

INSECTICIDE SUSCEPTIBILITY OF THE ADULT DARKLING BEETLE, *ALPHITOBIUS*  
*DIAPERINUS* (COLEOPTERA: TENEBRIONIDAE): TOPICAL TREATMENT WITH  
BIFENTHRIN, IMIDACLOPRID, AND SPINOSAD

by

WHITNEY ELIZABETH BOOZER

(Under the Direction of Nancy C. Hinkle)

ABSTRACT

*Alphitobius diaperinus* is a worldwide pest of poultry. Loss of insecticide susceptibility has been observed in darkling beetle populations worldwide. Topical bioassays were performed using technical grade spinosad (90% active ingredient by weight), bifenthrin (94.88% active ingredient by weight), and imidacloprid (95% active ingredient by weight) to determine susceptibility status of beetle populations in Georgia. LD<sub>50</sub>s were determined and compared to the LD<sub>50</sub> of a susceptible laboratory colony to ascertain resistance ratios. A discriminating dose based on the LD<sub>99.9</sub> of the susceptible population (Denmark) was also estimated. Varying levels of resistance to bifenthrin and imidacloprid were observed, with highest resistance occurring to imidacloprid (>3000-fold). Populations treated with spinosad showed only slight tolerance. Data indicate that resistance to bifenthrin is occurring in populations with prior pyrethroid exposure, and that efficacy of imidacloprid may be severely limited due to significant resistance occurring in beetle populations.

INDEX WORDS: *Alphitobius diaperinus*, darkling beetle, insecticide resistance, bifenthrin, spinosad, imidacloprid, broiler house, topical application

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## DEDICATION

This work is dedicated to my family and friends who stuck by me through the good times and the bad. To Sonya, Bobby, Taylor, Morgan, and Jacob Boozer who were my built-in support system and never let me quit. To Eric who picked me up when I was down, and to Su Yee who shared in my walk through the valleys and over the mountains of graduate student life. We finally made it! And lastly to all of the darkling beetles who (unwillingly) gave their lives for the sake of my research.

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## CHAPTER 1

### INTRODUCTION

Major transformations have arisen in the poultry industry worldwide to meet the demands of a growing human population (Axtell 1999). In order to meet these needs, poultry production systems, in the last few decades, have made the move from highly integrated farms to intensive systems utilizing confined rearing methods (McCrary and Hobbs 2001). These changes have led to a rapid increase in the production of poultry meat, which currently represents almost one-third of the meat produced and consumed worldwide (Scanes 2007). Poultry meat production and trade have shown extraordinary vitality in the last 35 years (Windhorst 2006). With the industry thriving and expanding on a global scale, poultry meat production has surpassed that of veal and beef, and has continued to increase, surpassing pork in 2005 (Windhorst 2006). However, numerous impediments for poultry producers and integrators have arisen in direct correlation with these changes, such as waste management and disposal, welfare concerns, and environmental issues. Along with the evolution of the commercial poultry industry we also see the expansion of the arthropod pests that beleaguer these systems (Axtell 1999). One insect pest in particular has presented an enormous challenge for poultry producers on an international level.

The darkling beetle, *Alphitobius diaperinus* Panzer (Coleoptera: Tenebrionidae), is a significant pest in poultry worldwide. Though these beetles are small, their importance as a poultry pest is enormous. Some of the major concerns associated with these beetles are the impact that they can have on bird health, food safety, and occupational sensitivity of personnel in direct contact with them. The darkling beetle is a known reservoir and vector of various food-

borne disease agents, along with numerous poultry pathogens and parasites, which are acquired by the bird through consumption of adult and immature beetle stages (Axtell and Arends 1990, Despins and Axtell 1995). Consumption of this insect pest by broiler chicks can also lead to decreased feed conversion efficiency and weight gain (Despins and Axtell 1995). The darkling beetle not only threatens the health and production of the birds, but it is also thought to be a health risk to humans, producing allergenic sensitivity in individuals who have been in close contact with it for extended periods of time (Schroeckenstein et al. 1988). In conjunction with the health risks associated with *A. diaperinus*, it is also considered a primary structural pest in the poultry industry, causing extensive damage to broiler housing, which has led to increased heating and repair costs for poultry producers (Axtell and Arends 1990). However, the problems associated with this insect pest are not limited to broiler facilities. In addition to the immense problems associated with *A. diaperinus* inside poultry houses, these beetles have also been known to cause concern in residential areas. Adult beetles, attracted to lights, will migrate into residential areas following litter distribution as fertilizer on pastures or fields near human habitation (Gall 1980, Axtell 1999). These intrusions by darkling beetles have led to costly lawsuits and highly irritated neighbors (Hinchev 1997). High costs associated with control of the darkling beetle, along with subsequent heating and repair expenditures due to damages previously mentioned, make this insect the number one pest in broiler production in the state of Georgia, with estimated cost of damage and control equating to around \$12 million in 2006 (Guillebeau et al. 2008). This is an increase from 2005 estimations, which were around \$9.9 million (Guillebeau et al. 2008).

The ability of the darkling beetle to achieve immense populations, survive harsh environments, and occupy hard to reach niches gives them the upper hand when it comes to

control, which in turn presents a vast challenge for poultry producers. The struggle to control *A. diaperinus* has become an almost impossible task. With past and current control measures for this pest relying heavily on insecticide use, the fear of resistance is incessantly present. These fears, which are well placed, are now becoming a reality.

Insecticide resistance is an on-going battle when dealing with insect pests, and the poultry industry is in no way exempt from the challenges that arise with resistance. Darkling beetle populations, both in the U.S. and in Australia, have exhibited resistance to several commonly used insecticides (Lambkin 2005, Lambkin and Rice 2006, Lambkin and Rice 2007, Hamm et al. 2006, Steelman 2008, Tomberlin et al. 2008). At present, there are five classes of chemicals approved for use in broiler facilities in the United States. These chemical classes are organophosphates, pyrethroids, spinosyns, neonicotinoids, and boric acid; with the most frequently used products consisting of pyrethroids and organophosphates (Szczepanik et al. 2008). With the narrowing availability of products, suppressing resistance is becoming progressively more exigent.

The testing of technical grade bifenthrin, spinosad, and imidacloprid to determine the baseline response of *A. diaperinus* will provide vital information on the levels of resistance occurring in the field. These tests will also permit subsequent monitoring for incipient resistance development, elucidate prevalence of resistant populations, as well as grant producers the ability to tailor their insecticide rotation schemes to best suit their particular needs. With increasing apprehensions concerning insecticide resistance, determining baseline susceptibility of darkling beetle populations is crucial in sustaining population suppression.

## CHAPTER 2

### LITERATURE REVIEW

#### *Alphitobius diaperinus*

##### **Taxonomy**

*Alphitobius diaperinus* has been referred to by a multitude of common names including darkling beetle, litter beetle, shining black wheat beetle, black fungus beetle, black poultry bug, Schmittle beetle, and shiny black moldy grain beetle (Swatonek 1970, Nolan 1982). *A. diaperinus* belongs to the order Coleoptera, family Tenebrionidae, and is a member of the tenebrionid tribe Alphitobiini (Doyen 1989). The Alphitobiini tribe is composed of four genera worldwide, of which two occur in the United States (Aalbu et al., 2002). The generic name *Alphitobius* was first published by Stephens in 1829 with the specific epithet *diaperinus*, which was established by Panzer in 1794 (Spilman 1966, Poole and Gentili 1996). Panzer was also credited with the naming of *Alphitobius picipes* and *Helops picipes*, now known as *Alphitobius laevigatus* (Spilman 1966). Both of these species belong to the genus *Alphitobius*, which comprises around eleven known species worldwide (Dunford and Kaufman 2006). Of these eleven species only two are found in the United States, *Alphitobius diaperinus*, or the darkling beetle, and *Alphitobius laevigatus*, the black fungus beetle (Dunford and Kaufman 2006). These two species can be separated morphologically by characters described by Preiss and Davidson (1970). Like its relative the darkling beetle, the black fungus beetle has also gained recognition as a cosmopolitan pest (Rees 2004). However, this beetle is primarily associated with stored products, and is considered to be an inconsequential pest (Rees 2004).



