IDENTIFYING PATTERNS AND DRIVERS OF MOVEMENT OF LESSER MEALWORM *ALPHITOBIUS DIAPERINUS* (COLEOPTERA: TENEBRIONIDAE)

by

Ben Sammarco

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Entomology

Fall 2023

© 2023 Sammarco
All Rights Reserved

IDENTIFYING PATTERNS AND DRIVERS OF MOVEMENT OF LESSER MEALWORM *ALPHITOBIUS DIAPERINUS* (COLEOPTERA TENEBRIONIDAE)

by

Ben Sammarco

Approved: _____
Michael Crossley, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____
Jacob Bowman, Ph.D.
Chair of the Department of Entomology and Wildlife Ecology

Approved: _____
Calvin L. Keeler, Ph.D.
Interim Dean of the College of Agriculture and Natural Resources

Approved: _____
Louis F. Rossi, Ph.D.
Vice Provost for Graduate and Professional Education and
Dean of the Graduate College

ACKNOWLEDGMENTS

Michael Crossley, Ph.D. for his continuous guidance, direction, academic and financial support during the past two years.

Deborah Delaney, Ph.D. and David Owens, Ph.D. for their advice and assistance in formulating the methods and goals of the project.

My graduate colleagues in the Agricultural Entomology Laboratory for their assistance and encouragement.

Chapter 1 “Biology and Management of Lesser Mealworm *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) in Broiler Houses” has been previously published in the Journal of Integrated Pest Management under the title “Biology and Management of Lesser Mealworm *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) in Broiler Houses”. The citation is Sammarco, B.C., Hinkle, N.C., Crossley, M.S. (2023). Biology and management of lesser mealworm *Alphitobius diaperinus* in broiler houses. *J. Integr. Pest Manag.*14(1). I am the primary author and originator of the work, which was completed as a part of this degree.

Chapter 2 “Lesser Mealworm *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) Displays Negative Phototaxis and Conditional Hygrotaxis” is currently in review for publication in the Journal of Environmental Entomology under the title Lesser Mealworm *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) Displays

Negative Phototaxis and Conditional Hygrotaxis". I am the primary author and originator of the work, which was completed as part of this degree.

TABLE OF CONTENTS

LIST OF TABLES vii
LIST OF FIGURES viii
ABSTRACT x

Chapter

1 BIOLOGY AND MANAGEMENT OF LESSER MEALWORM *ALPHITOBIUS DIAPERINUS* (COLEOPTERA: TENEBRIONIDAE) IN BROILER HOUSES 1

1.1 Introduction 1
1.2 Life History 2
1.3 Pest Impacts 5
1.4 Monitoring 7
1.5 Chemical Management 8
1.6 Cultural Management 10
1.7 Natural Enemies 12
1.8 Conclusion 13

2 LESSER MEALWORM *ALPHITOBIUS DIAPERINUS* (COLEOPTERA: TENEBRIONIDAE) DISPLAYS NEGATIVE PHOTOTAXIS AND CONDTIONAL HYGROTAXIS 15

2.1 Introduction 15
2.2 Materials and Methods 17

2.2.1 Beetles 17
2.2.2 Lights 17
2.2.3 Adult Phototaxis Experiment 18
2.2.4 Larval Phototaxis Experiment 19
2.2.5 Adult Burrowing Experiment 19
2.2.6 Adult Hygroreception Experiment 20
2.2.7 Comparative Light and Water Response Experiment 21
2.2.8 Statistical Analysis 21

2.3 Results 22

2.3.1 Adult Phototaxis 22
2.3.2 Larval Phototaxis 24
2.3.3 Adult Burrowing 26
2.3.4 Adult Hygroreception 28
2.3.5 Comparative Light and Water Response 31

2.4 Discussion 32

3 SURVEY OF COI DIVERSITY OF LESSER MEALWORM *ALPHITOBIUS DIAPERINUS* (COLEOPTERA: TENEBRIONIDAE) IN BROILER HOUSES IN EASTERN UNITED STATES AND EUROPE 37

3.1 Introduction 37
3.2 Materials and Methods 39

3.2.1 Beetle Sampling 39
3.2.2 DNA Extraction and Amplification 40
3.2.3 Analysis 40

3.3 Results 42
3.4 Discussion 45

REFERENCES 51

Appendix

A LIFE STAGE PHOTOGRAPHY OF *ALPHITOBIUS DIAPERINUS* 59
B PERMISSIONS 64

LIST OF TABLES

Table 2.1- Probability of adult beetles taking shelter relative to the no-shelter baseline at each minute under four light wavelengths and darkness. Significant p-values in bold print. 24

Table 2.2- Probability of larvae taking shelter relative to the no-shelter baseline at each minute under four light wavelengths and darkness. Significant p-values in bold print. 26

Table 2.3- Probability of adult beetles burrowing into substrate relative to a baseline of darkness at each minute under four light wavelengths. Significant p-values in bold print. 28

Table 2.4- Probability of hydrated and dehydrated beetles avoiding water relative to a baseline of random movement of hydrated beetles with no water present. Significant p-values in bold print..... 30

Table 3.1 Diversity statistics for sampled populations of *A. diaperinus* from the Eastern United States and Europe. 44

LIST OF FIGURES

Figure 1.1- Life cycle diagram of *A. diaperinus*. 3

Figure 2.1- Wavelengths of LED flashlight outputs measured with a Black-Comet CXR-SR-50 spectrometer. Each colored light had distinct peaks with minimal overlap of wavelengths. The white light encompassed the other three colors but lacked peaks outside the range of the other lights. 18

Figure 2.2- Average percentage of adult *Alphitobius diaperinus* hiding beneath shelter when exposed to different wavelengths over 5 minutes compared to a baseline of random movement (exposed to white light with no access to shelter). Error bars represent 1 standard deviation. The experiment was replicated 10 times. N per treatment per replicate per time point = 20 beetles. 23

Figure 2.3- Average percentage of larval *Alphitobius diaperinus* hiding beneath shelter when exposed to different wavelengths of light over 5 minutes compared to a baseline of random movement (exposed to white light with no access to shelter). Error bars represent 1 standard deviation. The experiment was replicated 10 times. N per treatment per replicate per time point = 20 larvae..... 25

Figure 2.4- Average percentage of adult *Alphitobius diaperinus* burrowed into substrate when exposed to different wavelengths of light over 5 minutes. Error bars represent 1 standard deviation. The experiment was replicated 10 times. N per treatment per replicate per time point = 20 beetles. 27

Figure 2.5- Average percentage of hydrated and dehydrated adult *Alphitobius diaperinus* moving or remaining within 2 cm of a water source over 30 minutes compared to baselines of random movement (No water present). Error bars represent 1 standard deviation. The experiment was replicated 10 times. N per treatment per replicate per time point = 20 beetles. 29

Figure 2.6- Average percentage of dehydrated adult *Alphitobius diaperinus* exposing themselves to white light with and without a water source in the illuminated section of the arena. Error bars represent 1 standard deviation. The experiment was replicated 10 times. N per treatment per replicate per time point = 20 beetles. 32

Figure 3.1- Haplotype network of COI sequences of *A. diaperinus* in the eastern United States and Europe. Each node represents a haplotype. Node size represents the number of individuals with the haplotype. Each tick on a line represents a nucleotide difference between connected haplotypes. 43

Figure 3.2- Rarefaction curves of the accumulation of haplotypes with increasing sampling of *A. diaperinus* populations. 45

Figure 3.3- Map of haplotype frequencies of *A. diaperinus* populations sampled from farms in the Eastern USA. 46

Figure 3.4- Map of haplotype frequencies of *A. diaperinus* populations sampled from farms in Europe. 47

Figure 3.5- The change in the number of farms producing chickens (right axis, dashed black line) and the number of broilers produced (left axis, solid red line) between 1930 and 1959 as tracked by the USDA Census of Agriculture. 50

Figure A.1- Dorsal, lateral, and ventral views of a second instar *A. diaperinus* larva. 59

Figure A.2- Dorsal, lateral, and ventral views of an early instar *A. diaperinus* larva. . 60

Figure A.3- Dorsal, lateral, and ventral views of an intermediate instar *A. diaperinus* larva. 60

Figure A.4- Dorsal, lateral, and ventral views of a late instar *A. diaperinus* larva. 61

Figure A.5- Dorsal, lateral, and ventral views of an *A. diaperinus* pupa. 62

Figure A.6- Dorsal, lateral, and ventral views of an *A. diaperinus* adult. 63

ABSTRACT

Alphitobius diaperinus is a ubiquitous pest of poultry houses found worldwide. They live in the litter of commercial poultry houses, especially broiler chicken houses. In high numbers they reduce poultry feed efficiency, cause stress and injury to birds, act as a reservoir for poultry diseases, and cause damage to structures and insulation in the house. Distribution and patterns of movement of *A. diaperinus* within broiler houses has been documented, but the specific environmental cues and behavioral drivers that govern those patterns are not well understood. I conducted a series of behavioral assays to evaluate the response of *A. diaperinus* to several wavelengths of light and the presence of water. I found that *A. diaperinus* adults and larvae are repelled by several wavelengths of light, but burrowing behavior is not connected to this response to light. I also found that *A. diaperinus* is repelled by water when in a normal, hydrated state, but is attracted to water when dehydrated, and this attraction is stronger than the repulsion from light. On a broader scale, *A. diaperinus* is believed to originate in Africa as a nest associate of several bird species, however the historical pattern of dispersal across the world is not known. As a preliminary survey to assess the viability of genetic tools to illuminate these historical patterns I sampled broiler house populations of *A. diaperinus* from several farms in the Eastern United States and Europe and sequenced the mitochondrial cytochrome oxidase subunit I gene. I analyzed the diversity of this gene between houses, farms, states, and nations, and found that broiler house *A. diaperinus* populations are very homogenous at the cytochrome oxidase subunit I gene.

Chapter 1

BIOLOGY AND MANAGEMENT OF LESSER MEALWORM *ALPHITOBIUS DIAPERINUS* (COLEOPTERA: TENEBRIONIDAE) IN BROILER HOUSES

1.1 Introduction

Alphitobius diaperinus (Coleoptera: Tenebrionidae: Alphitobiini) is a pest of stored grains and the poultry industry (Aalbu et al. 2002). *Alphitobius diaperinus* is believed to have originated in sub-Saharan Africa, but now occurs worldwide (Hopkins 1990). The beetle has been reported in Algeria, Argentina, Australia, Brazil, China, Denmark, France, Greece, India, Pakistan, Poland, and the United States, but likely occurs over an even broader distribution (Arshad et al. 1984, Geden et al. 1987, Bhattacharyya 1995, Salin et al. 2000, Bates et al. 2004, Skov et al. 2004, Lambkin et al. 2007, Amir and Nadir 2009, Hassemer et al. 2016, Li et al. 2016, Szczepanik et al. 2018, Arena et al. 2020, Dzik et al. 2022). Outside of anthropogenic environments *A. diaperinus* is a bird nest symbiont, feeding on scraps and detritus in the nests of a wide variety of species including pigeons and sparrows (Arshad et al. 1984, Bhattacharyya 1995). They have also been observed to have a similar life history feeding on detritus and guano among populations of bats in caves (McFarlane 1971). *Alphitobius diaperinus* is a highly resilient organism that persists despite management techniques and is a known reservoir of several avian diseases. In intensive broiler production their populations can reach staggering levels, with a single house hosting an estimated 34.7 million adults, pupae, and larvae in the litter (Singh 2011). *Alphitobius diaperinus* has been identified for use as feed in aquaculture, and is beginning to be cultivated in

several European countries for this purpose (Rumbos et al. 2019). However, this profile will focus on *A. diaperinus* as a pest of the poultry industry, and will examine its life history, impacts in stored grain and poultry facilities, and current and potential controls and management techniques.

1.2 Life History

Alphitobius diaperinus has 8-11 larval instars (Figure 1.1), with development time highly dependent on environmental temperature, from 10 days between instars at 20°C to 2 days between instars at 30°C (Wilson and Miner 1969). *Alphitobius diaperinus* larvae are creamy white in their early instars, later darkening to a yellow-brown hue (Figure A.1, Figure A.2, Figure A.3). They have segmented bodies measuring up to 11 mm in length during their last larval instar (Figure A.4), with three pairs of legs (Dunford and Kaufman 2006). Prior to pupation larvae seek isolation from other larvae and adults, dispersing and often finding substrate to burrow into. *Alphitobius diaperinus* pupae are exarate and resemble the adults, measuring 6 to 8 mm and are creamy white to tan colored (Dunford and Kaufman 2006) (Fig. A.5). Adults are roughly oval in shape, measuring between 5.8 and 6.3 mm, with brown to black shiny exoskeletons (Fig. A.6). The head is deeply emarginated and is often largely hidden by the pronotum. The pronotum is roughly twice as wide as it is long and textured with minute pits or punctures. The elytra completely cover the abdomen, are striated, and can open to allow for flight (Dunford and Kaufman 2006). The full life cycle from oviposition to adult eclosion takes between 34 and 38 days at the species' optimal temperature of 30°C (Rueda and Axtell 1996). Below 30°C development slows, to a maximum oviposition to adult eclosion time of 165 days when at 20°C, with growth and development ceasing completely below this

temperature (Rueda and Axtell 1996). Adults will mate as soon as exoskeleton tanning is complete, generally 5-8 days following eclosion (Hopkins et al. 1992). An adult female will live between four months and a year, laying eggs periodically throughout her adult life at an average rate of 3.5 eggs per day, laid singly on or within loose substrate, generally reaching a lifetime total of between 200 and 400 eggs, though individuals in laboratory settings have been observed to lay over 2,000 eggs in their lifetime (Hopkins et al. 1992, Rueda and Axtell 1996).

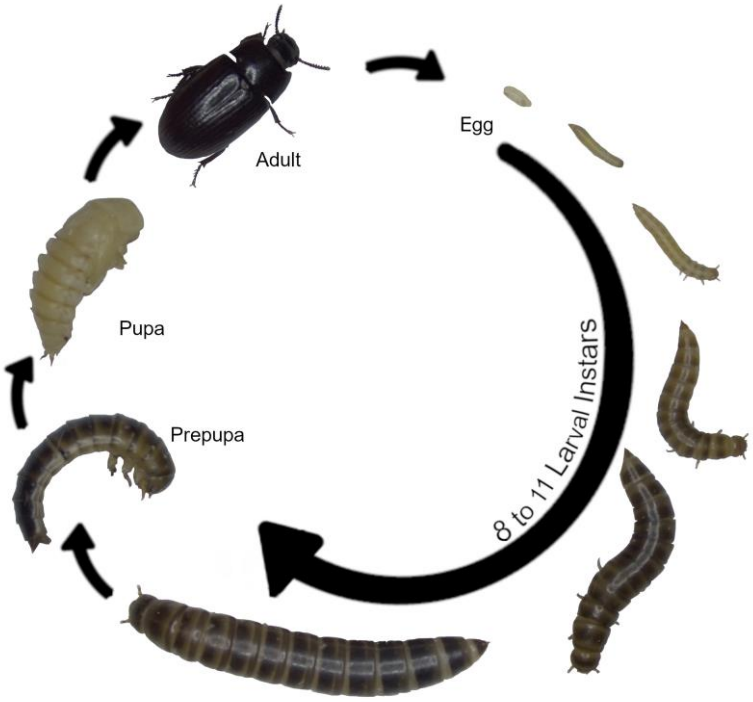

Figure 1.1- Life cycle diagram of *A. diaperinus*.

Alphitobius diaperinus adults and larvae are pests of stored grain products and chicken rearing facilities, feeding on different materials in each setting. In stored

grains *A. diaperinus* is a secondary pest on cereal grains, including wheat, barley, and rye, as well as associated products such as flours, bran, and hay (Rumbos et al. 2020). In broiler houses and caged layer facilities *A. diaperinus* lives in the litter and manure of the house, feeding on a combination of chicken manure and spilt feed (Dunford and Kaufman 2006). *Alphtiobius diaperinus* is a somewhat aggressive feeder, noted to quickly turn to cannibalism, or even to collectively attack and kill small vertebrates such as snakes when preferred food sources are unavailable (Crook et al. 1980). *Alphitobius diaperinus* produces a volatile alarm pheromone, and adult males produce an aggregation pheromone attractive to both sexes composed of limonene, (E)-β-ocimene, 2-nonanone, linalool, and daucene (Bartelt et al. 2009, Hassemer et al. 2019a). Populations in Brazilian poultry facilities exhibit a sixth component to the pheromone, (E,E)-α-farnesene, indicating that there may be variation in this pheromone between broad geographic areas, though this has not been investigated in other regions (Hassemer et al. 2019a).

Little is known about the movement and behavior of *A. diaperinus* outside of their preferred habitats. When litter and manure is removed and scattered across a field or buried in soil as an organic fertilizer, the beetles move upwards, out of the soil and disperse from the location, possibly in search of suitable habitat (Calibeo-Hayes et al. 2005, Kaufman et al. 2005). This behavior has led to *A. diaperinus* being considered a nuisance pest, as dispersing beetles have been reported making their way into residential areas in large numbers, prompting complaints and possible litigation from homeowners aimed at chicken farms (Hinchey 1997). Within manure piles of layer houses or litter of broiler houses *A. diaperinus* displays a tendency to aggregate along the walls of the facility and beneath feeder and drinker lines (Lambkin and Rice 2007,

Amir and Nadir 2009). These locations are thought to be preferred by the beetle as sources of shelter from predation by poultry, as experimental shelters placed on the litter surface also result in beetle aggregation (Lambkin et al. 2008). Within the litter substrate *A. diaperinus* displays vertical movement, burrowing deeper into substrate when unsheltered by other material and when ambient temperature decreases (Salin et al. 2000). Both adults and larvae display similar patterns of movement, with the exception of the climbing and boring behavior of pre-pupal larvae (Salin et al. 2000, Dunford and Kaufman 2006).

