CO_2) environments (Figure 9B). Mealworms spend more time in hypercarbic environments such as that of a burrow or stored grain facilities in which they tend to live; CO_2 levels between 2% and 3% can occur in infested granaries (Sinha *et al.*, 1986). My results are similar to the saw-toothed

grain beetle larvae (*Oryzaephilus surinamensis*) which can survive up to 2 days in modified atmospheres of 55%, 65%, 75% and 85% CO_2 (Hashema *et al.*, 2012). In addition, Harein & Press (1968) showed complete mortality of the wheat weevil (*Sitophilus granaries*) in larval and adult stages after 17 days of exposure to a CO_2 concentration of 40% and an O_2 concentration of 2% that is well above what one would find in a grain storage facility. Since my simulation is close to the CO_2 level one would find in a grain storage facility I can conclude that the mealworms can sense and are attracted to hypercarbic environments which supports my hypothesis that larval *T. molitor* are attracted to hypercarbia. Insects are able to tolerate elevated levels of CO_2 in part by the buffering capacity of their hemolymph. During the DGE closed phase CO_2 will accumulate and is buffered by the hemolymph. Once pCO_2 rises to 4-5 kPa the spiracles open which allows for respiration (Lighton, 1996).

Distance Traveled in Each Area for Oxygen and Carbon Dioxide Protocols

I observed that beetles traveled more in the normoxic area than the anoxic area of the O_2 gradient (Figure 10A). If the insect was traveling in an area of anoxia it would eventually need to respire; however, there could be no exchange of CO_2 and O_2 in an anoxic environment. Locomotion increases metabolic demand and the need for respiration, hence I can conclude they must be able to sense anoxia and avoid the area. This goes against my hypothesis that beetles would travel more in an area that is insalubrious for them.

I found no significant differences between the distances traveled in the three areas for the CO_2 gradient for beetles (Figure 11A). I am unable to make a direct comparison

to my data because there are no published works documenting total distance traveled in O_2 and CO_2 gradients. For beetles, however, Parde *et al.* (2004) has documented that *C. ferrugineus* moved towards elevated levels of CO_2 in one experiment up to 90cm. The way this distance was documented was total distance traveled away from the insertion point. It did not take into account the distance the insect may have moved back towards the insertion point or if it was crawling in circles.

I found no significant differences in distance traveled between areas for mealworms in the O_2 gradient (figure 10B). I found a significant difference in the distance traveled between the slightly hypercarbic (1%CO_2) and normocarbic areas (figure 11B) in the CO_2 gradient for mealworms. I could not find any published literature with which to compare these data. The increased distance traveled in the slightly hypercarbic (1%CO_2) area does not support my hypothesis that my animals would travel less in an area that is attractive to them.

Average Velocity in Each Area for Oxygen and Carbon Dioxide Protocols

I found no significant differences in velocity for both O_2 and CO_2 gradients for the beetles (figure 12A and figure 13A). The beetles moved with the highest velocity in the control protocol, and moved the second fastest in the sham protocol. Since this was the protocol with room air streamed through, it is possible that this is their normal speed in which they scavenge for food or seek shelter. In the O_2 and CO_2 protocols for my experiments beetles, average velocity was lower than my control and sham protocols. My results are similar to Pasche & Zachariassen's (1973) findings where increasing CO_2 concentrations over an extended period of time causes adult *Rhagium-Inquisitor* beetles

to become less active and eventually passive until normal atmospheric conditions are restored.

Mealworms tended to travel faster in the normoxic area than in the anoxic and hyperoxic areas in the oxygen gradient (figure 12B) which does not support my hypothesis that mealworms would travel with a lower velocity in areas that are attractive. Perhaps this was because the mealworm was searching to leave the area because these are normal conditions in which their predators live. They also traveled faster in the slightly hypercarbic (1% CO_2) area than the normocarbic and moderately hypercarbic (5% CO_2) areas (figure 13B) which does not support my hypothesis that my animals would travel slower in an environment they consider attractive. Larval velocity recorded in different atmospheric conditions remains under-studied. Conceivably mealworms can sense they are in an elevated CO_2 area and presume they are within their burrow. There are obvious functions associated with burrowing such as concealment from predators and oviposition (Anderson & Ultsch, 1987).

Limitations and Future Directions of Study

I can only infer from my results that beetles and mealworms preferred the areas they did based on time, distance traveled, and velocity recorded in the different areas of my test arena. A limitation of my study could be that the beetles and mealworms could either choose to leave an environment or they could simply close their spiracles and not respire. When looking at the physiology of insects, their hemolymph and tissues have a capacity to buffer CO_2. When insects are in areas of elevated CO_2 this buffering capacity may help decrease the partial pressure gradient between the CO_2 inside the insect and the

CO_2 in the atmosphere. When the insect eventually respires, CO_2 can still be released from the spiracles even in elevated atmospheric CO_2 because there is still a partial pressure gradient for CO_2. Another physiological response that I did not test was that my animals may have been able to slow their metabolic rate and decrease O_2 consumption and CO_2 production. Future studies should focus directly on Darkling beetle and mealworm spiracles under a microscope where different gas mixtures could be streamed across to quantify spiracle closure under varying gaseous environments.

Another limitation of this study could be that the CO_2 gradient was simply not large enough. If burrow concentrations of CO_2 could reach as high as 6-8% (Anderson & Ultsch, 1987), then a concentration of 5% CO_2 may not be great enough to provide a stimulant. Future studies could increase the high end of the gradient to a minimum of 10% CO_2 or possibly higher. I could develop a gradient from atmospheric CO_2 to maximum CO_2 before anesthetization of test beetles and mealworms. I could also give my animals more time to become acclimated to the test arena. I could let the animal take as much time as it needs to become adapted before turning on the gases and starting the experiment. I had wide standard deviations because some of the animals would not move as much as others did. Perhaps if they had more time to adapt to the arena before the test began my standard deviation would decrease.

Additionally, I could increase the duration of each trial. Since mealworms' locomotion is considerably less, with extra time it is plausible they could spend more time, travel farther or faster in different areas of the O_2 and CO_2 gradients than reported in my data shown here. From the literature I know that some of the behavioral responses

that were observed in modified atmospheres took anywhere from one day up to 7 days; therefore, I would increase each trial to a minimum of 8 days.

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