## **Life Stages: Description and Biology**

All life stages of *A. diaperinus* can be found inhabiting litter in poultry houses (Watson et al., 2003) year round. The developmental stages of the darkling beetle have been thoroughly documented under controlled and varied temperature conditions in the laboratory (Barké and Davis 1969, Wilson and Miner 1969, Preiss and Davidson 1971). *A. diaperinus* developmental rate and survival have been shown to be heavily influenced by temperature, along with nutritional quality and food availability, humidity, and effects of microorganisms (Wilson and Miner 1969, Rueda and Axtell 1996). The optimal temperature for darkling beetle development is approximately 30 to 35°C. At this temperature the mean *A. diaperinus* development time from egg to adult emergence is around 37.9 to 29 d respectively (Rueda and Axtell 1996). At lower temperatures, such as 21°C, the developmental time from egg to adult can last as long as 60-85 d (Barké and Davis 1969).

### Adult

Darkling beetle adults are shiny brown to black in color, with a broadly-oval and moderately convex profile (Fig. 4). A newly eclosed adult darkling beetle is soft bodied and reddish brown in color. In laboratory conditions it has been shown to take an average of 7d for the cuticle of the darkling beetle to harden and darken to its characteristic color (Wilson and Miner 1969, Preiss and Davidson 1971). However, Hopkins et al. (1992) found that in their laboratory environment an average of only 5 d was required for completion of this tanning process. Adults are small in size, ranging in length from 5.1-6.1mm (Wilson and Miner 1969) with evenly spaced depressions on the elytra. The pronotum is twice as broad as it is long, and is deeply emarginated anteriorly (Preiss and Davidson 1970). The sex ratio observed in darkling beetle populations obtained from laboratory cultures was approximately 1:1 (Preiss 1969,

Falomo 1986). The sex of the darkling beetle adult can be determined based on the shape of the metathoracic tibial spines (Fig. 9) (Barké and Davis 1967). The male has one straight and one curved metathoracic tibial spine, while both spines are straight on a female. A male can also be recognized by the deeply emarginate posterior edge of the 8<sup>th</sup> sternite, which is straight on a female (Barké and Davis 1967). Adult female darkling beetles have been shown to mate with males of the same age after a pre-oviposition period of about 10 to 13 d (Wilson and Miner 1969, Preiss and Davidson 1971). Hopkins et al. (1992) demonstrated that male and female pairs of the same age would mate 7 d post-emergence, while females placed with older males would mate as early as 3 d post-emergence. Mating rituals observed by Falomo (1986) consisted of “calling”, mate recognition, mate grooming, and copulation. The “calling” behavior was achieved in both sexes by exhibiting headstands, wing fanning, abdominal tip wiping and dragging (Falomo 1986). The mean life-span of *A. diaperinus* has been reported as greater than 400 d (Preiss and Davidson 1971), with female fecundity as high as 3.6 to 7.3 eggs per day (Rueda and Axtell 1996). The longevity of adult *A. diaperinus* and long oviposition period, with high female fecundity, allows for immense population build up in broiler facilities.

### Egg

The egg of the darkling beetle, when first laid, is creamy white in color and darkens with age. It is oval in shape and ranges in length from around 1.0 to 1.4 mm, with average width approximately 0.44 mm (Preiss 1969). As development progresses the egg shape alters from oval to slightly concave (Fig. 5) (Wilson and Miner 1969). When laid, the eggs are anchored into cracks and crevices by the female darkling beetle, via a clear sticky substance (Wilson and Miner 1969). The eggs are often laid in clusters. Barké and Davis (1969) observed 1 to 28 eggs per cluster with 1, 2, and 3 eggs per cluster accounting for almost 50% of all the clusters

collected. Temperature is an important factor in egg development. Egg hatch can occur anywhere from around 3-13 d after oviposition, with the highest rate of egg hatch occurring at 30°C (Rueda and Axtell 1996). The median times for egg development when placed at temperatures of 20, 25, 30, 35, and 38°C were found to be 13.4, 6.0, 4.4, 2.6, and 2.6 d respectively, with no egg hatch occurring at 17°C (Rueda and Axtell 1996). Along with temperature, relative humidity also plays a role in egg hatch. Barké and Davis (1969) found that the highest percent of egg hatch occurred at a relative humidity of 70%, which corresponds with the data collected by Preiss and Davidson (1968) who reported that maximum hatch was accomplished with relative humidity of 68-71%.

### Larva

Darkling beetle larvae ostensibly resemble true mealworms (Fig. 6) (*Tenebrio* spp.). They have three pairs of legs and segmented bodies that taper posteriorly (Fig. 7) (Dunford and Kaufman 2006). A newly hatched larva is about 1.5 mm in length and white in color (Wilson and Miner 1969, Francisco and Prado 2001). As it grows and the cuticle hardens, the larva darkens to a brownish color (Francisco and Prado 2001). The larvae grow to about 10 mm in length before pupating. The duration of the larval stage is heavily dependent on temperature, and can last from 22.4 to 133 d at temperatures ranging from 35 to 20°C (Rueda and Axtell 1996). No larval development is observed at temperatures as low as 17°C (Rueda and Axtell 1996). The number of larval instars is also highly variable, ranging from 6 to 11 instars. Wilson and Miner (1969) observed 11 larval instars at a temperature of 15.5°C; however, at 26.6°C only a single larva reached the 9<sup>th</sup> instar. Francisco and Prado (2001) used mean head capsule widths to characterize larval instars and found that at 27°C and 70% relative humidity only 8 larval instars were observed. The mean head capsule measurements obtained for each of the 8 larval

instars were 0.228 ( $\pm$  0.0192), 0.228 ( $\pm$  0.0225), 0.348 ( $\pm$  0.0433), 0.478 ( $\pm$  0.0433), 0.721 ( $\pm$  0.0216), 1.061 ( $\pm$  0.0536), 1.208 ( $\pm$  0.0769), 1.339 ( $\pm$  0.0436) mm respectively (Francisco and Prado 2001). When the final instar is achieved, the larvae will seek out pupation substrates in the earth floor or insulation for protection against predators and cannibalism from adult and larval conspecifics (Ichinose et al. 1980, Despins et al. 1987, Geden and Axtell 1987). Geden and Axtell (1987) evaluated this behavior of the larvae in both field and laboratory conditions and found that climbing occurred primarily at night between 2000 and 2400 h, and that it is influenced by both soil availability as a pupation substrate and larval density. While these are the primary factors influencing climbing behavior, under field conditions factors such as litter moisture, temperature, management practices, bird presence and density, and bird age also play a role in the inception of this behavior.

### Pupa

*A. diaperinus* pupae are exarate and initially white in color, but change to tan within a day (Fig. 8) (Barké and Davis 1969). Female pupal length was found to be significantly different from that of the male, with female lengths averaging 5.9 mm while males averaged 5.5 mm (Preiss 1969). Sexual dimorphism occurs in the pupal stage of *A. diaperinus* (Barké and Davis 1967). The difference between sexes can be observed on the ventral posterior section of the abdomen, where the female has a pair of non-sclerotized fleshy projections that are not observed in the male (Barké and Davis 1967, Preiss 1969). These projections are second valvifers (Barké and Davis 1967). The pupal stage of *A. diaperinus* can be found in the soil floors of broiler houses, as well as in the insulation, particularly when population numbers are high (Safrit and Axtell 1984, Geden and Axtell 1987). The pupal stage lasts about 4 to 17 d, again depending on temperature (Rueda and Axtell 1996). Mean pupal development times observed by Rueda and

Axtell (1996) under lab conditions were 17.0, 8.0, 5.5, 4.0 and 4.1 d at temperatures of 20, 25, 30, 35, and 38°C respectively.