1.3 Pest Impacts

Alphitobius diaperinus is near omnipresent in intensive chicken rearing facilities. At low population levels their impacts are considered negligible, but their high fecundity means that an uncontrolled population can quickly grow large enough to cause significant damage to birds and structures. Of major concern in broiler houses is the capacity for *A. diaperinus* to act as a reservoir for a variety of avian diseases, including *Salmonella typhimurium, Escherichia coli*, avian leucosis, Marek's disease, and Turkey Coronavirus (Eidson et al. 1966, de las Casas et al. 1968, Goodwin and Waltman 1996). *Alphitobius diaperinus* is especially effective as a reservoir in broiler houses because they live within the litter and manure on which the chickens spend their entire lives, consuming the feces of the chickens and in turn being consumed by the chickens. Beetles with pathogens on their exoskeleton or within their guts can transmit those pathogens to chickens when ingested, especially to young chicks, which have been documented to consume between 300 and 500 beetle larvae in a single day (Despins and Axtell 1995, Skov et al. 2004, Hazeleger et al. 2008, Roche et al. 2009). Microbial communities can persist in the gut of *A. diaperinus* for 5 weeks or more

when removed from contaminated environments, potentially allowing them to vector pathogens between flocks (de las Casas et al. 1968, Crippen et al. 2022).

In cases of extreme infestation *A. diaperinus* has been known to burrow into and partially feed on vertebrates, including dead and moribund chicks (Crook et al. 1980). Furthermore, chicks feeding on *A. diaperinus* are incapable of properly digesting the insect's exoskeleton, resulting in watery feces containing masses of undigested beetles and larvae. Chicks feeding on *A. diaperinus* consume less feed, which together with the digestive stress of consuming darkling beetles results in decreased growth and weight gain (Despins and Axtell 1995). These losses in weight and impacts to health remain even if chicks later switch to an all-feed diet, resulting in delayed growth compared to chicks fed exclusively starter feed their entire lives (Despins and Axtell 1995). However, turkey poults of a similar age can seemingly be fed *A. diaperinus* larvae without significant impact to growth and feed efficiency (Despins and Axtell 1994). The reduced growth and feed efficiency of broilers feeding on darkling beetles leads to more feed being required to bring a flock to salable weight, resulting in estimated losses of between $2,649.90 and $4,262.90 per 100,000 birds, with exact numbers varying based on the price of feed being used (Karen et al. 2018).

Extensive infestation can also lead to damage to insulation materials due to the burrowing of larvae seeking pupation sites, the initial tunneling exacerbated by the wider adults leaving following emergence (Fairchild et al. 2005). This damage can greatly reduce the effectiveness of the insulation and compromise the carefully controlled airflow in and out of the house, resulting in increased loss of heat in cold months and influx of heat in warm months. This forces heating and venting

mechanisms to be more active and increases energy costs for the grower. However, adoption of fiberglass insulation instead of spray foam may reduce the likelihood of *A. diaperinus* feeding damage resulting in substantial modifications to airflow (M. Czarick, personal communication). *Alphitobius diaperinus* also occurs in caged layer houses in manure piles accumulating below the hens. In these houses the beetle is a structural pest, with prepupal larvae climbing and boring into wooden support beams and pupating inside them, degrading the beam as they do insulation and over time threatening the structural integrity of the house (Kaufman et al. 2005). In both layer and broiler houses beetle activity results in reduced efficacy of house structures and eventually a need for replacement of structural components. Ultimately, because of its pervasive presence in poultry production, it is difficult to exactly quantify the economic impacts of *A. diaperinus*. Parsing losses of birds, feed, and airflow, and temperature regulation due to beetle activity from losses due to other factors is a worthy goal that would nonetheless require an intensive and far-reaching study.

1.4 Monitoring

Infestations of *A. diaperinus* are all but impossible to fully remove from a broiler house, and the benefits of complete eradication are not worth the cost, especially when the vast majority of issues arising from infestations are the result of massive populations and become negligible with smaller infestations. Populations of *A. diaperinus* are rarely intensively monitored by farmers, but when they are checked, the method generally involves setting traps. The most effective traps for collection of *A. diaperinus* use corrugated cardboard as a substrate for beetles to climb and seek shelter within (Safrit and Axtell 1984). The Arends tube trap, consisting of sections of cardboard rolled up and placed within short lengths of PVC pipe, is a commonly used

design that captures large numbers of beetles while still being simple to place and retrieve. Sampling efforts for *A. diaperinus* typically consist of traps being distributed across the litter of the house and left for 1 week, after which they are collected and frozen to kill the captured beetles, which are then removed and counted. Due to the general lack of monitoring practice, control methods are applied identically over time, with management changing only when issues begin to occur. A recent improvement to monitoring practice involves deployment of the synthetic aggregation pheromone in conjunction with pitfall traps, increasing their efficacy (Hassemer et al. 2019a). With more precise monitoring techniques, more targeted or adjusted controls could be implemented, potentially saving growers control expenditure.

1.5 Chemical Management

Treatment for *A. diaperinus* is typically conducted proactively, seeking to keep populations low instead of responding to problematic population levels. Frequency of treatment varies among growers, up to treating after every flock, but most will conduct treatment once every 3-4 flock cycles. Chemical treatment for *A. diaperinus* consists of spraying of litter, floors, and walls with insecticides between flock cycles, when no chickens are present in the house. A variety of insecticides are available for use: pyrethroids (bifenthrin, cyfluthrin, β-cyfluthrin, γ-cyhalothrin, cypermethrin, permethrin), organophosphates (fenitrothion, tetrachlorvinphos), neonicotinoids (acetamiprid, imidacloprid), insect growth regulators (methoxyfenozide, pyriproxyfen, teflubenzuron), and spinosyns (spinosad) (Lambkin 2005, Lambkin et al. 2007, Kozaki et al. 2008, Tomberlin et al. 2008, Boozer 2011, Lambkin and Furlong 2011, Zorzetti et al. 2015, Hickmann et al. 2018, Renault and Colinet 2021). The organophosphate chlorpyrifos was often used, but as of February 2022, it is no longer

registered for use in poultry in the U.S. (EPA 2021). Organic farms restricted from the use of synthetic pesticides will use boric acid instead (G. Cartanza, personal communication). *Alphitobius diaperinus* has been shown to develop resistance to most types of insecticide after prolonged use (Lambkin 2005, Hamm et al. 2006, Lambkin and Rice 2006, Kaufman et al. 2008, Steelman 2008, Tomberlin et al. 2008, Lambkin et al. 2010, Lambkin and Furlong 2011, 2014, Hickmann et al. 2018, Renault and Colinet 2021).

Alphitobius diaperinus has also been found to develop cross resistance to insecticides exhibiting the same mode of action. For example, populations in commercial broiler houses in Australia that had evolved field resistance to cyfluthrin also exhibited resistance to γ-cyhalothrin, despite never being exposed to the latter chemical prior to testing (Lambkin et al. 2010). Populations have been shown to become highly resistant to fenitrothion after 10 to 20 years of consistent use (Lambkin 2005). *Alphitobius diaperinus* has not yet exhibited cross-resistance between different chemical modes of action, such as pyrethroids and spinosad (Lambkin and Rice 2007, Singh and Johnson 2015). Rotating modes of action could therefore be expected to delay resistance and maintain control of beetle populations. The mechanisms of resistance to various insecticides are not fully understood. Metabolic mechanisms appear to play at least a partial role in pyrethroid resistance, but are not the only factor, evidenced by incomplete sensitivity restored to resistant beetles following addition of the enzymatic inhibitor piperonyl butoxide (Lambkin and Furlong 2011). Analysis of acetylcholinesterase genes in tetrachlorvinphos-resistant and susceptible populations of *A. diaperinus* showed that point mutations in the gene are insufficient to explain resistance (Kozaki et al. 2008). Issues of insecticide resistance may be exacerbated by

lack of monitoring practice, as lack of insecticide efficacy may go unnoticed until visible issues occur. Some control methods, such as insect growth regulators, go underutilized despite demonstrated efficacy, likely due to the delayed effect on beetle mortality resulting in a perception that these chemicals are less effective (Zorzetti et al. 2015). Combinations of insecticides may have the potential for synergistic effects that increase effectiveness. For example, spinosad has been shown to increase sensitivity to cyfluthrin and other pyrethroids (Lambkin and Furlong 2014). Alternative methods of insecticide application, such as applying insecticide to the materials of house walls, may provide effective control while using less material, reducing management costs (Kaufman et al. 2008).

Alternative chemical control methods to synthetic insecticides include application of diatomaceous earth and plant essential oils. Diatomaceous earth can decrease population growth of *A. diaperinus* compared to untreated houses but does not display the lethality or population reduction of synthetic insecticides and may be more suited to maintaining control of already low populations (Oliveira et al. 2017). Select essential oils such as lemon and citronella have been shown to repel (but not directly kill) *A. diaperinus* and could be paired with trapping methods that utilize a push-pull system (Francikowski et al. 2019). The options available to control *A. diaperinus* are limited and as resistance to synthetic insecticides grows more common, more diverse and effective methods are becoming necessary.

1.6 Cultural Management

Removal and replacement of litter is one of the most effective means of controlling *A. diaperinus* populations in broiler houses. Changing the litter removes the bulk of the population, including larvae, adults, and eggs deposited in the litter.

However, frequent litter removal (e.g., after each flock cycle) is expensive, both in material and labor, and is not considered financially viable by some growers. The practice of windrowing, piling the litter in rows to allow for fermentation and heating of manure for sanitation purposes, has an additional benefit of causing beetles to vacate the manure, reducing the resident population in the litter between flocks (Barker et al. 2013). However, this reduction is less than that of fully removing and replacing litter, as a portion of the population may take refuge by burrowing or hiding in crevices. Manipulation of house and litter temperature between flocks can be a means of reducing the population directly. Warming litter to 45°C or hotter successfully kills beetles and can be used in conjunction with insecticides and litter drying for greater effect (Wolf et al. 2015). Exposing the interior of the house to winter temperatures by opening doors between flocks, lowering the temperature below 20°C, can help reduce beetle population sizes as well (Singh 2011). When litter is spread in fields, mechanical incorporation of manure into soil via plowing can decrease beetle re-emergence by over 50%, though manure spreading is generally not performed with cognizance of darkling beetle presence (Calibeo-Hayes et al. 2005).

Control is more difficult while a flock is present in the house, as the use of insecticides and the manipulation of temperature and litter is more restricted. During a flock cycle proper maintenance of litter and house conditions, such as keeping litter dry, can slow the growth of *A. diaperinus* populations (Wolf et al. 2015). Some animal welfare standards, such as those recommended by the Global Animal Partnership, stipulate that broiler house interiors be illuminated by lamps or sunlight via windows (Global Animal Partnership 2020). It is unknown what effect this has on overall beetle populations, but there is some evidence that *A. diaperinus* is repelled by certain

wavelengths of light (unpublished data). In caged layer houses, damage to support beams by boring larvae and emerging adults can be prevented by wrapping strips of polyethylene around the beams (Kaufman et al. 2005). The beetles cannot grip and climb the material, and so are prevented from climbing and burrowing into the beam, protecting the structure. Another potential avenue of cultural control is the use of attractants and repellants to influence the movement and aggregation of *A. diaperinus* within houses. a push and pull system using synthetic *A. diaperinus* alarm and aggregation pheromones to direct beetles and larvae into pitfall traps, dramatically improving the yield compared to un-baited traps (Hassemer et al. 2019a). Lemon and citronella essential oils have also been found to have a repelling effect on *A. diaperinus* and could serve a similar function (Francikowski et al. 2019).

1.7 Natural Enemies

Biological control is a less common method of controlling *A. diaperinus,* with only one biological control agent, the entomopathogenic fungus *Beauveria bassiana*, currently marketed as a treatment for *A. diaperinus* populations. *B. bassiana* has been shown to be capable of infecting and killing *A. diaperinus* and has the potential to be even more effective as a control agent when deployed in an attract-and-kill strategy (Santoro et al. 2008). Pitfall traps baited with aggregation pheromone and designed to inoculate *A. diaperinus* with spores of *B. bassiana* have shown success in inducing lateral spread of *B. bassiana* across *A. diaperinus* populations, creating a more efficient method of application for longer-term control than sprays of *B. bassiana* spores alone (Hassemer et al. 2020). However, several other natural enemies have been identified and are being investigated as potential control agents. The parasitic mite *Acarophenax mahunkai* Steinkraus & Cross (Acari, Tarsonemina:

Acarophenacidae) is found with *A. diaperinus* in wild bird nests and feeds on the eggs of *A. diaperinus* (Steinkraus and Cross 1993). Introduction of this mite species to broiler houses could potentially serve as a long-term passive control method that increases the pressure on broiler house *A. diaperinus* populations. In addition to parasitic mites, entomopathogenic nematodes in the genus *Steinernema* may potentially serve as biological control agents (Szalanski et al. 2004). In laboratory settings, *Steinernema* species have been shown to cause between 60 and 95% mortality in populations of larvae and adults within a few days of exposure (Szalanski et al. 2004, Del Valle et al. 2016, Kucharska et al. 2016). In field trials in broiler litter, *Steinernema* caused a ca. 70% mortality rate for up to 7 weeks, after which mortality fell to between 0 and 30%, as the nematode population was potentially disrupted by litter manipulations between flocks (Geden et al. 1987). Without repeated applications, beetle populations quickly recovered to peak size.

1.8 Conclusion

The impacts of *Alphitobius diaperinus* are numerous but generally minor, amounting to an economic drain on a grower rather than a pressing concern that will ruin a given flock. This is further emphasized by the prevalence and near omnipresence of *A. diaperinus*, as few farms will be entirely devoid of the species. As such, management of the species must be conducted with the intent to mitigate these losses, and management regimes must remain affordable and viable over the long-term. One of the largest obstacles to creating a true integrated pest management regimen for *A. diaperinus* is our lack of ability to fully assess the economic damage inflicted by the beetle, preventing the determination of an economic injury level and economic threshold. To surmount this issue more research is needed on the economic

costs of bird injury, feed loss, and insulation damage. Another obstacle is the looming specter of insecticide resistance. Long term integrated pest management programs will need to account for changes in insecticide efficacy, and new, field-tested control methods such as cultural and biological control need to be developed to augment or replace insecticide regimes and slow or manage the growth of resistance. These alternative control methods may be discovered or augmented by a greater understanding of the patterns and behavioral drivers of movement of *A. diaperinus* within houses and on regional scales, as well as the mechanism of colonization of new and cleared houses. With better informed and more effective management strategies, *A. diaperinus* can be greatly diminished as an economic pest of poultry production.

Chapter 2

LESSER MEALWORM *ALPHITOBIUS DIAPERINUS* (COLEOPTERA: TENEBRIONIDAE) DISPLAYS NEGATIVE PHOTOTAXIS AND CONDTIONAL HYGROTAXIS

2.1 Introduction

Patterns of movement in arthropods are governed by environmental cues and signals and the behavioral responses to those cues. This is not only limited to host detection and navigation, but also behaviors that dictate where individuals forage and rest, with responses varying with the condition and life stage of an individual. For example, terrestrial isopods are attracted to areas of higher humidity, lower temperature, and lower light intensity (Warburg 1968). These behavioral patterns can be reversed by acanthocephalan parasites, driving isopods out of shelter and exposing them to predation by starlings, promoting the continuation of the parasites' life cycle (Moore 1983). It has also been shown that mosquito larvae exhibit alternating attraction or repulsion to light at different ages (Holmes 1911). Mosquito larvae are broadly indifferent to light but become negatively phototaxic in response to shadows passing overhead, such as those created by a low-flying bird, while adults exhibit positive phototaxis. Ectoparasites such as the rabbit tick *Haemaphysalis leporispalustris* use signals of light and humidity to position themselves on vegetation at the ideal height to latch onto their preferred host, with positive phototaxis and negative hygrotaxis driving larval ticks out of moist undergrowth and onto exposed stalks (Camin and Drenner 1978). So, both internal and external factors alter the

strength and directionality of responses to environmental cues, creating the potential for manipulation of arthropod response.

Understanding of environmental cues and their resulting behaviors can inform management practices to reduce or inhibit arthropod pest activity. Manipulation of environmental cues in the form of repellents and synthetic pheromones is a common practice in livestock agriculture but is not the only avenue of cultural control available (Sonenshine 2004, Reeves et al. 2010). Light has been found to be an effective signal for altering the behavior of many lepidopterous crop pests (Wen et al. 2018, Uehara et al. 2019, Liu et al. 2021). Several species of stored grain pests have been shown to exhibit attraction or repulsion to specific wavelengths of light (Park and Lee 2017). Phototaxis has also been observed in multiple species of ectoparasitic and scavenging beetles (Rakowski 1988, Wang et al. 2021). Light thus represents a potential tool with which to promote or suppress arthropod movement to enhance pest management.

In the case of *Alphitobius diaperinus*, an arthropod pest of broiler house chicken production, the patterns of movement within broiler houses are well documented (Salin et al. 2000, Chernaki-Leffer et al. 2007, Lambkin et al. 2007, Amir and Nadir 2009). The population of beetles tends to accumulate near the walls of the house beneath condensed, packed litter, and along feeder and drinker lines, with transitional movement between these hotspots observed. This pattern has been partially attributed to these locations being sheltered from predation by poultry within the house (Lambkin et al. 2008). However, this only explains why the behavior is selected for in *Alphitobius* populations, and the environmental cues that drive this behavior have not been confirmed. In order to identify these cues and the dynamics that may govern movement behaviors in *A. diaperinus*, we conducted a series of

behavioral assays assessing responses of *A. diaperinus* adults and larvae to two environmental cues: 1) the presence or absence of several wavelengths of light and 2) the presence or absence of water. Light and water were selected as environmental cues because the yellow mealworm *Tenibrio molitor*¸ a close relative with a similar life history to *A. diaperinus*, has been shown to be sensitive to specific wavelengths of light and the presence of water (hygroreception) (Harbach and Larsek 1977, Sharkey et al. 2017).

2.2 Materials and Methods

2.2.1 Beetles

Adult and larval *A. diaperinus* were obtained from laboratory colonies maintained by the University of Delaware Agricultural Entomology Lab. The colonies were originally sourced from commercial broiler houses in Delaware (collected by D. Owens). Colonies were kept in a growth chamber (kept at 25ºC, 16:8 light:dark, and ~70% humidity) in 32 x 17 cm ventilated plastic containers provisioned with ~1,088 cm^3 of chicken layer pellets (Purina® Start & Grow® Non-Medicated, Nestlé Purina Petcare, St. Louis, MO) and pieces of apple and carrot replaced weekly to serve as a water source. Pieces of Styrofoam were provided to serve as shelter and pupation sites. Individuals used in experimental trials were placed in a new colony to prevent the reuse of individuals across experiments.

2.2.2 Lights

Battery powered LED flashlights were acquired from RaySoar (Jiaxing, Zhejiang, China). Each flashlight had an output of 200 lumens. The four colors used for phototaxis treatments were red (600-640nm), green (500-560nm), blue (440-

470nm) and white (440-700nm) (Fig. 2.1). Output wavelengths were confirmed with a Black-Comet CXR-SR-50 Spectrometer from Stellar Net Inc. (Tampa, Florida, USA).

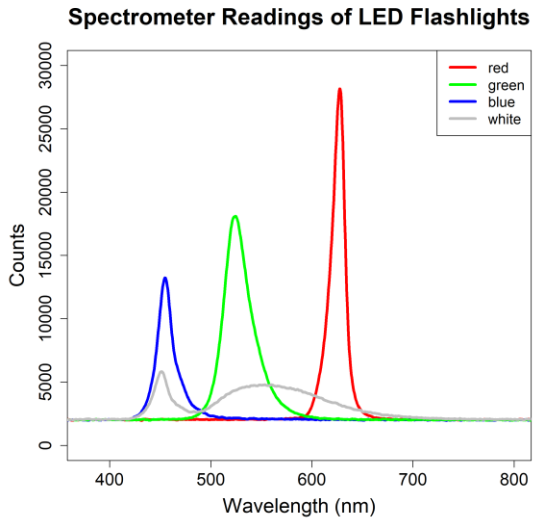

Figure 2.1- Wavelengths of LED flashlight outputs measured with a Black-Comet CXR-SR-50 spectrometer. Each colored light had distinct peaks with minimal overlap of wavelengths. The white light encompassed the other three colors but lacked peaks outside the range of the other lights.

2.2.3 Adult Phototaxis Experiment

A sample of 1,200 adult beetles was collected from *A. diaperinus* colonies. Twenty beetles were randomly selected from the subsample and placed in one half of an 8.5 cm diameter petri dish. A flashlight was mounted 30 cm above the petri dish, the beam focused onto the petri dish, and all other light sources switched off. A cardboard cover was placed over the side of the petri dish without beetles, creating a shaded half of the arena. Each minute for five minutes the number of beetles beneath the shelter was recorded. This procedure was repeated 10 times for each treatment with the sequence of treatments randomly determined using the *sample* function in R

version 4.2.1 (R Core Team 2021). There were 6 treatments: red light, green light, blue light, white light, darkness, and a no-shelter control. The no-shelter control used white light but provided no shelter to act as a baseline of random movement for comparison with the responses to other treatments.

2.2.4 Larval Phototaxis Experiment

The same experimental design that was used to examine adult phototaxis was repeated with late instar *A. diaperinus* larvae. Preliminary experiments showed that small larvae were unable to move effectively on the surface of the petri dish, but larger larvae between 1.5 cm and 2 cm were capable of movement. As a result, larvae in this size range were sampled from a mixed age colony.

2.2.5 Adult Burrowing Experiment

A subsample of 1,000 adult beetles was collected from *A. diaperinus* colonies. Twenty beetles were randomly selected from the subsample and placed in one half of an 8.5 cm diameter x 5 cm deep flat-bottomed bowl filled with 2.5 cm of loose substrate (Chicken litter sampled from broiler houses blended for 30s in a food processor). A flashlight was mounted 30 cm above the bowl, the beam focused onto the substrate, and all other light sources switched off. Each minute for five minutes the number of beetles burrowed into the substrate was determined (20 minus the number of beetles not burrowed) and recorded. This procedure was repeated 10 times for each treatment with the sequence of treatments randomly determined using the *sample* function in R. There were 6 treatments: red light, green light, blue light, white light, and darkness.

2.2.6 Adult Hygroreception Experiment

A subsample of 800 adult beetles was collected from *A. diaperinus* colonies. Four hundred beetles of this subsample were selected for controlled desiccation. Beetles were placed in a plastic petri dish which was then sealed in a plastic container alongside a reusable silica desiccant. The collective beetles were weighed daily to track weight loss. Preliminary experiments showed that mortality occurred once beetles had lost 50% of their original mass. We considered beetles to be "dehydrated" when they exceeded 20% loss of mass. Non-desiccated beetles were placed in an identical setup without a desiccant and provisioned with water via single-ply paper towel folded 4 times and moistened with 10ml of water daily. Twenty beetles were randomly selected from the desiccated or hydrated subsample and placed in one half of an 8.5 cm diameter petri dish with a piece of paper towel either dry or moistened on the other half of the dish. Paper towel pieces consisted of a rectangular piece of single-ply paper towel measuring 7.5 cm by 5 cm folded in half three times in alternating directions, resulting in a 2.5 cm by 2 cm rectangle with a thickness of 0.25 cm. For treatments with wet towels the pieces were initially moistened with 6 ml of water from a syringe, with 2 ml of water added after each hour. All light sources were switched off. Every minute for 5 minutes, and then every 5 minutes for a further 25 minutes (total time 30 minutes), a flash photograph was taken of the petri dish and the number of beetles within 2 cm of the paper towel was counted. There was one control treatment, hydrated beetles without a water source, and three experimental treatments: 1) hydrated beetles with a water source, 2) dehydrated beetles with a water source, and 3) dehydrated beetles without a water source. The procedure was repeated 10 times for each treatment.

2.2.7 Comparative Light and Water Response Experiment

A subsample of 400 adult beetles was collected from *A. diaperinus* colonies. All beetles were desiccated as described for the adult hygroreception experiment. Twenty beetles were randomly selected from the subsample and placed in one half of an 8.5 cm diameter petri dish. A white flashlight was mounted 30 cm above the petri dish, the beam focused onto the petri dish, and all other light sources switched off. A cardboard cover was placed over the side of the petri dish with beetles, creating a shaded half of the arena. A piece of dry or moistened paper towel prepared in the manner described in the adult hygroreception experiment was placed in the illuminated half of the dish. Each minute for five minutes the number of beetles exposing themselves to light was recorded. There were two treatments, an experimental treatment in which the moistened paper towel was placed on the exposed half of the petri dish, and a control treatment in which the paper towel was dry, providing no water. This procedure was repeated 10 times for each treatment.

2.2.8 Statistical Analysis

All calculations and analyses were conducted in R version 4.2.1. For each treatment, the mean response and standard deviation at each time point was calculated for visualization. A binomial generalized linear model was constructed using treatment as the independent variable for each time point to determine the probability of beetles exposing themselves to light or water. For the adult and larval phototaxis experiments, the no-shelter control treatment was designated the baseline. For the adult burrowing experiment, darkness was used as the baseline. For the adult hygroreception experiment, the hydrated beetles without a water source treatment was used as the baseline. For the comparative light and water response experiment, the no-water

control treatment was used as the baseline. After reviewing the results of the comparative light and water experiment, a second analysis was performed comparing the no-water control treatment to the white light treatment of the adult phototaxis experiment, with the white light treatment used as the baseline. Because beetles have had no time to respond to stimuli at time point zero and all responses are identical across treatments and experiments, time point zero was excluded from analysis in all experiments.

2.3 Results

2.3.1 Adult Phototaxis

Adult beetles displayed a statistically significant response to the presence of light (Fig. 2.2). In darkness, adult beetles moved between the sheltered and unsheltered halves if the petri dish, but the probability of sheltering was not significantly different from the baseline (Table 2.1). Under white light when no shelter was provided, beetles moved randomly about the dish. At minute 1 under white, blue, and red light, the probability of sheltering was not significantly different from the baseline (Table 2.1). At minute 1 under green light, the probability of sheltering was significantly greater than the baseline (Table 2.1). From minute two and onwards, the probability of hiding was significantly greater than the baseline for all light colors (Table 2.1). By minute 5, up to 80% of beetles had taken shelter from light. (Table 2.1).

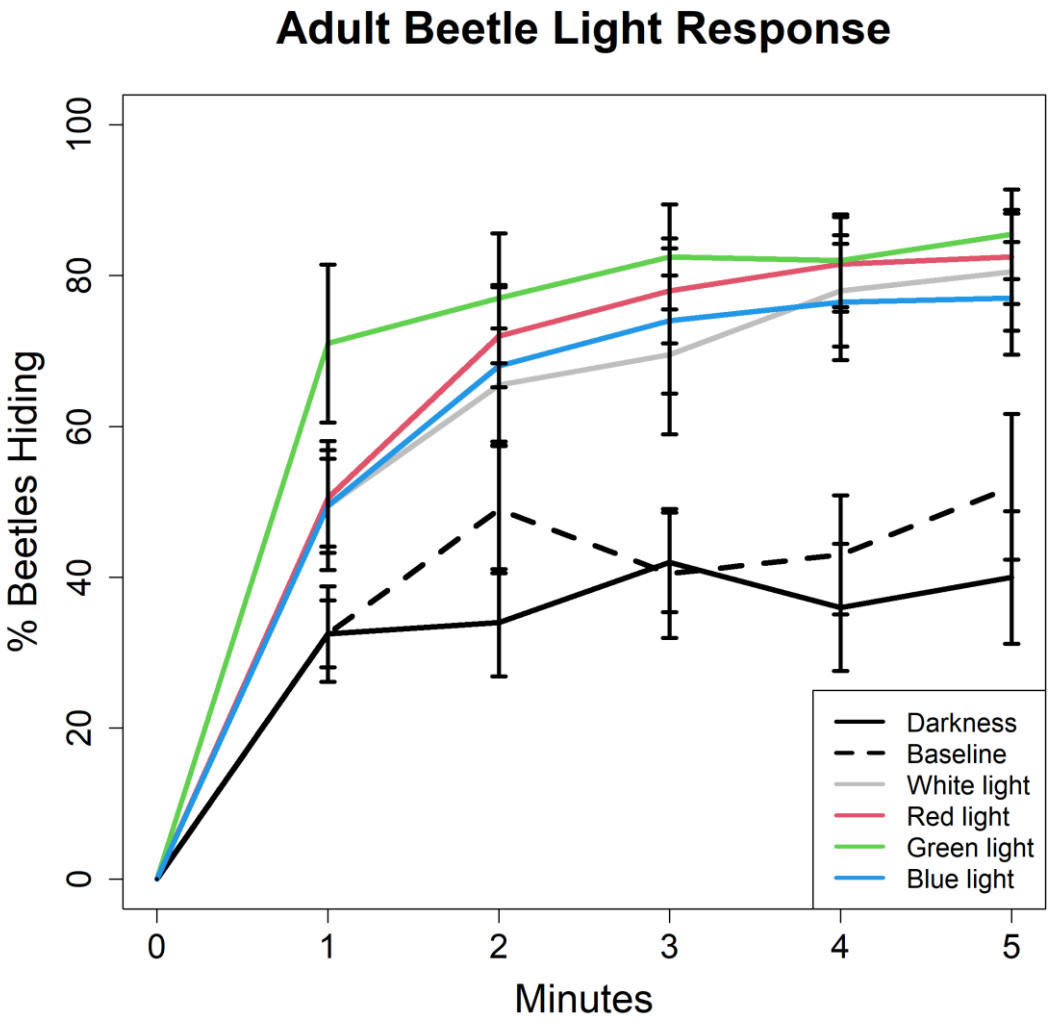

Figure 2.2- Average percentage of adult *Alphitobius diaperinus* hiding beneath shelter when exposed to different wavelengths over 5 minutes compared to a baseline of random movement (exposed to white light with no access to shelter). Error bars represent 1 standard deviation. The experiment was replicated 10 times. N per treatment per replicate per time point = 20 beetles.

Table 2.1- Probability of adult beetles taking shelter relative to the no-shelter baseline at each minute under four light wavelengths and darkness. Significant p-values in bold print.

Treatment	Minute	Probability	p-value
Darkness	1	0.50	1.00
	2	0.44	0.12
	3	0.51	0.88
	4	0.47	0.48
	5	0.44	0.19
White	1	0.57	7.91E-02
	2	0.60	**3.48E-02**
	3	0.66	**3.41E-04**
	4	0.72	**2.96E-06**
	5	0.61	**2.70E-05**
Green	1	0.70	**5.66E-06**
	2	0.69	**9.34E-05**
	3	0.77	**1.64E-08**
	4	0.76	**9.16E-08**
	5	0.77	**3.24E-07**
Blue	1	0.57	7.91E-02
	2	0.61	**1.33E-02**
	3	0.69	**1.98E-04**
	4	0.71	**9.53E-06**
	5	0.68	**3.39E-04**
Red	1	0.58	6.15E-02
	2	0.65	**2.00E-03**
	3	0.73	**9.30E-07**
	4	0.75	**1.46E-07**
	5	0.73	**5.15E-06**

2.3.2 Larval Phototaxis

Late instar larvae displayed a statistically significant response to white, green, and blue light (Fig. 2.3). In darkness and under red light, the probability of hiding was not significantly different from the baseline at any time point. Under white light, larvae had a significantly greater probability of hiding after 2 minutes (Table 2.2).

Under green and blue light, the response was slower, with a significantly greater probability of hiding after 4 minutes (Table 2.2).

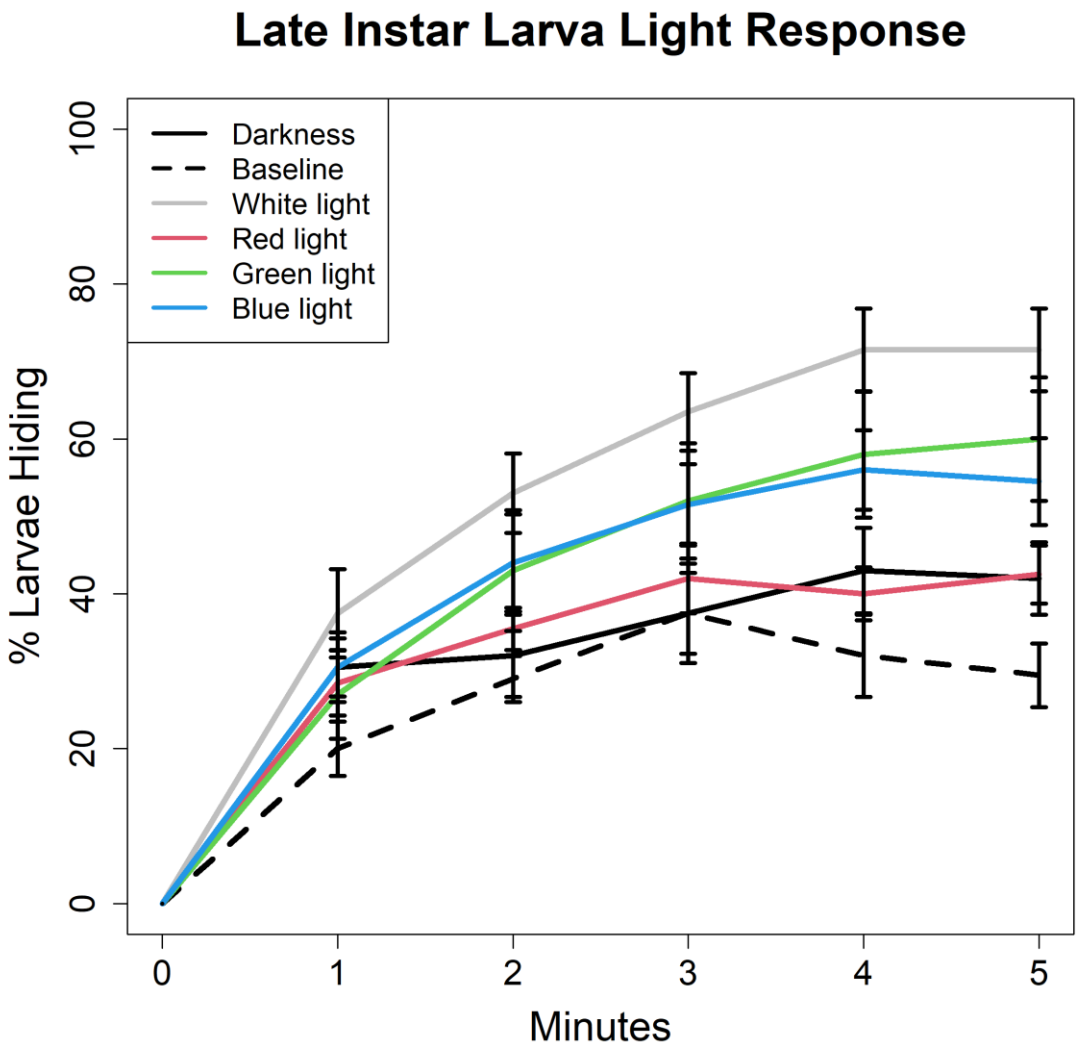

Figure 2.3- Average percentage of larval *Alphitobius diaperinus* hiding beneath shelter when exposed to different wavelengths of light over 5 minutes compared to a baseline of random movement (exposed to white light with no access to shelter). Error bars represent 1 standard deviation. The experiment was replicated 10 times. N per treatment per replicate per time point = 20 larvae.