### Habitats and Distribution

*A. diaperinus* have been found in a vast array of habitats worldwide including bird nests, bat caves, piggeries and dairy farms. It has been found inhabiting Saker nests in Hungary, pigeon nests in Sudan and Texas, and sparrow and purple martin nests in Wisconsin (Levi 1957, Thompson 1966, Yagi and Razig 1972, Merkl et al. 2004). The darkling beetle has also established itself in bat caves in Texas and Kenya (Reddell 1966, McFarlane 1971) and has been found living in a piggery in Ireland (O'Connor 1987), as well as on dairy farms in the United States. However, its most notable conquest recorded was its residence in the scrotum of a Norway rat, in which 4 adults and 16 larvae were living (Crook et al. 1980). Despite some of these more intriguing habitats, the darkling beetle has the greatest impact economically as an invader of poultry houses, where temperature, moisture, food, and modern poultry practices have created an ideal environment for the survival and proliferation of this pest.

Within poultry houses populations of *A. diaperinus* tend to increase during the warm seasons, such as summer and autumn, while numbers generally decline during the colder seasons (Pfeiffer and Axtell 1980). Lambkin et al. (2007) observed the distribution of darkling beetles found in broiler houses in Australia. Their findings indicated that adult darkling beetles emerge from the compact dirt floor soon after a new flock is brought into the house (Lambkin et al. 2007). These newly emerged adults allow for the perpetuation of beetle populations in poultry facilities (Axtell and Arends 1990, Lambkin et al. 2007). Darkling beetle populations usually peak when the birds are at about 3 wk of age, and larvae begin to burrow into the earthen floor or

insulation when the flock is 6 wk old. Pupation then occurs and adults will emerge with the next flock (Lambkin et al. 2007).

Darkling beetles often amass in areas of higher temperature, suitable moisture, and adequate nutrients within a broiler house (Axtell and Arends 1990). Strother and Steelman (2001) used geographic information systems (GIS) to provide a spatial display of adult and larval populations in broiler houses. They found that beetle populations built at one end of the broiler house during the beginning of flock grow-out and gradually spread toward the other end of the house with each consecutive week. Lambkin et al. (2007) attempted to identify population distribution of darkling beetles in broiler houses in Australia. The outcome showed highly uneven distribution of darkling beetle adults and larvae in the broiler houses, with lower concentrations of beetles occurring in the open areas and under drinker lines, while higher densities were found under feed pans and along the edges of the house. Other studies concerning distribution have found similar results, with highest densities concentrated under feed pans and along the walls of broiler houses (Safrit and Axtell 1984, Salin et al. 2000, Lambkin et al. 2008). Spatial distribution studies conducted by Salin et al. (2000) demonstrated that late instar larvae, adults, and pupae tend to occur under feeders in the top 0-10 cm layer of soil at the end of a broiler grow-out period, with soil surface compactness and density being the most discriminate factors influencing spatial distribution.

### Diet

Darkling beetles have the ability to find nourishment in diverse places; they can be voracious predators as well as proficient scavengers. *A. diaperinus* are known pests of stored products feeding on poor quality cereals and grains (Harding and Bissell 1958) and have been reported feeding on detritus and guano in bat caves and bird nests in eastern Africa (McFarlane

1971, Vaughan et al., 1984). The darkling beetle was previously thought to be exclusively phytophagous or saprophagous until 1958 when it was observed attacking dead and moribund birds (Harding and Bissell 1958). Harris (1966) further tested the darkling beetle's carnivorous habits by placing two snakes and a salamander in a container with several hundred beetles. Within 12 h all three specimens were attacked and eaten. In poultry houses the darkling beetle has been regarded as the best-adapted scavenger (Pfeiffer and Axtell 1980), where, in addition to consuming feed and manure, adults and late-instar larvae will prey on other insects, and dead or dying birds (Axtell and Arends 1990). A laboratory study conducted by Despins et al. (1988) investigating the role of the darkling beetle as a predator of the house fly (*Musca domestica*) indicated that *A. diaperinus* had a significant impact on house fly emergence. Both adult and larval beetles were observed feeding on the maggots and puparia of the house fly, with the late instar larvae consuming significantly more maggots than the adult beetle. Hulley and Pfeleiderer (1988) also reported darkling beetles feeding on house fly (*Musca domestica*) eggs, small larvae, and freshly-killed adults when the cuticle was damaged. The darkling beetle has been found to prey on *Tenebrio molitor* eggs and pupae in laboratory cultures (Harris 1966). In addition to feeding on other insect species, *A. diaperinus* will also become cannibalistic, particularly in conditions of starvation and overcrowding (Parween and Begum 2001), with late instar larvae and adults readily feeding on pupae and other larvae (Vaughan et al. 1984, Axtell and Arends 1990). Sarin (1973) tested the enzymes present in the alimentary canal of *A. diaperinus* and found that, with the exception of lactase which is present only in the hindgut of adults, both the larva and adult have the same digestive enzymes. These enzymes present in the digestive system of *A. diaperinus* are a combination of those found in omnivorous and phytophagous insects (Sarin 1973). This species has the enzymes to digest proteins, fats, starch, sucrose, maltose,

lactose, and cellulose (Sarin 1973). The darkling beetle's mouthparts were examined by Leschen and Steelman (1988). Their observations led them to the conclusion that the adult darkling beetle is a general feeder, while the larva possesses planar molar surfaces that are adapted for feeding on "cemented" food material. When examining the adult digestive system of *A. diaperinus*, McAllister et al. (1995a) found no midgut caeca or regenerative crypts in either the larvae or adults. This substantiates omnivorous or scavenging feeding habits. They also noted the lack of a crop, indicative of continuous feeding since the crop's primary function is food storage (McAllister et al. 1995a). The darkling beetle's propensity for scavenging and continuous feeding could have significant implications concerning their potential as disease vectors (McAllister et al. 1995a).

#### Survival and Longevity

*Alphitobius diaperinus* is believed to have originated in sub-Saharan Africa in association with bird nests and bat caves (McFarlane 1971, Vaughan et al. 1984, Lambkin 2001). It has been imported into temperate regions via commerce, in stored food products (Crook et al. 1980). Cosmopolitan in distribution, *A. diaperinus* was first known as a secondary pest usually found in flour-mill basements infesting damp or musty flour or grain, preferring cereal products that are slightly out of condition (USDA 1953). It is believed to have first infested Indiana brooder houses from crushed corn cobs that were used as insulation for the walls (Gould and Moses 1951) as well as in Maryland from corn cob litter (Harding and Bissell 1958). Although *A. diaperinus* is well known as a pest of seeds, grain, feed, and cereal, this beetle has a long list of hosts worldwide, including an assortment of other plant and animal matter (Crook et al., 1980). When it comes to broiler facilities, litter and environmental conditions provide an optimal habitation for the darkling beetle.