Table 2.2- Probability of larvae taking shelter relative to the no-shelter baseline at each minute under four light wavelengths and darkness. Significant p-values in bold print.

Treatment	Minute	Probability	p-value
Darkness	1	0.54	3.58E-01
	2	0.51	7.82E-01
	3	0.50	1.00
	4	0.54	2.75E-01
	5	0.55	2.24E-01
White	1	0.56	1.13E-01
	2	0.60	**1.31E-02**
	3	0.63	**2.52E-03**
	4	0.70	**3.24E-06**
	5	0.71	**1.14E-06**
Green	1	0.52	5.47E-01
	2	0.55	1.72E-01
	3	0.57	1.12E-01
	4	0.62	**4.85E-03**
	5	0.64	**9.86E-04**
Blue	1	0.54	3.58E-01
	2	0.56	1.41E-01
	3	0.56	1.32E-01
	4	0.61	**1.02E-02**
	5	0.61	**8.97E-03**
Red	1	0.53	4.61E-01
	2	0.52	5.42E-01
	3	0.52	6.47E-01
	4	0.52	4.35E-01
	5	0.55	2.04E-01

2.3.3 Adult Burrowing

On average, 90% of beetles were burrowed into the substrate after one minute under all wavelengths of light tested (Fig. 2.4). Light wavelength had a statistically significant effect on beetle response at minute 2, but not for minutes 1, 3, and 5 (Table

2.3). At five minutes there was no significant difference in the number of beetles burrowed into the substrate for any treatment.

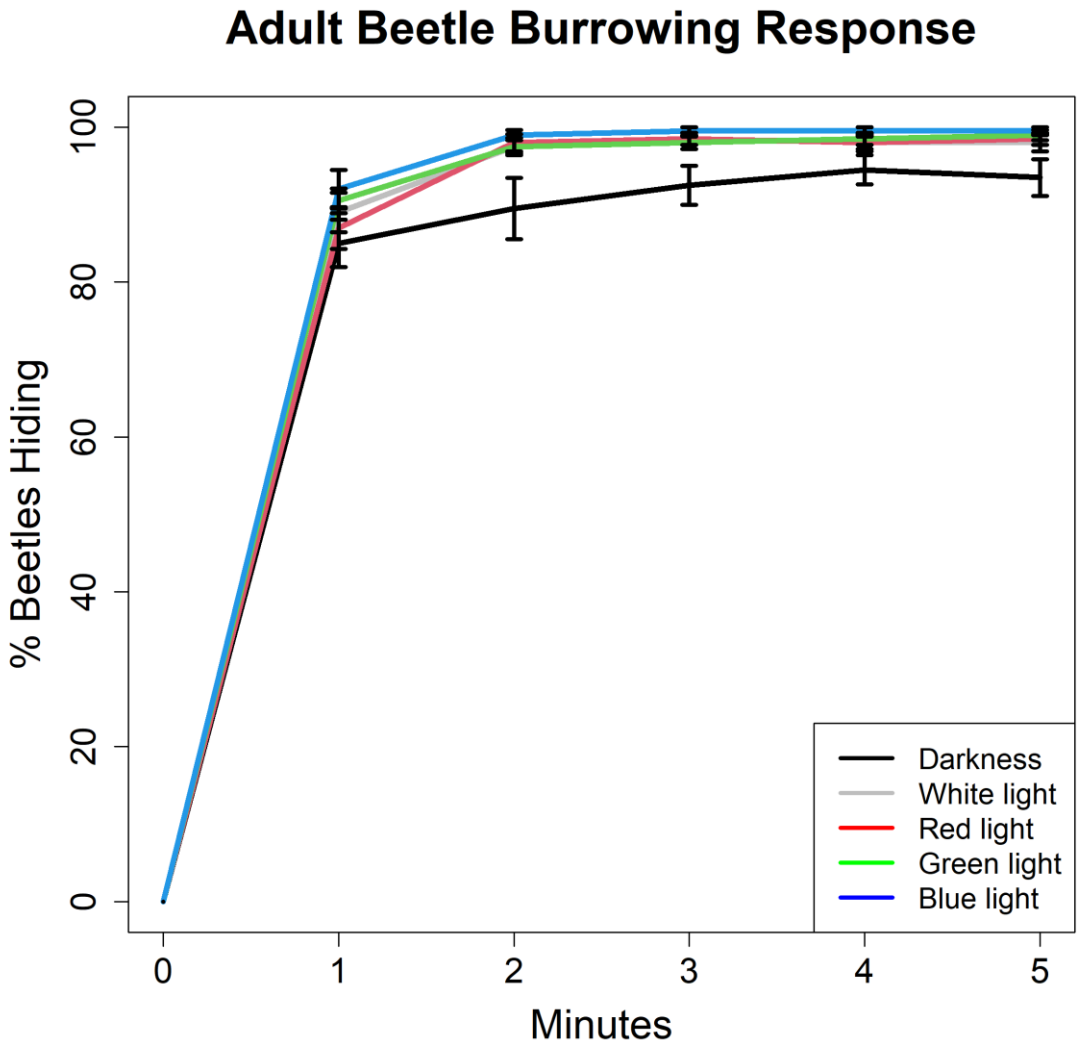

Figure 2.4- Average percentage of adult *Alphitobius diaperinus* burrowed into substrate when exposed to different wavelengths of light over 5 minutes. Error bars represent 1 standard deviation. The experiment was replicated 10 times. N per treatment per replicate per time point = 20 beetles.

Table 2.3- Probability of adult beetles burrowing into substrate relative to a baseline of darkness at each minute under four light wavelengths. Significant p-values in bold print.

Treatment	Minute	Probability	p-value
White	1	0.57	2.98E-01
	2	0.81	**4.69E-03**
	3	0.83	**1.19E-02**
	4	0.73	8.77E-02
	5	0.76	**4.23E-02**
Red	1	0.54	6.17E-01
	2	0.84	**2.79E-03**
	3	0.83	**1.19E-02**
	4	0.73	8.77E-02
	5	0.81	**2.37E-02**
Green	1	0.61	1.40E-01
	2	0.81	**4.69E-03**
	3	0.79	**2.07E-02**
	4	0.79	**4.86E-02**
	5	0.87	**1.45E-02**
Blue	1	0.65	5.33E-02
	2	0.91	**1.64E-03**
	3	0.94	**9.06E-03**
	4	0.92	**2.23E-02**
	5	0.93	**1.39E-02**

2.3.4 Adult Hygroreception

Beetles displayed a statistically significant response to the presence of water that changed with beetle condition (Fig. 2.5). From 4 minutes onwards, dehydrated beetles that were placed in an arena with a water source had a significantly greater probability to be within 2 cm of the water source than in any other treatment (Table 2.4). In contrast, hydrated beetles that were placed in an arena with a water source had a significantly higher probability of avoiding the water source compared to all other treatments for the full 30-minute duration (Table 2.4).

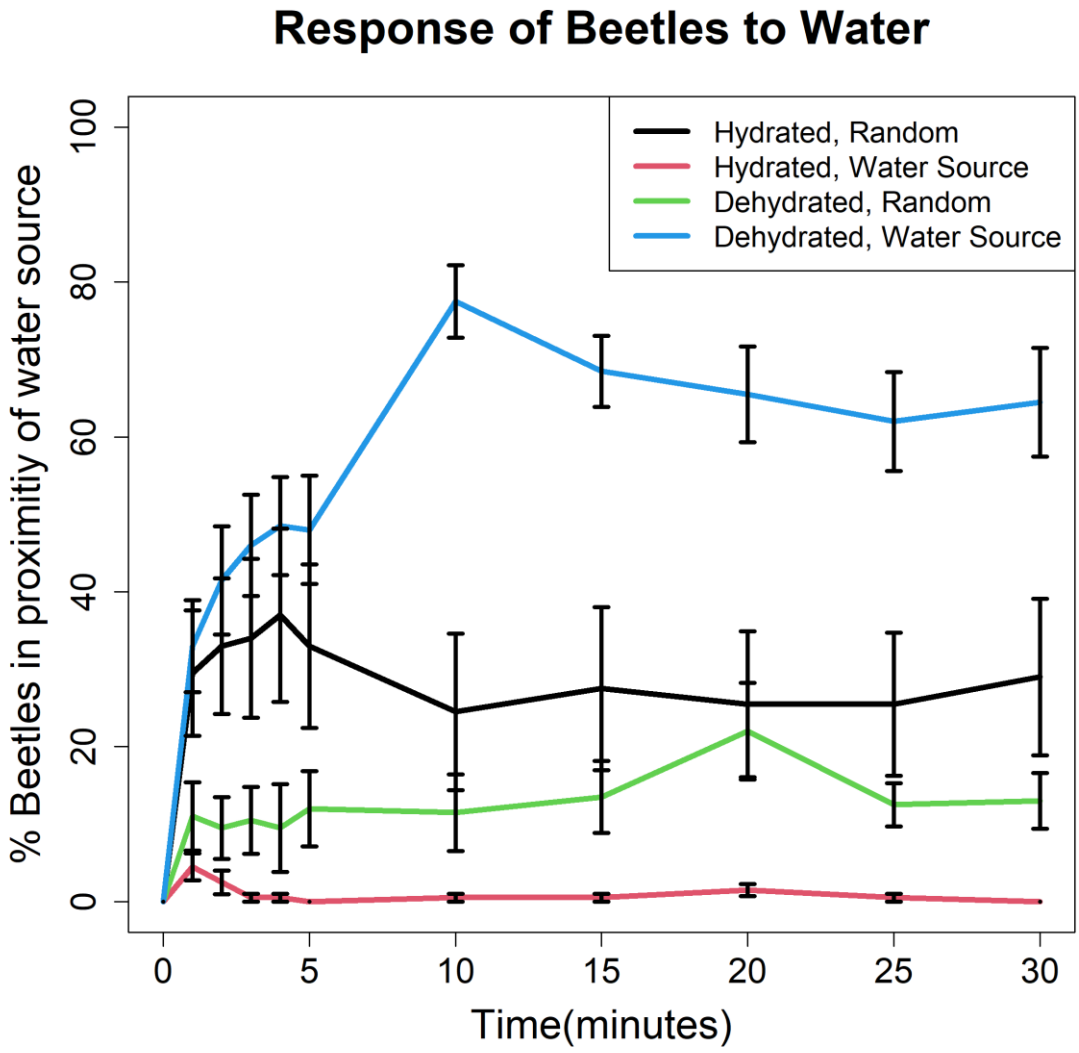

Figure 2.5- Average percentage of hydrated and dehydrated adult *Alphitobius diaperinus* moving or remaining within 2 cm of a water source over 30 minutes compared to baselines of random movement (No water present). Error bars represent 1 standard deviation. The experiment was replicated 10 times. N per treatment per replicate per time point = 20 beetles.

Table 2.4- Probability of hydrated and dehydrated beetles avoiding water relative to a baseline of random movement of hydrated beetles with no water present. Significant p-values in bold print.

Treatment	Minute	Probability	p-value
Hydrated, Water Present	1	0.84	**8.65E-05**
	2	0.97	**4.35E-04**
	3	0.94	**1.14E-04**
	4	0.97	**4.85E-04**
	5	0.97	**4.85E-04**
	10	1.00	9.92E-01
	15	1.00	9.89E-01
	20	1.00	9.89E-01
	25	1.00	9.93E-01
	30	1.00	9.93E-01
Dehydrated, Water Present	1	0.51	8.28E-01
	2	0.54	4.94E-01
	3	0.57	3.36E-01
	4	0.70	**9.00E-03**
	5	0.63	8.19E-02
	10	0.67	**1.55E-02**
	15	0.73	**1.56E-03**
	20	0.77	**3.42E-04**
	25	0.81	**6.45E-05**
	30	0.76	**1.77E-03**
Dehydrated, Water Absent	1	0.84	**8.65E-05**
	2	0.92	**4.52E-05**
	3	0.94	**1.14E-04**
	4	1.00	9.89E-01
	5	0.95	**9.61E-05**
	10	0.81	**1.29E-04**
	15	0.89	**1.38E-05**
	20	0.84	**4.26E-05**
	25	0.88	**1.06E-05**
	30	0.92	**6.99E-05**

2.3.5 Comparative Light and Water Response

When illuminated with white light, the presence of water did not have a significant effect on beetle response at any time point (Time=1, log odds=0.47, $p=5.52x10^{-1}$; Time=2, log odds=0.49, $p=7.74x10^{-1}$; Time=3, log odds=0.46, $p=3.41x10^{-1}$; Time=4, log odds=0.42, $p=4.32x10^{-2}$; Time=5, log odds=0.78, $p=7.36x10^{-1}$). However, in both treatments dehydrated beetles exposed themselves to light at a greater rate than was observed in hydrated beetles during the adult phototaxis experiment (Fig. 2.6). Analysis of the control (water absent) group compared to the results of the white light treatment from the adult phototaxis experiment showed that dehydrated beetles were significantly less likely to hide than hydrated beetles after three minutes (Time=1, log odds=0.45, $p=1.96x10^{-1}$, Time=2, log odds= 0.57, $p=7.22x10^{-2}$, Time=3, log odds= 0.59, $p=2.23x10^{-2}$, Time=4, log odds=0.60, $p=1.62x10^{-2}$, Time=5, log odds=0.69, $p=4.80x10^{-6}$).

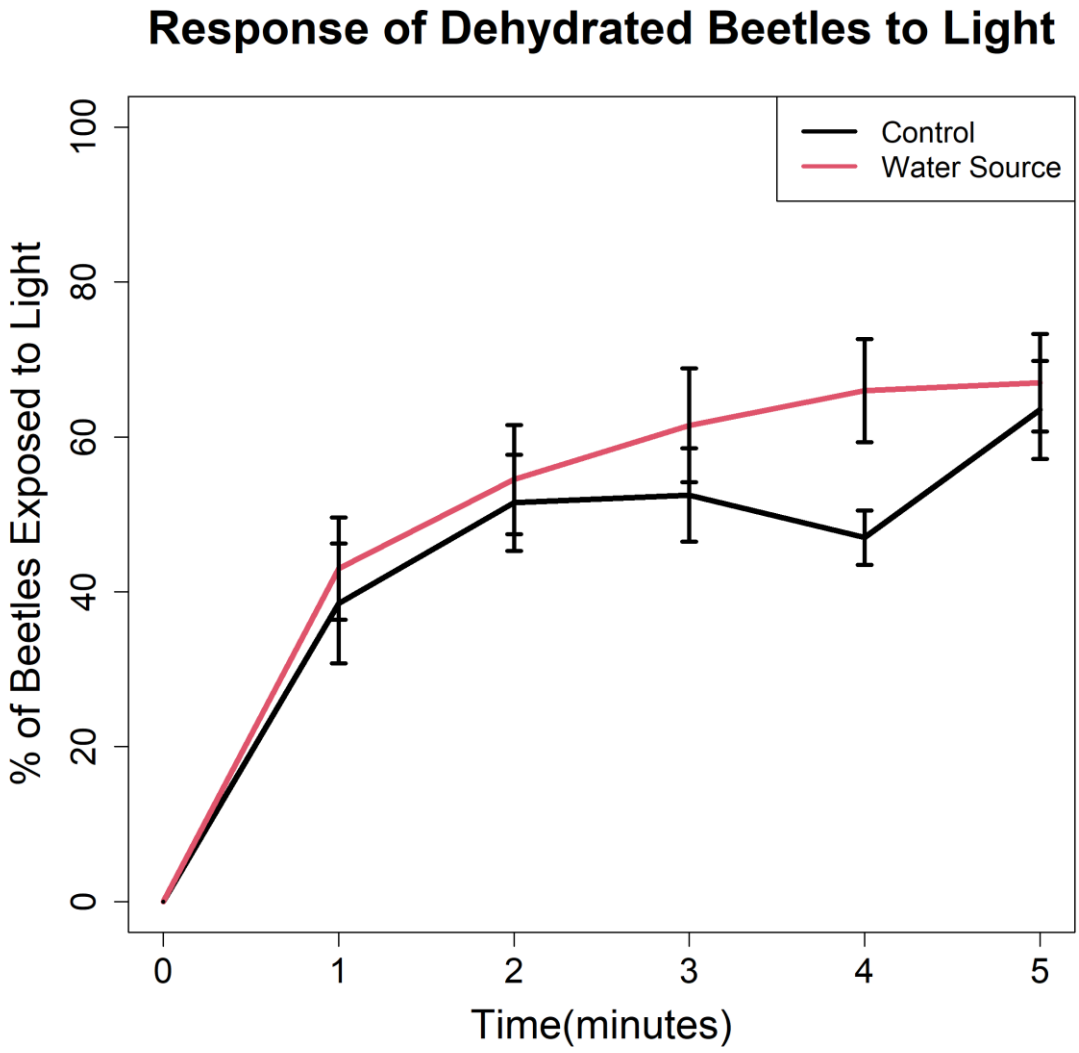

Figure 2.6- Average percentage of dehydrated adult *Alphitobius diaperinus* exposing themselves to white light with and without a water source in the illuminated section of the arena. Error bars represent 1 standard deviation. The experiment was replicated 10 times. N per treatment per replicate per time point = 20 beetles.

2.4 Discussion

The responses exhibited by adult beetles to light indicate that *Alphitobius diaperinus* exhibits negative phototaxis to all tested wavelengths of light (Fig. 2.2).

When exposed to green, blue, or red wavelengths of light, beetles hid at a greater rate than they would if moving randomly. This broadness of response is in contrast to work conducted on *Tenebrio molitor*, which has been found to respond only to green wavelengths of light (Sharkey et al. 2017). Beetle movement in darkness did not significantly differ from that of beetles that were illuminated with white light and provided with no shelter. This could suggest that the beetles do not discriminate between an open air and a covered space significantly larger than their body. Phototaxis thus appears to be a main driver of hiding behavior in adult *A. diaperinus*, but the possibility of other drivers such as tactile response cannot be discounted without further experimentation.

Late instar larvae had a different response to light compared to the adults. Overall movement from larvae was lower, even for the baseline (white light, no shelter) treatment. Larvae displayed a response to white, green, and blue light, though the response was weaker overall than that of adult beetles, and there was no significant response to red light (Fig. 2.3). This difference in behavior could be attributed to a difference in morphology: Adults beetles have more developed eyes than larvae, while the larvae have larger and more numerous setae than adults (Dunford and Kaufman 2006). It is possible that larvae are using other environmental signals, such as tactile senses, to locate and navigate to shelter in addition to photoreception. Therefore, despite exhibiting nearly identical patterns of movement and behavior within broiler houses, *A. diaperinus* adults and larvae may be using different environmental cues to drive that behavior.

When placed on a substrate that facilitated burrowing, a higher proportion of beetles took shelter within the substrate than was observed in the hard surface and

shelter trials (Fig. 2.4). In addition, the proportion of burrowing beetles was the same for all wavelengths of light, the only difference between illuminated trials and darkness being that the maximum proportion of beetles burrowing was reached faster in light than in darkness. The presence or absence of light did not increase or decrease the total number of beetles burrowing. This seems to indicate that "burrowing" and "sheltering" are two different behaviors for *A. diaperinus* adults, with different environmental drivers. Burrowing does not seem to be tied to phototaxis in a significant way. It is possible that the proportion of beetles in a population that are burrowed is a function of available substrate and density of beetles, but further testing would be required to confirm this.

Our results indicate that *A. diaperinus* exhibits both hygroreception and hygrotaxis (Fig. 2.5). However, the directionality of hygrotaxis depends on the condition of the beetles. Compared to expected random movement, dehydrated beetles exhibit an attraction to water, and hydrated beetles exhibit a repulsion from water. Hydration condition also seems to have an effect on phototactic response of beetles. During the light and hydration cross test, dehydrated beetles were more likely to expose themselves to white light compared to the hydrated beetles of the adult photoreception experiment, though the presence of water still has an effect on beetle behavior. When water was present, dehydrated beetles moved to the paper towel and remained in contact with it. When water was absent, the beetles moved aimlessly around the exposed portion of the dish, occasionally returning to the darkened half of the dish. This distinction can be seen in the fluctuation of the control treatment at minute 4 (Fig. 2.6). It is possible that severe dehydration causes adult beetles to

engage in searching behavior to seek moisture sources that overrides the repulsion of light, emboldening the beetles to venture out of shelter in search of water.

Taken together, these results indicate that adult *A. diaperinus* will hide from sufficiently intense sources of light unless they are dehydrated, and they can detect a water source. This explains why the highest densities of *A. diaperinus* can be found beneath the shelter of crusted litter and feed trays, as these locations are well sheltered from light. It also explains why exposed beetles are most likely to be seen beneath drinker lines or around recently deceased poultry, as these are rare sources of water available to the beetles. By leveraging these signals, we can improve trapping and management of *A. diaperinus* in poultry production. Due to the vital role played by light in physiological development of broilers, manipulation of light while birds are present is limited (Olanrewaju et al. 2006). Phototactic response could potentially be exploited to influence beetle movement through manipulation of lighting regimes when birds are absent between flocks, or by the creation of low, darkened spaces to concentrate beetles in locations of the grower's choosing without interfering with the exposure of broilers to light. Insecticide-treated traps could be baited with a moisture source to draw out and kill adult beetles. Alternatively, greater emphasis could be placed on drying houses prior to insecticide treatments, altering the behavior of the beetles to improve the chance of exposure to the insecticide. Yields of monitoring and research traps can be improved using water as an attractant or light in the house as a repellent, creating a push-pull system to drive beetles where a grower would prefer to have them, perhaps in conjunction with other attractants and repellents (Hassemer et al. 2019b). If a house is illuminated entirely, then intentionally sheltered areas could be created as attractants and traps for the beetles, where they can be destroyed or

removed from the house. However, we still only have a partial understanding of the internal and external factors that control the movement of *A. diaperinus*. With a more complete understanding of the environmental cues governing burrowing behavior, larval movement, and pupation site location for *A. diaperinus*, methods can be expanded to account for and manage all life stages of the pest.

Chapter 3

SURVEY OF COI DIVERSITY OF LESSER MEALWORM *ALPHITOBIUS DIAPERINUS* (COLEOPTERA: TENEBRIONIDAE) IN BROILER HOUSES IN EASTERN UNITED STATES AND EUROPE

3.1 Introduction

Agricultural insect pests arise when a species escapes from environmental population controls and balloons into quantities capable of major damage to crops and livestock, impacting grower profits. Escape from control can occur in several forms, such as access to abundant food resources not found in their natural environment or the loss of a controlling predator. One way pest species can escape from predators and access new food resources is through range expansions into agricultural settings where normal environmental controls are no longer present, becoming an outbreak pest in their new range such as in the case of the brown marmorated stinkbug *Halyomorpha halys* (Hemiptera: Pentatomidae) (Bariselli et al. 2016) or recently in the case of the spotted lanternfly *Lycorma deliculata* (Hemiptera: Fulgoridae) (Ladin et al. 2023). Understanding how insect populations have spread in the past and tracing the origins of introduced species can inform new practices for preventing further expansions and potential biological or chemical control techniques.

One method of investigating the origin and dispersal of outbreaking pest populations is the use of genetic tools and markers to infer past patterns of population movement that we can no longer observe. Sections of DNA that code for traits not subject to selection can be expected to drift over time due to mutations, creating

deviations in sequences between individuals that grow larger after long periods of reproductive isolation at a calculable rate (Tajima 1983). Cytochrome c oxidase subunit I (COI) is a mitochondrial gene that is commonly used for genetic barcoding in insects, especially beetles, because the gene is well understood and well differentiated among insect taxa. These techniques can be used to examine old, gradual changes such as the expansion of the Colorado potato beetle *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) from heterogeneous plains ecosystems to homogeneous potato monocultures (Izzo et al. 2018), or they can be used to examine recent, abrupt expansions such as the introduction of the yellow legged hornet *Vespa velutina* (Hymenoptera: Vespidae) from a single population in China to Korea and France (Arca et al. 2015). Using analysis of COI and microsatellite markers, these studies identified the likely genetic origins of populations in the expanded range and the path they took to their current range.

Alphitobius diaperinus is a generalist bird nest associate that has expanded into the anthropogenic environment of deep-litter broiler (chickens raised for meat) houses worldwide (Hopkins, 1990). It has become a pest of the poultry industry, causing damage to both livestock and structures (Despins and Axtell 1995, Aalbu et al. 2002, Fairchild et al. 2005). While the likely origin of the species is known, the details of how and when the species spread to poultry facilities across the world is has not been investigated. The full mitochondrial genome of *A. diaperinus* has been sequenced (Hong et al. 2020), but no survey of genetic diversity has been conducted. This preliminary study sampled broiler house populations of *A. diaperinus* from several locations in the Eastern United States and Europe and sequenced a section of the COI gene to survey the genetic diversity of *A. diaperinus* populations and explore the

potential for genetic tools to be used to investigate patterns of genetic differentiation and population dispersal.

3.2 Materials and Methods

3.2.1 Beetle Sampling

Specimens were sampled from 11 locations in the United States and Europe: two farms in Delaware, USA, two farms in Maryland, USA, one farm in Georgia, USA, three farms in Alabama, USA, one colony from Italy, one colony from the Czech Republic, and one colony from Turkey. Delaware samples were collected in the summer of 2022. 30 adult beetles were collected from each house on the two farms (4 houses on DE-Farm-1, 2 houses on DE-Farm-2, total 180 beetles). Maryland samples were collected from two laboratory colonies established from two farms in Maryland. 20 adult beetles were collected from the MD-Farm-1 colony and 19 adult beetles were collected from the MD-Farm-2 colony (39 beetles total). MD-Farm-1 beetles had been kept in colony for seven months prior to collection, while MD-Farm-2 beetles had been kept in colony for nine months prior to collection. Georgia samples were collected in the spring of 2022. 43 adult beetles were collected from a single farm. Alabama samples were collected in the spring of 2022. Over 30 beetles were collected from each house (2 houses on AL-Farm-1, 1 house on AL-Farm-2, 1 house on AL-Farm-3). Italy, Czech Republic, and Turkey samples were collected in the spring of 2021. 30 *A. diaperinus* larvae were collected from Italy, 15 larvae were collected from the Czech Republic, and 30 larvae were collected from Turkey. Italian beetles were maintained in a laboratory colony for three years prior to collection. Turkish beetles were maintained in a laboratory colony for five years prior to collection. Czech

Republic beetles were maintained in a laboratory colony for three months prior to collection.

3.2.2 DNA Extraction and Amplification

DNA was extracted from samples using a Qiagen DNEasy Blood and Tissue Kit (Germantown, Maryland, USA). DNA was extracted from 20 individuals from each source, except for the MD-Farm-2 sample which had only 19 individuals available and the Czech Republic sample, which had only 15 individuals available. DNA was thus extracted from a total of 314 *A. diaperinus* individuals. The cytochrome oxidase subunit I gene was then amplified using a touchdown polymerase chain reaction using paired primers: Pat (5'-TCCAATGCACTAATCTGCCATATTA-3') and Jerry (5'-CAACATTTATTTTGATTTTTTGG-3') to obtain forward and reverse sequences for COI (Simons et al. 1994). The touchdown PCR protocol was as follows: Denaturation at 94°C for 2 minutes; 25 step-down PCR cycles, each consisting of denaturation at 94°C for 30 seconds, followed by annealing for 30 seconds at 55°C reduced by -0.4°C each cycle, and elongation at 72°C for 90 seconds. 15 low-hold PCR cycles, each consisting of denaturation at 94°C for 30 seconds, annealing at 45°C for 30 seconds, and elongation at 74°C for 90 seconds, adding 3 seconds each cycle; elongation at 72°C for 7 minutes. Amplified sequences were sent to Eurofins Genomics (Louisville, Kentucky, USA) for Sanger sequencing.

3.2.3 Analysis

Successful sequence pairs were aligned in Geneious Prime (Biomatters Limited 2023) and ambiguous sites corrected by inspection of read signal strength. Consensus sequences for all samples were combined in a multiple sequence alignment

in Geneious and trimmed to the shortest sequence to achieve a uniform length of 515 nucleotides. The sequences were confirmed to be *A. diaperinus* COI via a nucleotide BLAST search in the National Institute for Biotechnology Information database. After sequences were organized, rarefaction analysis of the accumulation of haplotypes at each site with increased sampling was performed using the "vegan" package in R (R Core Team 2021, Oksanen et al. 2022).

Consensus sequences for all samples were aligned and the ends of the sequences trimmed to the length of the shortest sequence, resulting in a final sequence length of 515 base pairs. A haplotype network was constructed using POPart (Leigh and Bryant 2015). Haplotype diversity (h) and nucleotide diversity (π) were calculated for the overall sequences and each site using the "pegas" package in R (Paradis 2010, R Core Team 2021). Haplotype diversity is a measure of the number of different haplotypes in a population calculated as

$$h = n(1 - \sum_{i=1}^{l} x_i^2)/(n-1)$$

where n is sample size and x_i is the sample frequency of the ith haplotype. Nucleotide diversity is a measure of how different haplotypes are from each other in a population calculated as

$$\pi = \sum_{ij} \left(\frac{\pi_{ij}}{l}\right) / \left(\frac{n(n-1)}{2}\right)$$

where π_{ij} is the number of differences between the ith and jth sequences and n is the total number of sequences (Nei 1987). Tajima's D was calculated for all regions using Arlequin 3.5.2.2 (Excoffier and Lischer 2010). Tajima's D is a neutrality statistic that indicates whether there are a greater or fewer number of rare haplotypes in a

population than would be expected under neutral selection, calculated as the standardization of *d*, calculated as

$$D = \frac{d}{\sqrt{V(d)}} \quad d = \pi - \frac{S}{\sum_{i=1}^{n-1} \frac{1}{i}}$$

where $V(d)$ is the variance of d, π is the observed nucleotide diversity, S is the number of segregating sites, and n is the number of sequences.

3.3 Results

We collected 267 sequences across all sites and found 12 haplotypes with 11 polymorphic sites. Haplotype 1 (H1) was found at each location and was either the dominant or only haplotype at each location (Fig 3.1). All other haplotypes were represented in only a single population. Haplotypes 2, 3, 4, 5, 6 ,7, 8, 10, and 11 differed from haplotype 1 by one nucleotide. Haplotypes 9 and 12 differed from haplotype 1 by two nucleotides. Rarefaction analysis of the accumulation of haplotypes at each site suggested there would be minimal increase in observed diversity from further sampling, except in the case of DE-Farm-2 house 1 (Fig 3.2). The number of polymorphic sites ranged from 0 to 4. Nucleotide diversity ranged from 0 to 0.001, and haplotype diversity ranged from 0 to 0.462, with the highest overall diversity observed in DE-Farm-2 house 1. Tajima's D was negative for all sites with more than one haplotype but was not significantly outside the expected range (2 to -2) for a neutrally evolving population for any location (Table 3.1). When calculated for all samples as a single population Tajima's D was -2.11, below the expected range of neutrality, indicating a greater proportion of rare haplotypes than expected given the number of polymorphic sites.

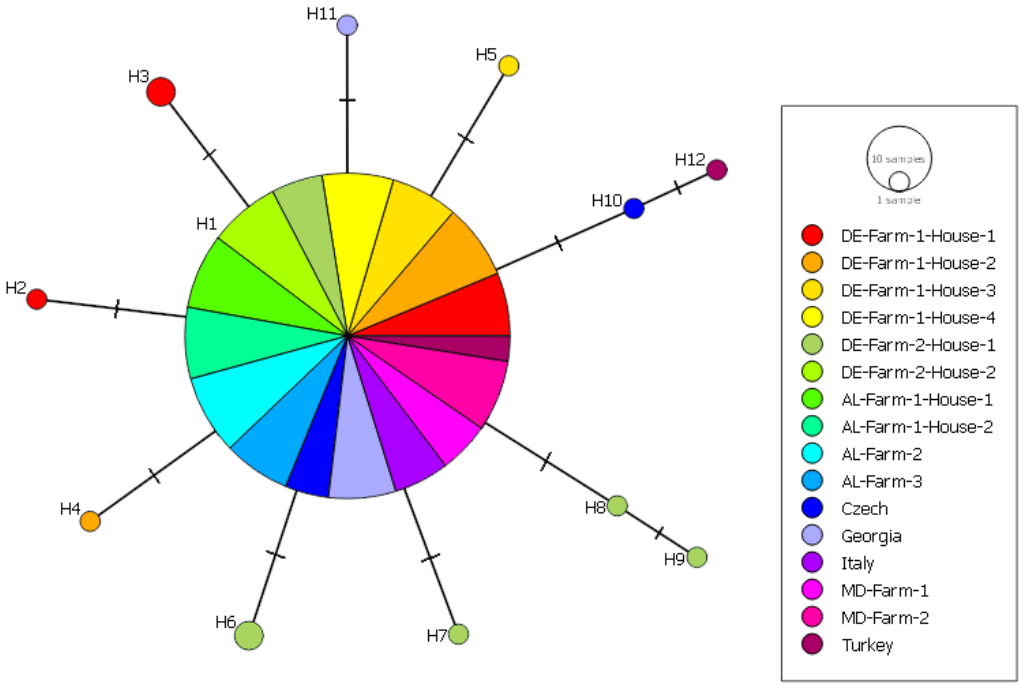

Figure 3.1- Haplotype network of COI sequences of *A. diaperinus* in the eastern United States and Europe. Each node represents a haplotype. Node size represents the number of individuals with the haplotype. Each tick on a line represents a nucleotide difference between connected haplotypes.

Table 3.1 Diversity statistics for sampled populations of *A. diaperinus* from the Eastern United States and Europe.