As a tropical species adapted to high temperatures and low humidity (Salin et al. 1998), the darkling beetle thrives in broiler house conditions, which mimic their natural environment (Renault et al. 1999). In spite of their ability to prosper in poultry houses, darkling beetles do have some limitations. The darkling beetle requires relatively high moisture, particularly in the immature stages. Sarin and Saxena (1973) determined the optimum conditions for growth of the darkling beetle to be 30°C at 90% relative humidity. Female adults do not lay eggs below 50% relative humidity, and reproduction is repressed at temperatures ranging from 15-17°C (Farkas 1966, Rueda and Axtell 1996). Renault et al. (1999) found that the ability of the darkling beetle to perform motile activity decreases at temperatures below 15°C. In the same study a temperature of 6°C was increasingly lethal to the darkling beetle, which goes into a chill coma, or cessation of activity, at 5.8°C (Renault et al. 1999). However, *A. diaperinus* has adapted to survive in slightly less extreme conditions. When held at 10°C for a month, 70% of the adult darkling beetles were still alive and active (Renault et al. 1999). Both male and female adult beetles have the ability to avoid freezing through a process called supercooling (Salin et al. 1998), which is a condition in which their body fluids cool below freezing but do not change phase to solid. The mean supercooling points (SCPs) of *A. diaperinus* populations when held at 100% relative humidity were around -9.5°C and -9.2°C for males and females respectively, with no significant differences exhibited between sexes (Salin et al. 1998). However, when adults were held at 0% relative humidity a significant difference in mean SCPs was observed, with males exhibiting lower supercooling points at -14.9°C than females at -11.3°C (Salin et al. 1998). The principal elements determining mortality at these low temperatures is the duration and intensity of the cold (Renault et al. 1999). This plays a major role in the adult darkling

beetle's ability to survive during winter cleanout, which is a vital factor in understanding the population dynamics of the darkling beetle.

### **Pest Status**

While the darkling beetle is considered a pest of stored products, it does not pose a significant economic threat to this industry. The grains these beetles feed on are often already damaged. However, the darkling beetle is considered the foremost premise pest in the poultry industry (Axtell 1999). Simco et al. (1966) found *A. diaperinus* to be the most commonly observed insect pest in poultry houses, detecting few houses free of infestation. The problems caused by the darkling beetle can culminate in substantial economic losses to poultry producers. In 2006 the state of Georgia reported losses and control costs associated with the darkling beetle to be over \$12,000,000 million (Guillebeau et al. 2008). Other states have also estimated high monetary losses due to damage by *A. diaperinus* (Turner 1986).

### **Insulation and Structural Damage**

Damages accrued in broiler houses by the darkling beetle generally arise when final instar larvae, looking for a protective pupation site, tunnel into insulation and wooden support structures when unable to find suitable soil (Vaughan et al. 1984, Despins et al. 1987). Geden and Axtell (1987) examined the climbing propensity of larval and adult darkling beetles in a commercial broiler house, observing highest activity at night. The amount of damage caused by the darkling beetle is often dependent on population density and availability of pupation sites, with damage by late instar larvae increasing 4-fold with lack of soil and higher larval densities (Geden and Axtell 1987). In only 3-4 years, darkling beetles can cause extensive damage to styrofoam insulation, effectively destroying its insulative capability (Hinkle and Hickle 1999), reducing effectiveness of insulating material by as much as 20–30% (Vaughan et al. 1984,

Despins et al. 1987). Other insulating materials such as polyisocyanurate, polyurethane, and fiberglass are also vulnerable to darkling beetle destruction (Vaughan et al. 1984, Axtell and Arends 1990). Increased production costs are directly correlated with damages caused by the darkling beetle and occur as a result of material replacement and increased labor. Other monetary setbacks, indirectly related to darkling beetle destruction, arise due to energy loss, market loss while houses are out of production for repairs, and losses to bird growth efficiency due to poor environmental conditions (Vaughan et al. 1984). Houses with severe beetle damage have exhibited as much as 67% higher energy costs compared to houses with little to no damage (Geden et al. 2001).

### Bird Performance

There are many components involved in achieving good bird performance. In broiler production, feed is the most costly of these elements (May et al. 1998). Efficient feed utilization by a flock can be of considerable economic importance to a broiler producer (Vest 1999). Nevertheless, broiler chicks are often found feeding on darkling beetle larvae in litter and feed pans, rather than the feed provided (Despins and Axtell 1995). Despins and Axtell (1995) assessed the effects that larval consumption has on chick growth. Chicks that fed only on larvae for 6 d weighed less than chicks that fed on starter feed. These larval-fed chicks were unable to achieve the same weight gain as chicks fed only starter feed, even after being placed on feed for 9 d. The consumption of darkling beetle larvae had a number of other negative impacts on the chicks. They began showing signs of stress, having problems defecating and producing watery stool. Whole larval cuticles were present in the chicks' feces, confirming that the larval cuticle was indigestible (Despins and Axtell 1995).

Darkling beetles can also have a negative impact on bird performance when humidity is very low. In such situations darkling beetles become desperate for water, requiring them to look for it in less than ideal places. *A. diaperinus* have been observed crawling up the feathers of resting birds and biting the skin around feather follicles for moisture (Savage 1992). This behavior can cause weeping lesions or areas of pink and swollen skin resembling skin leukosis. When harassed by these biting beetles, birds will move around to avoid the attack, resting only briefly. Repeated disturbances cause birds to expend unnecessary energy, which lowers their overall performance and feed conversion efficiency (Savage 1992).

### Medical Importance

Pathogen transmission by insects is an enormous concern worldwide, which can have negative implications to both human and animal health. Numerous studies have established insects as key components in the transmission of disease causing agents, including bacterial pathogens. *Salmonella* spp. have been experimentally transmitted by a number of different types of insects including fleas, flies, and cockroaches (Mackerras and Mackerras 1948, Eskey et al. 1949, Greenberg et al. 1963), while Crumrine et al. (1971) showed that *Salmonella* Montevideo could be carried by a number of stored product pests when exposed to contaminated wheat.

The ecological conditions and omnivorous eating habits associated with the darkling beetle augment its ability to vector pathogenic organisms. *A. diaperinus* adults and larvae are known reservoirs of a variety of bacteria, viruses, and protozoa, which can affect both bird and human health. Goodwin and Waltman (1996) collected darkling beetles from seven poultry farms and tested them for common avian pathogens, concluding that darkling beetles do serve as vectors for various pathogens including immunosuppressive viruses and bacteria such as

*Salmonella*. Therefore they present a significant risk to the health of birds that come into contact with them (Goodwin and Waltman 1996).

Several genera of bacteria have been isolated from *A. diaperinus* including *Micrococcus*, *Streptococcus*, *Staphylococcus*, *Serratia*, *Klebsiella*, *Pseudomonas*, and *Salmonella* (De las Casas et al. 1968, De las Casas et al. 1972, Olsen and Hammack 2000). Harein et al. (1970) isolated twenty-six pathogenic serotypes of *Escherichia coli* from darkling beetle adults acquired from a turkey brooder house. *A. diaperinus* adults and larvae were found to harbor *E. coli* both externally and internally for 12 d, and were able to release *E. coli* in their feces for up to 6 to 10 d (McAllister et al. 1996). One-day-old chicks that fed on these contaminated beetles tested positive for *E. coli*, substantiating the belief that the darkling beetle may play a part in the transmission and spread of *E. coli* in broiler production systems (McAllister et al. 1996). *A. diaperinus* has also been implicated in the dissemination of *Salmonella* in broiler facilities. Following a single 24 h feeding, *Salmonella* Typhimurium was detected in darkling beetle feces for 28 d (McAllister et al. 1994). Surface swabs and whole body homogenates were positive for *S. Typhimurium* 16 d post-exposure (McAllister et al. 1994). *S. Typhimurium* can also persist on non-living darkling beetles for at least 45 d (De las Casas et al. 1968). One-day-old chicks were found to be contaminated with *S. Typhimurium* within 24 h of ingesting a single inoculated adult or larval beetle (McAllister et al. 1994). Roche et al. (2009) evaluated the ability of *A. diaperinus* to transmit *Salmonella* Typhimurium to day-of-hatch chicks, and its spread to nonchallenged pen mates. Findings demonstrated that three weeks after chicks were gavaged with larvae exposed to *Salmonella*-inoculated feed, 25-33% of the challenged birds and 45-58% of pen mates tested positive for *Salmonella*. Pens challenged with contaminated adult beetles showed a lower percent of *Salmonella*-positive broilers, with 0-57% of the challenged birds and

20-40% of pen mates testing positive. Along with *E. coli* and *Salmonella* the darkling beetle has also been considered a competent reservoir and vector of *Campylobacter jejuni*, which is one of the foremost causes of food-borne disease in most parts of the developed world (Altekruse et al. 1997). Chicks fed insects that had been inoculated with the pathogen on the day of feeding showed colonization with *Campylobacter* at levels of 50-100% (Hazeleger et al. 2008). *A. diaperinus* larvae inoculated with *C. jejuni* harbored it exteriorly for 12 h, internally for 72 h, and in fecal matter for 12 h post exposure (Strother et al. 2005). Strother et al. (2005) showed that with increased consumption of contaminated adults and larvae the percent of birds testing positive for *Campylobacter* also increased. Consumption of a single adult or larval beetle resulted in 90% of birds testing positive for *Campylobacter*, while consumption of 10 adults or larvae produced 100% *Campylobacter*-positive birds (Strother et al. 2005).

Along with bacteria, some viruses are also able to be sequestered and transmitted by the darkling beetle. Healthy birds developed symptoms of infectious bursal disease (IBD) after being fed beetles collected from a broiler house contaminated with the virus (Snedeker et al. 1967). Further studies evaluating the role of *A. diaperinus* as a reservoir for infectious bursal disease virus demonstrated that adult darkling beetles could harbor the virus for up to 14 d after ingestion and that it could be found on the mouthparts, digestive tract, and in the hemolymph of adult beetles 24 h post ingestion (McAllister et al. 1995b). These findings elucidate the darkling beetle's role as a competent reservoir for infectious bursal disease virus, rather than a fomite. Eidson et al. (1966) observed the relationship between Marek's disease and the darkling beetle, demonstrating that adults and larvae found in contaminated litter could acquire the leukosis virus, making them complicit in its environmental survival. Transmission of certain turkey viruses has also been linked to the darkling beetle. Despins and Axtell (1994) evaluated the role

of darkling beetle larvae as mechanical vectors for enteric pathogens of turkey by exposing them to turkey feces from an enteritis-affected flock and then feeding them to turkey poults. After exposure to the contaminated feces, the larvae tested positive for turkey enterovirus and rotavirus, and turkey poults that fed on them expressed clinical signs of enteritis.

The darkling beetle also acts as an intermediate host for some protozoans. The protozoa (*Eimeria* spp.) that cause avian coccidiosis have been found to survive when ingested by darkling beetles (Reyna et al. 1983). Alicata (1939) first reported that *A. diaperinus* was naturally infected with encysted larvae of *Subulura brumpti*, a common cecal worm of poultry in Hawaii. Adult darkling beetles were originally reported as an intermediate host for the poultry tapeworm, *Choanotaenia infundibulum*, by Elowni and Elbihari (1979) and have also been found to serve as an intermediate host for other helminths such as *Raillietina* spp. (Gogoi and Chaudhuri 1982).

Of course, not all poultry pathogens are capable of being transmitted by *A. diaperinus*. De las Casas et al. (1976) demonstrated that fowl pox virus did not multiply in darkling beetles and would persist for only a maximum of 6 d; however, darkling beetle excrement can be a source of contamination (De las Casas et al. 1976). Darkling beetles are also unsuccessful vectors of Newcastle disease virus, which was recovered only from adults fed highly infected chorioallantoic membranes for up to 2 d post inoculation (De las Casas et al. 1976). De las Casas et al. (1973) also evaluated the importance of the darkling beetle as a carrier and vector of reovirus 24. They concluded that while reovirus 24 can survive in the darkling beetle for at least 9 d, the low titers obtained render them ineffective carriers (De las Casas et al. 1973). Although the darkling beetle does not serve as a primary reservoir for these pathogens, the causative agents

of these diseases have all been recovered from this beetle pest, providing a potential source of contamination in the poultry environment.

There are also more direct concerns, related to human health, that are associated with the presence of *A. diaperinus*. In 1988 the first known cases of occupational sensitivity to the darkling beetle were discovered (Schroeckenstein et al. 1988). Three individuals were found exhibiting symptoms of asthma, rhinitis, conjunctivitis, urticaria, and angioedema when exposed to the darkling beetle (Schroeckenstein et al. 1988). Further tests determined that these individuals had developed sensitivity to antigens produced by *A. diaperinus* due to occupational exposure (Schroeckenstein et al. 1988). Some of these health issues are attributable to the production by the darkling beetle of benzoquinones, which are used as a defense mechanism against predators (Tschinkel 1975). Health ailments such as conjunctivitis and corneal ulcerations can occur when eyes are exposed to quinone vapors (Tseng et al. 1971).

### Nuisance

Problems associated with the darkling beetle can spread far beyond the walls of a broiler facility. Most concerns arise following broiler house clean-out, when litter is stored in stack houses or applied to pastures and crop fields as inexpensive fertilizer. Although litter removal is an important aspect in darkling beetle control, it can also serve as a means for dispersion and reinfestation of this pest (Calibeo-Hayes et al. 2005) due to large numbers of living darkling beetles being contained in the litter. Darkling beetles are nocturnal, with greatest activity occurring shortly after dark (Geden and Axtell 1987). The adult darkling beetle, attracted to artificial lights, is capable of flying to residential areas located near fields on which beetle-contaminated manure has been spread (Axtell 1999). The en masse movement of *A. diaperinus* adults into these residential areas can result in expensive litigations and poor community



relations (Hinchey 1997, Miller 1997). Studies have been conducted in an attempt to suppress the emergence of *A. diaperinus* from field applied litter. Kaufman et al. (2005b) examined the use of mechanical incorporation of litter into field soils in New York. Their results showed that moldboard plowing did significantly reduce beetle emergence compared to no tillage. A similar study, using mechanical incorporation of poultry litter into fields, was conducted by Calibeo-Hayes et al. (2005). They found that incorporation of poultry litter in clay field soils through disking, mulching and plowing caused significant reductions in beetle emergence relative to no tillage. They also studied incorporation of litter into sandy field soil and found that disk and plow treatments had a significant impact on reducing adult emergence. They also observed that natural reduction of adult emergence occurred when litter was land applied during the winter mo.

### **Control Measures**

There is no simple one step process that will completely eliminate darkling beetle populations from broiler facilities. Proper control measures require a combination of practices to be effective. Control practices should be implemented in a timely and practical manner and should include biological, cultural, mechanical, and chemical control strategies, as well as monitoring. Lambkin and Cameron (2000) outlined a proposed IPM method that included conducting further studies to better understand darkling beetle behavior and population dynamics, implementation of insecticide rotation programs, development of an insecticide resistance management protocol and the utilization of more innovative insecticides and alternative control strategies. Implementation of such practices could prove to be essential for current and future control of *A. diaperinus*.

## Biological Control

While biological control is an attractive alternative to insecticide use, it has not successfully been put into practice for control of *A. diaperinus*. Several natural enemies of the darkling beetle have been discovered and examined as possible biological control agents. The most promising of these is the fungal pathogen *Beauveria bassiana* (Balsamo) Vuillemin (Geden et al. 1998). The ability of this fungus to thrive in the warm and humid earth floor of a broiler house makes *B. bassiana* a potential biocontrol agent for *A. diaperinus* (Steinkraus et al. 1991). High susceptibility was exhibited by early instars of the darkling beetle when exposed to a natural epizootic strain of this fungal pathogen isolated from *A. diaperinus* (Geden et al. 1998). However, in the same study adult beetles were 1000 times less susceptible than their young larvae (Geden et al. 1998). Geden and Steinkraus (2003) found that when *B. bassiana* was prepared as a granular formulation, 60-90% suppression of darkling beetle larvae was obtained. Although this seems promising, the effects were transitory, lasting only 2 wk after treatment (Geden and Steinkraus 2003). Castrillo and Brooks (1998) conducted genetic tests on *B. bassiana* collected from various darkling beetle populations to determine any genetic variation within the species. Twenty-four strains were identified, all of which showed considerable variability in their relative virulence to *A. diaperinus* larvae (Castrillo and Brooks 1998). Although most have found this fungal pathogen to be the most capable as a biological control agent, Gindin et al. (2009) also examined the pathogenic potential of *Metarhizium anisopliae* (Metschn.) Sorokin isolates against *A. diaperinus*. Their findings showed that both *B. bassiana* and *M. anisopliae* produced 5-97% mortality, however, larval mortality of greater than 80% was obtained with virulent strains of *M. anisopliae* but not *B. bassiana* (Gindin et al. 2009).