Region	Population	*N*	No. of polymorphic sites	No. of haplotypes	Haplotype diversity (h)	Nucleotide diversity (π)	Tajima's D
All		267	11	12	0.102	0.0002	-2.11*
USA, Delaware	Farm 1 House 1	19	2	3	0.292	0.0006	-1.12
USA, Delaware	Farm 1 House 2	20	1	2	0.1	0.0002	-1.16
USA, Delaware	Farm 1 House 3	18	1	2	0.216	0.0002	-1.16
USA, Delaware	Farm 1 House 4	18	0	1	0	0	
USA, Delaware	Farm 2 House 1	18	4	5	0.462	0.001	-1.35
USA, Delaware	Farm 2 House 2	18	0	1	0	0	0
USA, Alabama	Farm 1 House 1	19	0	1	0	0	0
USA, Alabama	Farm 1 House 2	18	0	1	0	0	0
USA, Alabama	Farm 2	20	0	1	0	0	0
USA, Alabama	Farm 3	17	0	1	0	0	0
USA, Georgia	-	18	1	2	0.111	0.0002	-1.16
USA, Maryland	Farm 1	18	0	1	0	0	0
USA, Maryland	Farm 2	13	0	1	0	0	0
Italy	-	14	0	1	0	0	0
Turkey	-	7	2	2	0.286	0.0011	-1.24
Czech Republic	-	12	1	2	0.167	0.0003	-1.45

*Value of Tajima's D was statistically significantly different from zero. All other values were statistically indistinguishable from zero.

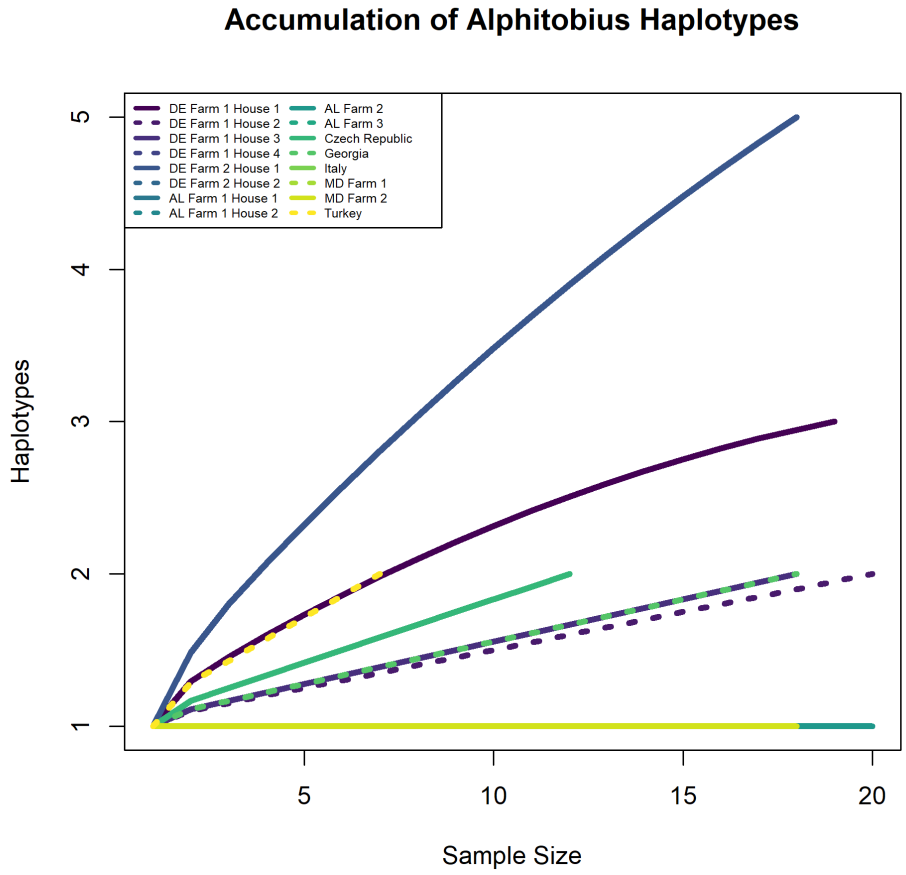

Figure 3.2- Rarefaction curves of the accumulation of haplotypes with increasing sampling of *A. diaperinus* populations.

3.4 Discussion

The overall diversity of COI in broiler house *A. diaperinus* populations was very low. All locations were dominated by the same haplotype despite being separated by hundreds of kilometers (Fig 3.3). Remarkably, the same haplotype was also dominant among sampled European populations (Fig. 3.4). No other haplotypes were shared between populations, even between houses on the same farm, and all haplotypes deviated from the dominant haplotype by no more than two nucleotides

(Fig 3.1). From the rarefaction results we feel confident that our sampling from United States populations provides representative samples of the diversity of each site (Fig 3.2). Rarefaction analysis suggested that there would be minimal return from further sampling of the European populations, but increasing the number of countries and farms visited might still reveal additional unique haplotypes.

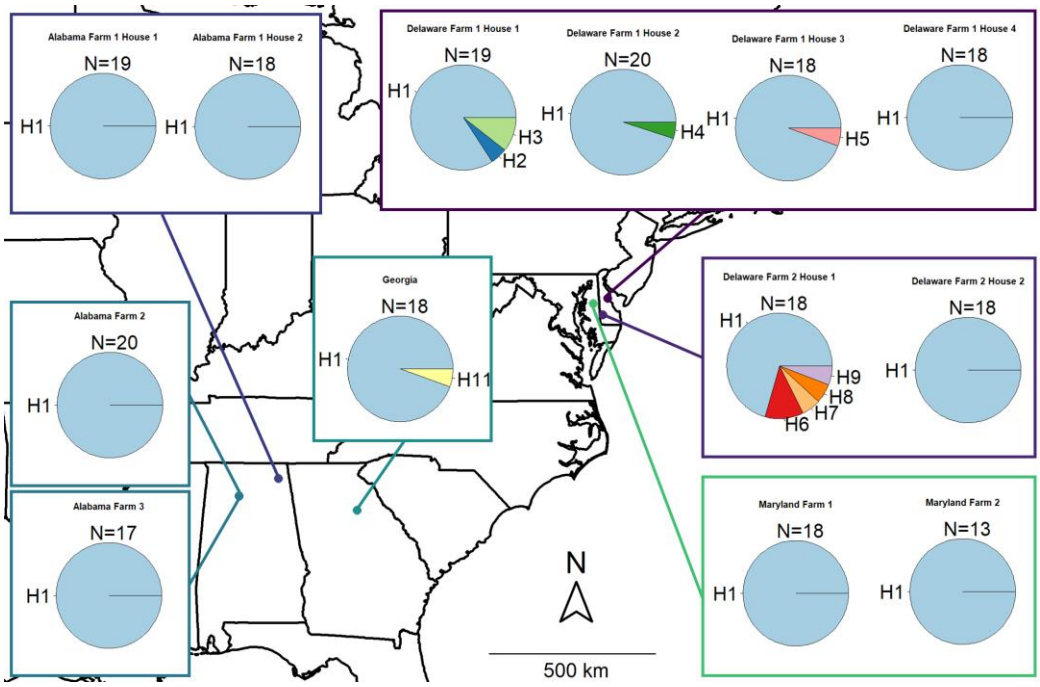

Figure 3.3- Map of haplotype frequencies of *A. diaperinus* populations sampled from farms in the Eastern USA.

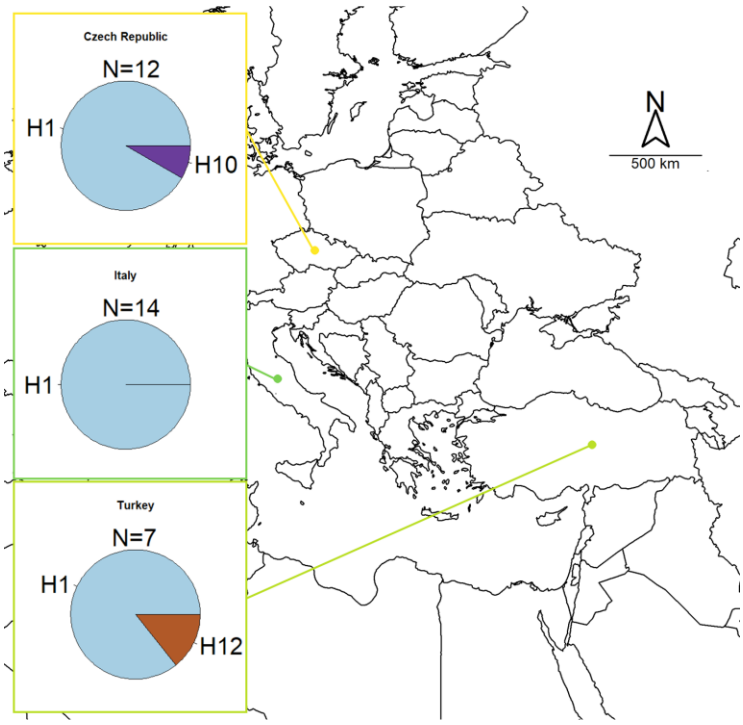

Figure 3.4- Map of haplotype frequencies of *A. diaperinus* populations sampled from farms in Europe.

These results imply that there is a high degree of genetic homogeneity in *A. diaperinus* populations. The homogeneity of *A. diaperinus* has multiple potential explanations. The prevalence of a single haplotype across locations could suggest that among wild populations of *A. diaperinus* existing as nest associates in their original ancestral range of Sub-Saharan Africa (Arshad et al. 1984), only a small number of individuals were successfully transported and ultimately established in commercial poultry environments, resulting in a major bottleneck event. This is supported by the overall Tajima's D value of -2.11 (Table 3.1), which we would expect to see in the case of population expansion following a bottleneck event. This pattern would resemble the distribution of haplotypes observed in *Vespa velutina*, where 11

haplotypes were observed in the native range but only one haplotype was observed in each invasive range (Arca et al. 2015). However, sampling of wild populations in the ancestral range of *A. diaperenis* is needed to confirm this.

A bottleneck event for broiler house *A. diaperinus* is further supported by the historical context of commercial poultry production. Mass rearing of chickens for meat production is a very recent practice. Prior to World War II, chickens were raised primarily for egg production, with only expired hens and culled roosters being sold for meat (Williams 1998). These chickens would be kept in small numbers in coops, often maintained alongside a variety of other livestock and crops. By the 1940's, however, a confluence of cultural and economic factors created high demand for chicken meat that propelled the growth of mass broiler production in the Delmarva peninsula (Delaware, Maryland, Virginia, USA) and eventually other hotbeds of poultry production across the USA (Williams 1998). These commercial broiler houses might contain 36,000-52,000 chickens and accumulate deep layers of plant-based litter mixed with chicken manure (Brothers 2022). So-called "deep-litter" broiler houses only came into being with the massive intensification of production that came with rearing chickens for sale as meat, evident by a steep decline in the number of farms rearing chickens simultaneous with an increase in the numbers of chickens being produced according to the USDA Census of Agriculture (USDA NASS 2019). (Fig 3.5). These deep-litter, insulated houses created a suitable environment for *A. diaperinus* in regions where the climate would otherwise be too cold for year-round survival (Wilson and Miner 1969). Though early records are scarce, by 1964 *A. diaperinus* was already noted as being an established pest of poultry houses and a frequently intercepted species in European grain imports (Freeman 1964). Ultimately,

this suggests that *A. diaperinus* has been present in United States poultry houses for less than 100 years, potentially colonizing them from grain storage facilities, which could explain the lack of differentiation among populations.

Further study could answer several remaining questions about the origin of *A. diaperinus*. Sampling and sequencing of COI or other genes in wild populations of *A. diaperinus* not associated with poultry production could distinguish whether the homogeneity of broiler house populations is representative of the species or the product of a bottleneck resulting from a subset of the population transitioning to anthropogenic settings. Sampling of populations from stored grain facilities, where *A. diaperinus* is also a pest, may provide insight into how genetic diversity of the species changed, if at all, as it moved between anthropogenic settings. More extensive sampling of European populations may also reveal diversity not captured by this study. However, while there is clear potential for genetic tools to illuminate details of the origin of *A. diaperinus*, we caution that the homogeneity displayed in COI of broiler house populations might also be evident in the nuclear genome, which could stymie efforts to parse recent changes and gene flow amongst *A. diaperinus* populations.

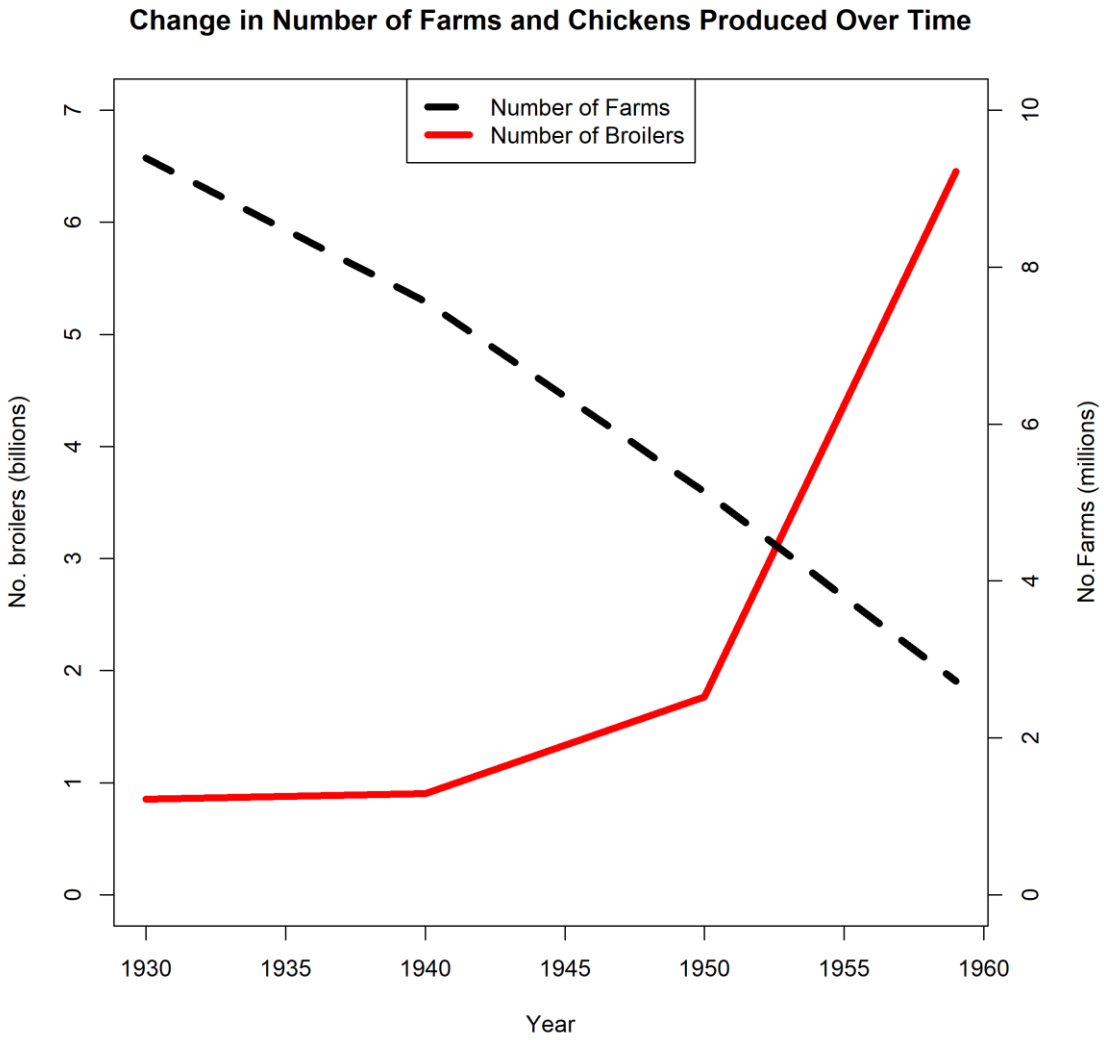

Figure 3.5- The change in the number of farms producing chickens (right axis, dashed black line) and the number of broilers produced (left axis, solid red line) between 1930 and 1959 as tracked by the USDA Census of Agriculture.

REFERENCES

Aalbu, R. L., C. A. Triplehorn, J. M. Campbell, K. W. Brown, Somerby R. A, and D. B. Thomas. 2002. Family 106. Tenebrionidae Latreille 1802, pp. 463–509. *In* Arnett Jr, R.H., Thomas, M.C., Skelley, P.E., Frank, J.H. (eds.), American Beetles Volume 2 Polyphaga: Scarabaeoidea through Curculionoidea. CRC Press, Boca Raton, FL.

Amir, A., and A. Nadir. 2009. Spatial distribution and population fluctuation of *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) during a flock cycle raised in a broiler house in the North-East of Algeria. Academic Journal of Entomology. 2: 88–91.

Arca, M., F. Mougel, T. Guillemaud, S. Dupas, Q. Rome, A. Perrard, F. Muller, A. Fossoud, C. Capdevielle-Dulac, M. Torres-Leguizamon, X. X. Chen, J. L. Tan, C. Jung, C. Villemant, G. Arnold, and J. F. Silvain. 2015. Reconstructing the invasion and the demographic history of the yellow-legged hornet, *Vespa velutina*, in Europe. Biol Invasions. 17: 2357–2371.

Arshad, M., G. A. Jan, and A. Khattack. 1984. Insect fauna of birds' nests of N.W.F.P. Bulletin of Zoology Pakistan. 2: 1–7.

Bariselli, M., R. Bugiani, and L. Maistrello. 2016. Distribution and damage caused by *Halyomorpha halys* in Italy. EPPO Bulletin. 46: 332–334.

Barker, K. J., C. D. Coufal, J. L. Purswell, J. D. Davis, H. M. Parker, M. T. Kidd, C. D. McDaniel, and A. S. Kiess. 2013. In-house windrowing of a commercial broiler farm during early spring and its effect on litter composition. Journal of Applied Poultry Research. 22: 551–558.

Bartelt, R. J., B. W. Zilkowski, A. A. Cossé, C. D. Steelman, and N. Singh. 2009. Male-produced aggregation pheromone of the lesser mealworm beetle, *Alphitobius diaperinus*. J Chem Ecol. 35: 422–434.