Adult and larval darkling beetles have also been observed infected with various protozoans such as *Gregarina alphetobii*, *Farinocystis tribolii*, and *Mattesia alphetobii* (Bala et al. 1990, Steinkraus et al. 1992). Although most entomophilic protozoa produce chronic infections, a few are extremely virulent (Brooks 1974). *M. alphetobii* is very pathogenic to darkling beetles and acts by destroying the fat body, which functions as a key center of metabolism and biochemistry in insects (Bala et al. 1990). *G. alphetobii* and *F. tribolii* are not usually found at high enough levels in broiler houses to greatly affect darkling beetle populations, however, it is possible that these protozoa can impact darkling beetle fecundity by augmenting the negative effects produced by other more virulent pathogens (Apuya et al. 1994).

Nematodes may also serve as potential biological control agents for *A. diaperinus*; however, populations of nematodes have to coincide with a high population of darkling beetles to be effective (Geden et al. 1987a). Initial laboratory studies, by Geden et al. (1985), showed that soil treated with entomogenous nematodes in the genera *Steinernema* and *Heterorhabditis* have some success in controlling *A. diaperinus*. *Steinernema feltiae* was infective against all stages of *A. diaperinus*, showing the highest levels of virulence against the larval stage. In a subsequent study conducted by Geden et al. (1987a), field evaluations with the All strain of *Steinernema feltiae* were conducted to determine if biological control with this nematode was possible. For three weeks, following house cleanout, nematode treated populations of *A. diaperinus* grew slower than the untreated populations (Geden et al. 1987a). Unfortunately, loss of persistence of *S. feltiae* 10-13 wk post-treatment resulted in essentially equal populations of beetles between treatments. *S. feltiae* did, however, persist in high numbers for 7 wk on two of the farms tested, providing 63-87% beetle mortality during this time (Geden et al. 1987a). Alternatively, less than 50% mortality was observed on the third farm only 3 wk post-treatment.

Szalanski et al. (2004) also examined the infectivity of *Steinernema* species. Of the three species observed, they found *Steinernema carpocapsae* and *S. feltiae* to be the most promising, however, infectivity of these two species varied greatly depending on the strain (Szalanski et al 2004).

Along with fungi, protozoans, and nematodes, a parasitic mite that attacks *A. diaperinus* eggs has also been evaluated as a possible control agent. *Acarophenax mahunkai* parasitized more than half of the egg masses in a beetle colony in a laboratory study conducted by Steinkraus and Cross (1993). This mite has many characteristics of an effective parasitic mite for biological control. It is very host specific, it has a shorter life cycle than *A. diaperinus*, and approximately 30 mite offspring can develop from one host egg (Steinkraus and Cross 1993).

#### Cultural and Mechanical Control

When used in combination with other control methods, cultural and mechanical control can be very effective at repressing *A. diaperinus* outbreaks and managing populations. Simple upkeep of equipment, such as maintenance of drinker lines, and other means of keeping the litter dry, reduces beetle numbers (Turner 1986). It has also been shown that treating the litter with alum (aluminum sulfate), a chemical used to control ammonia in litter, can reduce darkling beetle populations (Worley et al. 2000). In northern areas where sub-freezing temperatures persist, producers can reduce darkling beetle numbers by removing the litter and opening the broiler house up for a week or more (Dunford and Kaufman 2006). Frequent cleanouts and litter removal can assist in reducing beetle populations in a poultry facility (Hinton and Moon 2003). However, since litter removal can serve as a possible means of dispersal and reinfestation by *A. diaperinus* (Calibeo-Hayes et al. 2005), proper disposal of litter is essential. If the litter is applied to a field, as is often the case, mechanical incorporation may be necessary (Kaufman et al. 2005b). This has been shown in some cases to reduce beetle emergence, thereby reducing the

risk of disease transmission and poor public relations (Calibeo-Hayes et al. 2005, Kaufman et al. 2005b).

Mechanical barriers have also been tested in an attempt to deny late instar larvae access to vulnerable building structures, thus reducing damage to the facility (Geden and Carlson 2001). Field tests were conducted with polyethylene terephthalate (PET), which was used as a mechanical barrier. This barrier was >92% effective at 6 mo post-installation (Geden and Carlson 2001). However, when the PET was contaminated with fly fecal material, covering >80% of the plastic, efficacy of the barrier was reduced to 40-56% (Geden and Carlson 2001). Kaufman et al. (2005a) also evaluated the effectiveness of barriers against both adult and larval stages of *A. diaperinus* in caged-layer facilities. The plastic barrier was highly effective at preventing adult darkling beetle climbing; however, they also observed that its efficacy against larval climbing was reduced when fly specks increased (Kaufman et al. 2005a). Although this reduction in efficacy occurs, these barriers still serve as a fairly effective deterrent to beetle passage (Kaufman et al. 2005a).

### Monitoring

Monitoring is essential in a management program for darkling beetles. It is necessary in order to determine when control measures should be instigated (Axtell and Arends 1990) and it can be helpful when evaluating the efficacy of control practices. Facility premises can be monitored for darkling beetle activity by counting larvae on walls and posts just before dusk (Geden and Axtell 1987). Tube traps, which consist of corrugated cardboard inside a piece of plastic pipe, and Berlese funnels are both acceptable means of sampling beetle populations (Safrit and Axtell 1984, Stafford et al. 1988). While tube traps are commonly used for sampling, proper placement of the traps is crucial (Safrit and Axtell 1984). It is necessary to use a large

number of traps and make sure placement is consistent in order to get an accurate population estimation (Axtell and Arends 1990). There are some disadvantages to monitoring in this way. Tube traps often become inundated with beetles even when population densities are only moderately high, and there is a tendency to underrate young larvae when using this type of trap (Geden et al. 2001). This can lead to improper characterization of population age structure and, because of the saturation that occurs when darkling beetle densities are high, accuracy and sensitivity of the traps are lost (Geden et al. 2001). More research dealing with *A. diaperinus* pheromones and attractants could assist in creating a more sensitive monitoring method (Dunford and Kaufman 2006). Bartelt et al. (2009) did discover male specific compounds that are believed to act as an aggregation pheromone. However, the usefulness of this pheromone for monitoring and other management strategies remains to be determined (Bartelt et al. 2009).

### Chemical Control

The chemical warfare we wage against insect pests is not a novel act. It has been performed since the time of the ancient Romans who burned sulfur to kill insect pests (Delaplane 1996) and still plays an integral part of insect management today. However, chemicals were really not brought to the forefront of insect control until the advent of pesticides such as DDT, BHC, aldrin, dieldrin, and endrin (Delaplane 1996). Without pesticides the cornucopia of food and elevated standard of living that we benefit from would not exist (Delaplane 1996). We would experience dramatic losses in yield in many agricultural systems worldwide (Nauen and Bretschneider 2002).

Chemical applications have historically been found at the heart of most insect control programs, relying heavily on insecticides as their primary means of attack. With the development of DDT, the first insecticide to be used on a global scale (Denholm et al. 2002),

came the means necessary to increase crop and livestock production and decrease disease transmission. However, due to various reasons including environmental issues and resistance concerns, insecticide use is becoming less and less of the primary focus in insect control programs. Nevertheless, suppression of *A. diaperinus* populations in broiler facilities is still, in many ways, heavily reliant on insecticide use.

Historically the earliest recorded control measures for *A. diaperinus* in broiler facilities relied heavily on organophosphates, DDT, and carbamates (Harding and Bissell 1958, Simco et al. 1966). There are currently five classes of chemicals approved for use in broiler facilities in the United States. These chemical classes are organophosphates, pyrethroids, neonicotinoids, spinosyns and boric acid; with the most frequently used products consisting of pyrethroids and organophosphates (Szczepanik et al. 2008). Several pyrethroids are registered as premise treatments in broiler facilities (Salin et al. 2003), and boric acid is registered for use in some states as a soil and premise treatment (Geden et al. 2001). There are a variety of formulations of organophosphates, pyrethroids, and borates that have proven to be toxic to the darkling beetle when applied to structures or litter in laboratory tests; however, only temporary population reduction has been observed in the field (Vaughan et al. 1984, Arends 1987). This could be, in part, due to the fact that accumulation of dust on treated surfaces in broiler facilities reduces the effectiveness of premise treatments (Despins et al. 1991). Juvenile hormone analogues, insect growth regulators (IGRs), and avermectin have also shown promise in laboratory tests, but have yet to be verified as efficient in field use (Edwards and Abraham 1985, Weaver and Kondo 1987, Miller and Redfern 1988). The effects of lime hydrate have been evaluated in laboratory settings, providing increased mortality in adult and larval stages of *Alphitobius diaperinus*, however, no field trials have been conducted (Watson et al. 2003). Salin et al. (2003) tested the

effectiveness of a combined insecticide treatment consisting of cyfluthrin, an adulticide, and triflumuron, an insect growth regulator, against *A. diaperinus*. This combined treatment provided immense reduction in both adult and larval stages throughout the broiler grow-out period, achieving considerable control of populations by the end of the second treatment.

### Current Suppression Recommendations

With increasing demands for broiler meat, production systems have shifted to high density, intensive programs in controlled and confined housing systems (Axtell 1999). This shift not only benefits broiler production, but it also creates an ideal scenario for thriving darkling beetle populations (Axtell 1999). It is not unusual for populations in a typical broiler house to reach 1,000 beetles per square yard, which equates to over 2 million beetles in a single 20,000 ft<sup>2</sup> house (Rowland et al. 2007). Control methods of this particular pest are only partially successful (Axtell and Arends 1990), and long-term suppression is not very probable (Stringham and Watson 2008). In order to manage *A. diaperinus* populations it is essential that their behavior, biology, and ecology are well understood (Adams 2003). Recommended control measures consist of a combination of good management practices and informed insecticide use (Rowland et al. 2007).

Identification and management of potential infestation and re-infestation sites is a critical component in prevention of large beetle infestations. Locations of possible infestation include feed storage sites and areas of spilt feed. Sanitation around feed storage and cleaning of spilt feed will help to reduce this risk (Adams 2003). Litter stacking sheds can also serve as an area of re-infestation; this occurs when litter is removed from broiler houses during cake-out or complete cleanout and is temporarily stored in these facilities. Adult beetles infesting the litter are removed as well and immediately begin migrating back into houses that are closest to the



stack house (Rowland 2008). Within 2 d a large percentage will re-infest adjacent houses as well (Rowland 2008). To reduce the risk of this type of re-infestation, Rowland, director of Ivesco technical support, recommends 4 ft band application with permethrin under feed lines immediately after litter removal and that houses be closed for 24 h post-treatment. An alternative to this would be to chemically treat the stack shed. Rowland suggests utilization of the following products for this process: Ravap EC, StandGuard, Permethrin 10%, and Elector PSP. Application should be made immediately after litter is placed in the stack house and the entire surface of the litter should be treated using a high pressure/fine mist system.

Litter is also occasionally windrowed within the broiler houses. This is done to sterilize the litter for re-use. In this case, applying a registered insecticide in a 1 ft band down the sides of the windrow and over the top of the pile will provide chemical exposure to darkling beetles trying to escape (Rowland et al. 2007).

It is a common practice to apply the used litter from broiler houses to agricultural lands as a source of fertilizer; however, this can also serve as a means of re-infestation on a farm or invasion of other broiler houses and residential areas (Rowland 2008). Incorporation of the litter immediately after application can help reduce beetle emergence from the field, lowering the risk of their dispersal (Kaufman et al. 2005b).

Other litter management practices include more frequent cleanouts to prevent severe darkling beetle population buildup (Geden et al. 2001, Hinton and Moon 2003, Rowland et al. 2007) and reduction of litter moisture (Townsend 2010). However, increasing the frequency of complete cleanouts of broiler houses is not always practical due to the increased cost of shavings and limited means of litter disposal (Shah et al. 2006); consequently this practice has shifted from being performed annually to being done only once every two or more years (Stringham et

al. 1999). However, the benefits of increasing cleanouts may outweigh the issues if severe darkling beetle infestations occur (Rowland et al. 2007). Cleaning houses and leaving them opened in the winter may also help to drive the beetle population down if sub-freezing temperatures are sustained for at least a week (Dunford and Kaufman 2006). Controlling litter moisture is an easier and more manageable practice, which can be accomplished in part by routinely monitoring pipes and drinkers for any leaks and occasionally changing the position of drinkers to prevent extremely wet areas (Adams 2003, Townsend 2010). This will reduce the beetle's access to water, which is key to its survival (Townsend 2010).

Primary means of managing the darkling beetle rely heavily on insecticides (Axtell 1999). A list of currently approved insecticides can be found in the 2011 Georgia Pest Management Handbook (<http://www.ent.uga.edu/pmh>). While it is virtually impossible to completely eliminate darkling beetles from a broiler house through insecticide use (Townsend 2010), there are measures that can be taken to ensure maximal benefits from the treatments. When utilizing insecticides in a control program, timing of application, utilization of appropriate materials for type and location of treatment, identification of infested areas, and monitoring of product efficacy are crucial for a successful program (Cunningham et al. 2011).

The current regime for insecticide application after caking out is to treat immediately after litter removal, within 24-48 h following bird removal (Stringham and Watson 2007). This will provide higher percent mortality, since beetles remaining in the broiler house after cake out will be actively moving over the litter surface or just below it (Stringham and Watson 2007). There is some debate as to the best method of chemical application when all litter is removed from a house. One opinion is that best results can be obtained when insecticides are applied after new shavings are placed because beetles will crawl across the top of the litter to get to the feed

lines (Rowland et al. 2007). Others have found that application to the floor just before new litter is placed provides a higher percent mortality in beetle populations, reducing the number of darkling beetles for the next flock (Stringham and Watson 2007). However, it is agreed that mixing the insecticide in with the shavings is not advisable due to breakdown of the product by organic matter (J. Arends, personal communication, April 15, 2011). Also, if the litter being placed in the broiler house is actually recycled litter, chemical applications should be conducted after the litter is applied (Stringham and Watson 2007). Although these current recommendations are still being widely utilized, shifts in treatment regimes may be seen in the near future due to possible biological changes in the life-cycle of the darkling beetle, as it relates to broiler production cycles (J. Arends, personal communication, April 15, 2011).