Biomatters Limited. 2023. Geneious Prime 2023.1.2 (https://www.geneious.com).

Boozer, W. E. 2011. Insecticide susceptibility of the adult darkling beetle, *Alphitobius diaperinus* (Coleoptera: Tenebrionidae): topical treatment with bifenthrin, imidacloprid, and spinosad.

Brothers, D. 2022. New Farmer's Guide to the Commercial Broiler Industry: Poultry Husbandry & Biosecurity Basics. Extension: Alabama A&M $ Auburn Universities.

Calibeo-Hayes, D., S. S. Denning, S. M. Stringham, and D. W. Watson. 2005. Lesser mealworm (Coleoptera: Tenebrionidae) emmergence after mechanical incorporation of poultry litter into field soils. J Econ Entomol. 98: 229–235.

Camin, J. H., and R. W. Drenner. 1978. Climbing Behavior and Host-Finding of Larval Rabbit Ticks (*Haemaphysalis leporispalustris*). J Parasitol. 64: 905–909.

Chernaki-Leffer, A. M., L. M. Almeida, D. R. Sosa-Gómez, A. Anjos, and K. M. Vogado. 2007. Populational fluctuation and spatial distribution of *Alphitobius*

diaperinus (Panzer) (Coleoptera; Tenebrionidae) in a poultry house, Cascavel, Parana state, Brazil. Brazilian Journal of Biology. 67: 209–213.
Crippen, T. L., B. Singh, R. C. Anderson, and C. L. Sheffield. 2022. Adult *Alphitobius diaperinus* microbial community during broiler production and in spent litter after stockpiling. Microorganisms. 10.
Crook, P. G., J. A. Novak, and T. J. Spilman. 1980. The lesser mealworm, *Alphitobius diaperinus*, in the scrotum of *Rattus norvegicus*, with notes on other vertebrate associations (Coleoptera, Tenebrionidae; Rodentia, Muridae). Coleopt Bull. 34: 393.
Despins, J. L., and R. C. Axtell. 1994. Feeding behavior and growth of turkey poults fed larvae of the darkling beetle, *Alphitobius diaperinus*. Poult Sci. 73: 1526–1533.
Despins, J. L., and R. C. Axtell. 1995. Feeding behavior and growth of broiler chicks fed larvae of the darkling beetle, *Alphitobius diaperinus*. Poult Sci. 74: 331–336.
Dunford, J. C., and P. E. Kaufman. 2006. Lesser mealworm, litter beetle, *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae). Featured Creatures. (https://entnemdept.ufl.edu/creatures/livestock/poultry/lesser_mealworm.htm).
Eidson, C. S., S. C. Schmittle, R. B. Goode, and J. B. Lal. 1966. Induction of leukosis tumors with the beetle *Alphitobius diaperinus*. Am J Vet Res. 27: 1053–1057.
EPA. 2021. Tolerance Revocations: Chlorpyrifos.
Excoffier, L., and H. E. L. Lischer. 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Resour. 10: 564–567.
Fairchild, B., M. Czarick, and N. Hinkle. 2005. Poultry housing tips: darkling beetles... costs and control.
Francikowski, J., B. Baran, M. Cup, J. Janiec, and M. Krzyżowski. 2019. Commercially available essential oil formulas as repellents against the stored-product pest *Alphitobius diaperinus*. Insects. 10.
Freeman, J. A. 1964. Stored-products entomology in Britain, 1964. Annals of Applied Biology. 53: 200–215.
Geden, C. J., J. J. Arends, and R. C. Axtell. 1987. Field trials of *Steinernema feltiae* (Nematoda: Steinernematidae) for control of *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) in commercial broiler and turkey houses. J Econ Entomol. 80: 136–141.
Global Animal Partnership. 2020. 5-step animal welfare standards for chickens raised for meat v3.2. Global Animal Partnership.
Goodwin, M. A., and W. D. Waltman. 1996. Transmission of eimeria, viruses, and bacteria to chicks: darkling beetles (*Alphitobius diaperinus*) as vectors of pathogens. Journal of Applied Poultry Research. 5: 51–55.
Hamm, R. L., P. E. Kaufman, C. A. Reasor, D. A. Rutz, and J. G. Scott. 2006. Resistance to cyfluthrin and tetrachlorvinphos in the lesser mealworm, *Alphitobius diaperinus*, collected from the eastern United States. Pest Manag Sci. 62: 673–677.

Harbach, R. E., and J. R. Larsek. 1977. Humidity behavior and the identification of hygroreceptors in the adult mealworm Tenebrio Molitor. J. Insect Physiol. 23: 1121–1134.

Hassemer, M. J., M. Borges, D. M. Withall, J. A. Pickett, R. A. Laumann, M. A. Birkett, and M. C. Blassioli-Moraes. 2019a. Development of pull and push–pull systems for management of lesser mealworm, *Alphitobius diaperinus*, in poultry houses using alarm and aggregation pheromones. Pest Manag Sci. 75: 1107–1114.

Hassemer, M. J., M. Borges, D. M. Withall, J. A. Pickett, R. A. Laumann, M. A. Birkett, and M. C. Blassioli-Moraes. 2019b. Development of pull and push–pull systems for management of lesser mealworm, *Alphitobius diaperinus*, in poultry houses using alarm and aggregation pheromones. Pest Manag Sci. 75: 1107–1114.

Hassemer, M. J., R. B. Lopes, M. Borges, L. F. A. Alves, D. M. Withall, J. A. Pickett, R. A. Laumann, M. A. Birkett, and M. C. Blassioli-Moraes. 2020. Development of an attract-and-infect device for biological control of lesser mealworm, *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) in poultry houses. Biological Control. 149.

Hazeleger, W. C., N. M. Bolder, R. R. Beumer, and W. F. Jacobs-Reitsuma. 2008. Darkling beetles (*Alphitobius diaperinus*) and their larvae as potential vectors for the transfer of *Campylobacter jejuni* and *Salmonella enterica* serovar paratyphi B variant java between successive broiler flocks. Appl Environ Microbiol. 74: 6887–6891.

Hickmann, F., A. F. De Morais, E. S. Bronzatto, T. Giacomelli, J. V. C. Guedes, and O. Bernardi. 2018. Susceptibility of the lesser mealworm, *Alphitobius diaperinus* (Coleoptera: Tenebrionidae), from broiler farms of southern Brazil to insecticides. J Econ Entomol. 111: 980–985.

Hinchey, F. 1997. Bugged residents sue over beetles. The Columbus Dispatch. 1-2A.

Holmes, S. J. 1911. The Reactions of mosquitoes to light in different periods of their life history. Journal of Animal Behavior. 1: 29–32.

Hong, K. J., W. Ki, D.-S. Park, B.-K. Yang, H. Lee, J. Park, and W. Lee. 2020. The complete mitochondrial genome of *Alphitobius diaperinus* Panzer, 1797 (Coleoptera: Tenebrionidae). Mitochondrial DNA Part B. 5: 2291–2293.

Hopkins, J. D., C. D. Steelman, and C. E. Carlton. 1992. Anatomy of the adult female lesser mealworm *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) reproductive system. J Kans Entomol Soc. 65: 299–307.

Hopkins, J. W. 1990. A study of the reproductive anatomy and distribution of the adult male and female lesser mealworm *Alphitobius diaperinus* (Panzer) (Coleoptera:Tenebrionidae).

Izzo, V. M., Y. H. Chen, S. D. Schoville, C. Wang, and D. J. Hawthorne. 2018. Origin of Pest Lineages of the Colorado Potato Beetle (Coleoptera: Chrysomelidae). J Econ Entomol. 111: 868–878.

Karen, D., B. Grogan, and J. J. Arends. 2018. Darkling beetles and their economic impact on production.

Kaufman, P. E., C. Reasor, K. D. Murray, J. K. Waldron, and D. A. Rutz. 2005. Evaluation of a barrier to inhibit lesser mealworm (Coleoptera: Tenebrionidae) and Dermestidae movement in high-rise, caged-layer poultry facilities. J Econ Entomol. 98: 1744–1749.

Kaufman, P. E., C. Reasor, J. K. Waldron, and D. A. Rutz. 2005. Suppression of adult lesser mealworm (Coleoptera: Tenebrionidae) using soil incorporation of poultry manure. J Econ Entomol. 98: 1739–1743.

Kaufman, P. E., C. Strong, and D. A. Rutz. 2008. Susceptibility of lesser mealworm (Coleoptera: Tenebrionidae) adults and larvae exposed to two commercial insecticides on unpainted plywood panels. Pest Manag Sci. 64: 108–111.

Kozaki, T., B. A. Kimmelblatt, R. L. Hamm, and J. G. Scott. 2008. Comparison of two acetylcholinesterase gene cDNAs of the lesser mealworm, *Alphitobius diaperinus*, in insecticide susceptible and resistant strains. Arch Insect Biochem Physiol. 67: 130–138.

Kucharska, K., B. Zajdel, E. Pezowicz, J. Jarmul-Pietraszczyk, A. Mazurkiewicz, and D. Tumialis. 2016. Control of the lesser mealworm *Alphitobius diaperinus* using entomopathogenic nematodes combined with nanoparticles. Animal Science. 55: 57–67.

Ladin, Z. S., D. A. Eggen, T. L. E. Trammell, and V. D'Amico. 2023. Human-mediated dispersal drives the spread of the spotted lanternfly (*Lycorma delicatula*). Sci Rep. 13.

Lambkin, T. A. 2005. Baseline responses of Adult *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) to fenitrothion and susceptibility status of populations in Queensland and New South Wales, Australia. J Econ Entomol. 98: 938–942.

Lambkin, T. A., and M. J. Furlong. 2011. Metabolic mechanisms only partially explain resistance to pyrethroids in Australian broiler house populations of lesser mealworm (Coleoptera: Tenebrionidae). J Econ Entomol. 104: 629–635.

Lambkin, T. A., and M. J. Furlong. 2014. Application of spinosad increases the susceptibility of insecticide-resistant *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) to pyrethroids. J Econ Entomol. 107: 1590–1598.

Lambkin, T. A., R. A. Kopittke, S. J. Rice, J. S. Bartlett, and M. P. Zalucki. 2007. Distributions of lesser mealworm (Coleoptera: Tenebrionidae) in litter of a compacted earth floor broiler house in subtropical Queensland, Australia. J Econ Entomol. 100: 1136–1146.

Lambkin, T. A., R. A. Kopittke, S. J. Rice, J. S. Bartlett, and M. P. Zalucki. 2008. Factors affecting localized abundance and distribution of lesser mealworm in earth-floor broiler houses in subtropical Australia. J. Econ. Entomol. 101: 61–67.

Lambkin, T. A., and S. J. Rice. 2006. Baseline responses of *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) to cyfluthrin and detection of strong resistance in field populations in eastern Australia. J Econ Entomol. 99: 908–913.

Lambkin, T. A., and S. J. Rice. 2007. Baseline responses of *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) to spinosad, and susceptibility of broiler populations in eastern and southern Australia. J Econ Entomol. 100: 1423–1427.

Lambkin, T. A., S. J. Rice, and M. Furlong. 2010. Responses of susceptible and cyfluthrin-resistant broiler house populations of lesser mealworm (Coleoptera: Tenebrionidae) to γ-Cyhalothrin. J Econ Entomol. 103: 2155–2163.

de las Casas, E., B. S. Pomeeoy, and P. K. Haeein. 1968. Infection and quantitative recovery of *Salmonella typhimurium* and *Escherichia coli* from within the lesser mealworm, *Alphitobius diaperinus* (Panzer). Poult Sci. 47: 1871–1875.

Leigh, J. W., and D. Bryant. 2015. POPART: Full-feature software for haplotype network construction. Methods Ecol Evol. 6: 1110–1116.

Liu, Y., D. Zhang, L. Yang, Y. Dong, G. Liang, P. Donkersley, G. Ren, P. Xu, and K. Wu. 2021. Analysis of phototactic responses in *Spodoptera frugiperda* using *Helicoverpa armigera* as control. J Integr Agric. 20: 821–828.

Moore, J. 1983. Responses of an avian predator and its Isopod prey to an Acanthocephalan parasite. Ecology. 64: 1000–1015.

Nei, M. 1987. Molecular evolutionary genetics. Columbia University Press, New York.

Oksanen, J., G. L. Simpson, F. G. Blanchet, R. Kindt, P. Legendre, P. R. Minchin, R. B. O'hara, P. Solymos, M. H. H. Stevens, E. Szoecs, H. Wagner, M. Barbour, M. Bedward, B. Bolker, D. Borcard, G. Carvalho, M. Chirico, M. De Caceres, S. Durand, H. B. A. Evangelista, R. FitzJohn, M. Friendly, B. Ferneaux, G. Hannigan, M. O. Hill, L. Lahti, D. McGlinn, M.-H. Ouellette, E. R. Cunha, T. Smith, A. Stier, C. J. F. Ter Braak, and J. Weedon. 2022. Vegan: Community Ecology Package.

Olanrewaju, H. A., J. P. Thaxton, W. A. Dozier, J. Purswell, W. B. Roush, and S. L. Branton. 2006. A review of lighting programs for broiler production. Int J Poult Sci.

Oliveira, D. G. P., A. K. Bonini, and L. F. A. Alves. 2017. Field assessments to control the lesser mealworm (Coleoptera: Tenebrionidae) using diatomaceous earth in poultry houses. J Econ Entomol. 110: 2716–2723.

Paradis, E. 2010. Pegas: An R package for population genetics with an integrated-modular approach. Bioinformatics.

Park, J.-H., and H.-S. Lee. 2017. Phototactic behavioral response of agricultural insects and stored-product insects to light-emitting diodes (LEDs). Appl Biol Chem. 60: 137–144.

R Core Team. 2021. R: A Language and Environment for Statistical Computing.

Rakowski, G. 1988. Effect of illumination intensity on the response of the hide beetle, *Dermestes maculatus*, to aggregation pheromone. J Insect Physiol. 34: 1101–1104.

Reeves, W. K., J. E. Lloyd, R. Stobart, C. Stith, M. M. Miller, K. E. Bennett, and G. Johnson. 2010. Control of Culicoides sonorensis (Diptera: Ceratopogonidae)

blood feeding on sheep with long-lasting repellent pesticides. J Am Mosq Control Assoc. 26: 302–305.
Renault, D., and H. Colinet. 2021. Differences in the susceptibility to commercial insecticides among populations of the lesser mealworm *Alphitobius diaperinus* collected from poultry houses in France. Insects. 12.
Roche, A. J., N. A. Cox, L. J. Richardson, R. J. Buhr, J. A. Cason, B. D. Fairchild, and N. C. Hinkle. 2009. Transmission of *Salmonella* to broilers by contaminated larval and adult lesser mealworms, *Alphitobius diaperinus* (Coleoptera: Tenebrionidae). Poult Sci. 88: 44–48.
Rueda, L. M., and R. C. Axtell. 1996. Temperature-dependent development and survival of the lesser meaIworm, *Alphitobius diaperinus*. Med Vet Entomol. 10: 80–86.
Rumbos, C. I., I. Pantazis, and C. G. Athanassiou. 2020. Population growth of *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) on various commodities. J Econ Entomol. 113: 1001–1007.
Safrit, R. D., and R. C. Axtell. 1984. Evaluations of sampling methods for darkling beetles (*Alphitobius diaperinus*) in the litter of turkey and broiler houses. Poult Sci. 63: 2368–2375.
Salin, C., Y. R. Delettre, M. Cannavacciuolo, and P. Vernon. 2000. Spatial distribution of *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae) in the soil of a poultry house along a breeding cycle. Eur J Soil Biol. 36: 107–115.
Santoro, P. H., P. M. O. J. Neves, T. M. Alexandre, D. Sartori, L. F. A. Alves, and M. H. P. Fungaro. 2008. Selection of *Beauveria bassiana* isolates to control *Alphitobius diaperinus*. J Invertebr Pathol. 97: 83–90.
Sharkey, C. R., M. S. Fujimoto, N. P. Lord, S. Shin, D. D. McKenna, A. Suvorov, G. J. Martin, and S. M. Bybee. 2017. Overcoming the loss of blue sensitivity through opsin duplication in the largest animal group, beetles. Sci Rep. 7.
Simons, C., F. Frati, A. Beckenbach, B. Crespi, H. Liu, and P. Floors. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann Entomol Soc Am. 87: 651–701.
Singh, N. 2011. Chemical ecology, population dynamics and insecticide susceptibility of lesser mealworm *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae).
Singh, N., and D. Johnson. 2015. Baseline susceptibility and cross-resistance in adult and larval *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) collected from poultry farms in Arkansas. J Econ Entomol. 108: 1994–1999.
Skov, M. N., A. G. Spencer, B. Hald, L. Petersen, B. Nauerby, B. Carstensen, and M. Madsen. 2004. The role of litter beetles as potential reservoir for *Salmonella enterica* and thermophilic *campylobacter* spp. between broiler flocks. Avian Dis. 48: 9–18.
Sonenshine, D. E. 2004. Pheromones and other semiochemicals of ticks and their use in tick control. Parasitology. 129: S405–S425.

Steelman, C. D. 2008. Comparative susceptibility of adult and larval lesser mealworms, *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae), collected from broiler houses in Arkansas to selected insecticides. J Agric Urban Entomol. 25: 111–125.

Steinkraus, D. C., and E. A. Cross. 1993. Description and life history of *Acarophenax mahunkai*, n. sp. (Acari, Tarsonemina: Acarophenacidae), an egg parasite of the lesser mealworm (Coleoptera: Tenebrionidae). Ann Entomol Soc Am. 86: 239–249.

Szalanski, A. L., T. W. Palmer, T. McKay, and C. D. Steelman. 2004. Infectivity of *Steinernema* spp. (Nematoda: Steinernematidae) to adult litter beetles, *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) in the laboratory. Biocontrol Sci Technol. 14: 81–85.

Tajima, F. 1983. Evolutionary relationship of DNA sequences in finite populations. Genetics. 105: 437–460.

Tomberlin, J. K., D. Richman, and H. M. Myers. 2008. Susceptibility of *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) from broiler facilities in Texas to four insecticides. J Econ Entomol. 101: 480–483.

Uehara, T., T. Ogino, A. Nakano, T. Tezuka, T. Yamaguchi, Y. Kainoh, and M. Shimoda. 2019. Violet light is the most effective wavelength for recruiting the predatory bug Nesidiocoris tenuis. BioControl. 64: 139–147.

United States Department of Agriculture [USDA] National Agriculture Statistics Service (NASS). 2019. Census of Agriculture Historical Archive.

Del Valle, E. E., L. S. Frizzo, M. Malmierca, M. V. Zbrun, P. Lax, and M. E. Doucet. 2016. Biological control of *Alphitobius diaperinus* with *Steinernema rarum* CUL and *Heterorhabditis bacteriophora* SMC and feasibility of application in rice hull. J Pest Sci (2004). 89: 161–170.

Wang, Q., Z. Guo, J. Zhang, Y. Chen, J. Zhou, Y. Pan, and X. Liu. 2021. Phototactic behavioral response of the ectoparasitoid beetle *Dastarcus helophoroides* (Coleoptera: Bothrideridae): evidence for attraction by near-infrared light. J Econ Entomol. 114: 1549–1556.

Warburg, M. R. 1968. Behavioral Adaptations of Terrestrial Isopods. Am Zool. 8: 545–559.

Wen, C., Y. C. Ji, G. Y. Zhang, S. B. Tan, and J. B. Wen. 2018. Phototactic behaviour of Eucryptorrhynchus scrobiculatus and E. brandti (Coleoptera: Curculionidae) adults*. Biocontrol Sci Technol. 28: 544–561.

Williams, W. H. 1998. Delmarva's Chicken Industry: 75 Years of Progress. Delmarva Poultry Industry Inc.

Wilson, T. H., and F. D. Miner. 1969. Influence of temperature on development of the lesser mealworm, *Alphitobius diaperinus* (Coleoptera: Tenebrionidae). J Kans Entomol Soc. 42: 294–303.

Wolf, J., M. Potrich, E. R. Lozano, A. Gouvea, and C. S. Pegorini. 2015. Combined physical and chemical methods to control lesser mealworm beetles under laboratory conditions. Poult Sci. 94: 1145–1149.

Zorzetti J., Constanskit K., Santoro P. H., Fonseca I. C. B., and Neves P. M. O. J. 2015. Growth regulator insecticides for the control of the lesser mealworm beetle Alphitobius diaperinus (Coleoptera: Tenebrionidae. Recista Colombiana de Entomologia. 41: 24–32.

Appendix A

LIFE STAGE PHOTOGRAPHY OF *ALPHITOBIUS DIAPERINUS*

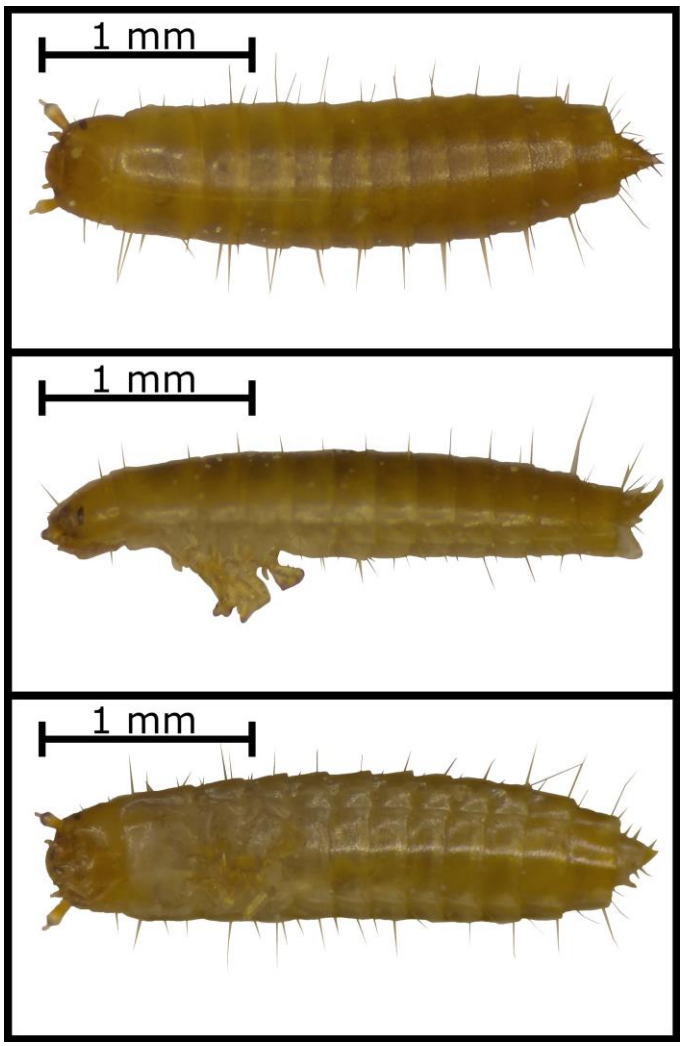

Figure A.1- Dorsal, lateral, and ventral views of a second instar *A. diaperinus* larva.

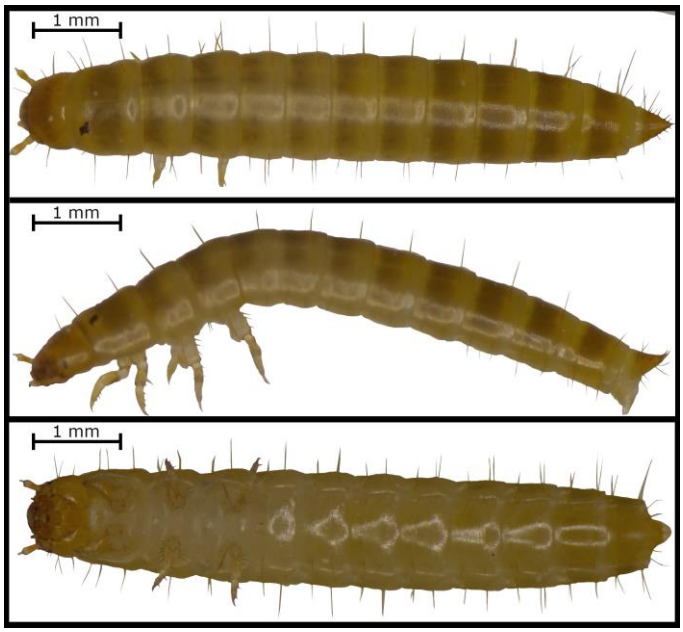

Figure A.2- Dorsal, lateral, and ventral views of an early instar *A. diaperinus* larva.

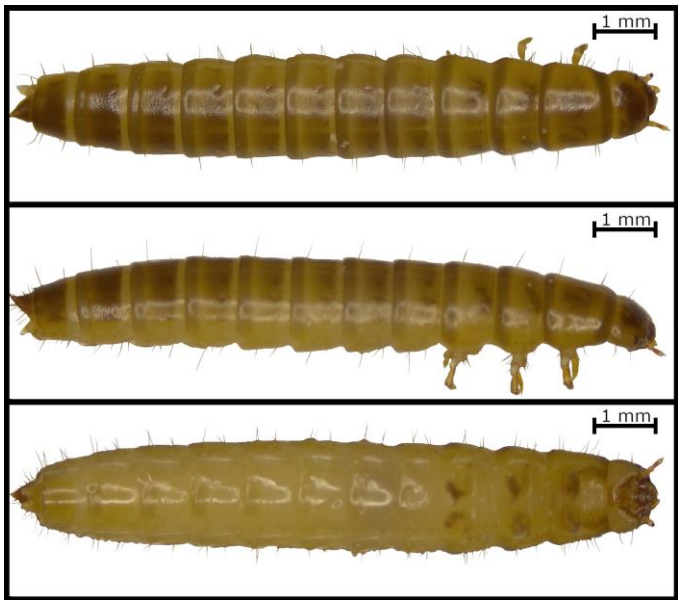

Figure A.3- Dorsal, lateral, and ventral views of an intermediate instar *A. diaperinus* larva.

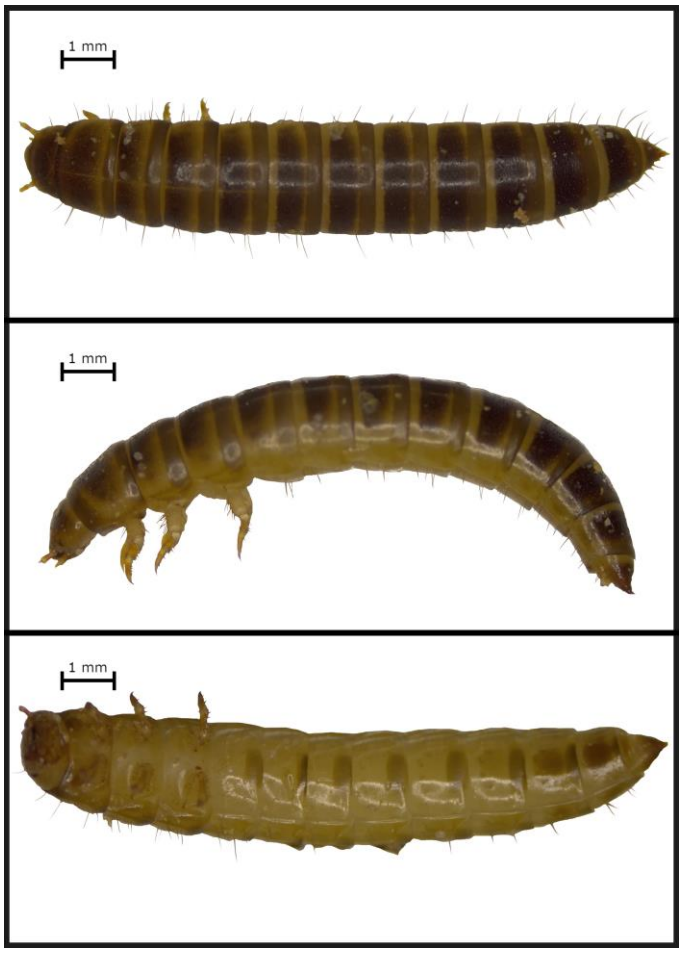

Figure A.4- Dorsal, lateral, and ventral views of a late instar *A. diaperinus* larva.

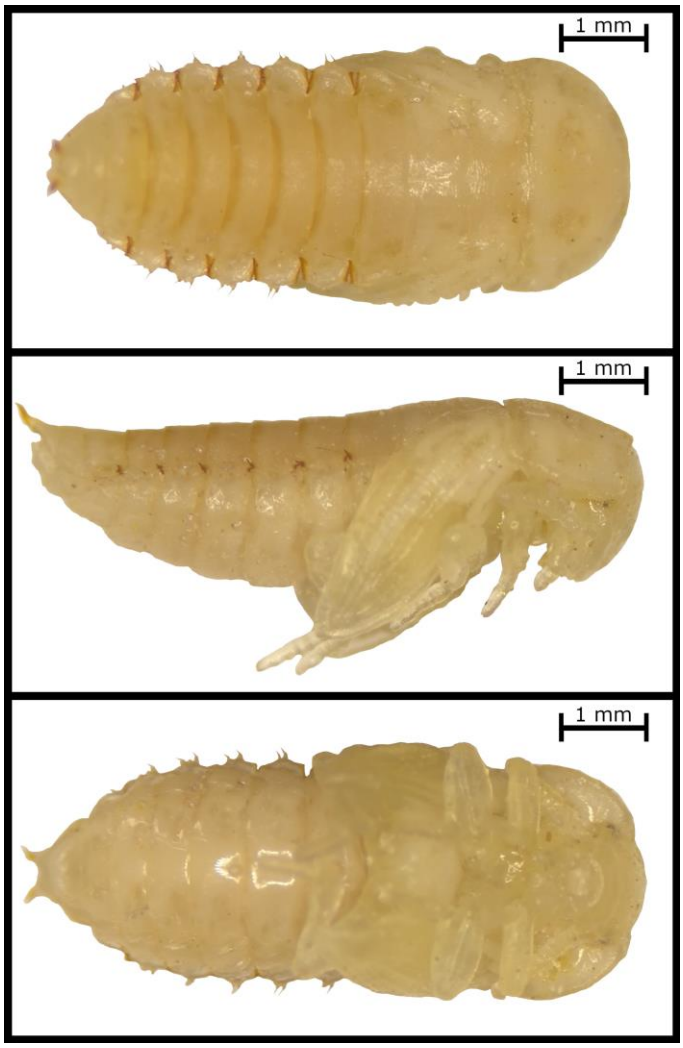

Figure A.5- Dorsal, lateral, and ventral views of an *A. diaperinus* pupa.

Figure A.6- Dorsal, lateral, and ventral views of an *A. diaperinus* adult.

Appendix B

PERMISSIONS

OXFORD UNIVERSITY PRESS LICENSE TERMS AND CONDITIONS

Sep 20, 2023 This Agreement between Ben Sammarco ("You") and Oxford University Press ("Oxford University Press") consists of your license details and the terms and conditions provided by Oxford University Press and Copyright Clearance Center.

License Number- 5633180009870

License date- Sep 20, 2023

Licensed content publisher- Oxford University Press

Licensed content publication- Journal of Integrated Pest Management

Licensed content title- “Biology and Management of Lesser Mealworm *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) in Broiler Houses”

Licensed content author- Sammarco, Ben C; Hinkle, Nancy C

Licensed content date- Jan 30, 2023

Type of Use- Thesis/Dissertation

Institution name-

Title of your work- Identifying Patterns and Drivers of Movement of Lesser Mealworm Alphitobius diaperinus (Coleoptera: Tenebrionidae)

Publisher of your work- University of Delaware 9/20/23, 1:36 PM

Expected publication date- Oct 2023

Permissions cost- 0.00 USD

Value added tax- 0.00 USD

Total- 0.00 USD

Title- Identifying Patterns and Drivers of Movement of Lesser Mealworm Alphitobius diaperinus (Coleoptera: Tenebrionidae)

Institution name- University of Delaware

Expected presentation date- Oct 2023

Requestor Location- Ben Sammarco 2 Brookside Dr COLTS NECK, NJ 07722 United States Attn: Ben Sammarco

Publisher Tax ID- GB125506730

Total- 0.00 USD

Terms and Conditions

STANDARD TERMS AND CONDITIONS FOR REPRODUCTION OF MATERIAL FROM AN OXFORD UNIVERSITY PRESS JOURNAL

1. Use of the material is restricted to the type of use specified in your order details.

2. This permission covers the use of the material in the English language in the following territory: world. If you have requested additional permission to translate this material, the terms and conditions of this reuse will be set out in clause 12.

3. This permission is limited to the particular use authorized in (1) above and does not allow you to sanction its use elsewhere in any other format other than specified above, nor does it 9/20/23, 1:36 PM RightsLink Printable License https://s100.copyright.com/AppDispatchServlet 3/4 apply to quotations, images, artistic works etc that have been reproduced from other sources which may be part of the material to be used.

4. No alteration, omission or addition is made to the material without our written consent. Permission must be re-cleared with Oxford University Press if/when you decide to reprint.

5. The following credit line appears wherever the material is used: author, title, journal, year, volume, issue number, pagination, by permission of Oxford University Press or the sponsoring society if the journal is a

society journal. Where a journal is being published on behalf of a learned society, the details of that society must be included in the credit line.

6. For the reproduction of a full article from an Oxford University Press journal for whatever purpose, the corresponding author of the material concerned should be informed of the proposed use. Contact details for the corresponding authors of all Oxford University Press journal contact can be found alongside either the abstract or full text of the article concerned, accessible from www.oxfordjournals.org Should there be a problem clearing these rights, please contact journals.permissions@oup.com

7. If the credit line or acknowledgement in our publication indicates that any of the figures, images or photos was reproduced, drawn or modified from an earlier source it will be necessary for you to clear this permission with the original publisher as well. If this permission has not been obtained, please note that this material cannot be included in your publication/photocopies.

8. While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by Oxford University Press or by Copyright Clearance Center (CCC)) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and Oxford University Press reserves the right to take any and all action to protect its copyright in the materials.

9. This license is personal to you and may not be sublicensed, assigned or transferred by you to any other person without Oxford University Press's written permission.

10. Oxford University Press reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted

in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

11. You hereby indemnify and agree to hold harmless Oxford University Press and CCC, and their respective officers, directors, employs and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

12. Unless otherwise specified, inclusion under a Creative Commons license or any other Open Access license allowing onward reuse is prohibited. 9/20/23, 1:36 PM RightsLink Printable License https://s100.copyright.com/AppDispatchServlet 4/4

13. Where permission to translate has been agreed, the credit line and any copyright/disclaimer notices provided by OUP shall be included on the same page as the Material, translated into the language of the new work, except for trademark names: Translated and reproduced by permission of Oxford University Press on behalf of the . Translation Disclaimer: OUP and the are not responsible or in any way liable for the accuracy of the translation. The Licensee is solely responsible for the translation in this publication/reprint.

14. Other Terms and Conditions: v1.5