Insecticide application rates and placements depend on the severity of the darkling beetle infestation. The new application recommendation is low volume, using a lawn or pull type 25 gal sprayer outfitted with a 7- to 8-ft adjustable articulated boom and low volume, flat fan nozzles (Stringham and Watson 2007, Stringham and Watson 2008). It is recommended not to exceed 12-15 gal in a 20,000 ft<sup>2</sup> house (Rowland et al. 2007, Stringham and Watson 2007). This new paradigm provides more flexibility in application and greater precision (Stringham and Watson 2007). The rate of application is also very important. Most products have a low and a high rate on the label, however, the difference between these rates really relates to the significance of the infestation (Stringham and Watson 2009). The low rate is not recommended even for only a moderate infestation (Stringham and Watson 2009). The placement of the insecticide should be targeted to treat areas where the highest numbers of beetles occur. This can vary depending on production style, temperature, and age of the flock (Stringham and Watson 2009); however, the greatest numbers of beetles will initially occur in the brooder area beneath

the feeders and along the walls (Stringham and Watson 2007). Therefore concentrating the treatment in the critical zones, from the walls to the inboard drinkers, will provide the greatest suppression of beetle populations (Stringham and Watson 2009). Insecticides should be applied in a 3 ft band under feeders and in a 3 ft wide band along the walls; this includes the footing and 2 ft up the sidewalls (Rowland et al. 2007). If labeling permits, secondary treatments using the highest acceptable rate can be applied 3 to 4 wk following the initial treatment in a band 18-21 in wide under feeders, which is where beetle numbers will be the highest (Stringham and Watson 2007). Seasonal adjustments to treatments, like reducing or halting treatment applications in the late summer to early fall and allowing natural suppression of populations by the cold, are commonly practiced (Stringham and Watson 2008). However, treating while numbers are low may actually assist in reducing the size of and impeding the onset of infestations occurring in the spring and early summer, when populations tend to explode (Stringham and Watson 2008).

Insecticides can lose their efficacy if not treated and handled properly. First of all, any control program utilizing insecticides should rotate between different chemical classes at least every two flocks to reduce or delay the onset of resistance (Rowland et al. 2007). Insecticides should be stored in a dark, cool and dry location, since extended exposure to sunlight and temperature extremes can diminish the efficacy of the product before the package is even opened (Stringham and Watson 2007). Efficacy can also be degraded when water soluble insecticides are mixed in neutral to alkaline water, which is known as alkaline hydrolysis (Stringham and Watson 2007). Adjusting the pH of the water for mixtures with pyrethroids and organophosphates by adding a packet of citric acid or PWT (sodium bisulfate) will help reduce this risk (Stringham and Watson 2007) and can improve the residual effects of these products by reducing the pH of the litter (Watkins and Donald 2002, Rowland et al. 2007). It has been found

that efficacy of an insecticide can also be compromised by tank mixing with a disinfectant (Geden et al. 1987b). Geden et al. (1987b) evaluated 56 different insecticide and disinfectant mixtures and out of those evaluated 24 showed reduced insecticidal activity. When it comes to chemical control, applying the label recommended amount of insecticide is crucial (Rowland et al. 2007). Application of more than the maximum amount of concentrate on the label is considered misuse of the product (Stringham and Watson 2007), however, it is also not recommended to use less than the labeled amount due to concerns of increasing resistance with this method of use (Rowland et al. 2007).

Monitoring darkling beetle populations to determine the efficacy of a beetle management program is crucial to ensuring the continuation of its success. This can be done by simply observing adults and larvae under feed pans when the birds are around 4-5 wk of age (Rowland et al. 2007). If few beetles are found it is a good indication that control measures are successful (Rowland et al. 2007). Since some insecticides take longer than others in providing control, looking at the number of beetles killed in the first 24 h is not always a good indicator of product effectiveness (Rowland et al. 2007).

While there is no “magic bullet” that is 100% effective at controlling *A. diaperinus* populations, centering control strategies around its behavioral and biological characteristics is an essential component of successful suppression strategies. Timely and appropriate incorporation of control strategies is important for successful suppression of *A. diaperinus* populations. However, successful suppression may require tailoring control programs specifically to the local beetle population.

## **Insecticide Resistance**

Insecticide resistance is a major concern when dealing with insect pests. Resistance is defined by the Insecticide Resistance Action Committee (IRAC) as “a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for the pest species”. Georghiou and Mellon (1983) defined resistance as “... a significant decrease in the normal level of response of a population toward an insecticide. Such change in sensitivity is understood to have resulted in diminished control ...”. The total costs associated with pesticide resistance are projected to range between 10 and 25% of the current treatment costs (Harper and Zilberman 1990), or around \$400 million annually in the United States (Pimentel et al. 1992). This implies that at least 10% of pesticide applications are conducted simply to fight increased resistance that has developed (Pimentel et al. 1992). Annual costs of resistance in the U. S. associated with pests of humans and livestock alone are approximately \$30 million (Pimentel et al. 1992). According to Gould (1991) there are at least 504 species of insects and mites that exhibit resistance to one or more common insecticides, and that number is continually increasing. Due to loss of insecticide efficacy, application frequencies and dosage are being increased (Pimentel et al. 1992). At the same time the effort to discover, develop, register, and manufacture new products is becoming increasingly more difficult, which leads to increased costs of new insecticides (Metcalf 1980). These heightened issues give rise to new and challenging complexities in all areas of pest control, and the poultry industry is by no means exempt from these challenges. Suppression of *A. diaperinus* populations in broiler facilities relies heavily on insecticide use due to the lack of alternative control measures available (Lambkin 2005). One major concern associated with the extensive use of insecticides is the development of resistance

in darkling beetle populations (Tomberlin et al. 2008). This concern is increasingly becoming a reality with recent detection of resistance in numerous *A. diaperinus* populations (Lambkin 2005, Hamm et al. 2006, Lambkin and Rice 2006, Lambkin and Rice 2007). High costs associated with insecticide use, short-term field efficacy of available products, and resistance development (Vaughan and Turner 1984, Weaver and Kondo 1987, Steinkraus et al. 1991, Tomberlin et al. 2008) make controlling the darkling beetle an almost impossible task.

While there are numerous products on the market that claim to drastically reduce darkling beetle populations, chemical control under field conditions is generally inadequate and transitory (Geden et al. 1998). Some of these products have also been shown to lose their insecticidal efficacy after years of repeated use. The first reported case of insecticide resistance in a field population of *A. diaperinus* was observed by Cogan et al. (1996). After a single application of iodofenphos SC (organophosphate), fenitrothion WP (organophosphate), permethrin WP (pyrethroid), and azamethiphos WP (organophosphate) to turkey broiler units in the UK, all but azamethiphos were found to be ineffective at suppressing beetle populations. In Australia Lambkin (2005) also examined fenitrothion, due to its apparent reduction in field efficacy, and found darkling beetles in broiler facilities to be highly resistant to this product. This work led to the decline of fenitrothion use in Australia (Lambkin and Rice 2006). With cyfluthrin (pyrethroid) replacing fenitrothion as the primary means of darkling beetle control in many parts of Australia, Lambkin and Rice (2006) examined *A. diaperinus* populations for any possible decline in response to this insecticide. Results from this study were not so promising. In almost all populations tested, resistance was detected with levels of up to 22 times that of the susceptible population (Lambkin and Rice 2006). The resistance level was found to be directly correlated with quantity of cyfluthrin applied (Lambkin and Rice 2006). They also found a strong

relationship between adult susceptibility to cyfluthrin and  $\gamma$ -cyhalothrin, indicating at least partial cross-resistance to  $\gamma$ -cyhalothrin in cyfluthrin-resistant populations (Lambkin et al. 2010). Lambkin and Rice (2007) also tested the efficacy of spinosad, one of the newer chemistries available against the darkling beetle. Spinosad was evaluated due to concerns of possible cross-resistance in populations of beetles showing resistance to cyfluthrin, however, no cross-resistance was observed (Lambkin and Rice 2007). Hamm et al. (2006) evaluated darkling beetles' susceptibility to cyfluthrin and tetrachlorvinphos (organophosphate) from various caged-layer poultry facilities. They found high levels of resistance to tetrachlorvinphos in two beetle strains that were tested. They also recorded cyfluthrin resistance in adult darkling beetles ranging from 1.7- to 9.5-fold, while larval resistance ranged from 0.5- to 29-fold. In Texas, four insecticides were evaluated for insecticidal efficacy against *A. diaperinus* (Tomberlin et al. 2008). Adult darkling beetles treated with Tempo SC Ultra (11.8%  $\beta$ -cyfluthrin) and Talstar WP (10% bifenthrin) showed the most susceptibility, with the greatest percentage of mortality being recorded (Tomberlin et al. 2008). Talstar Professional Insecticide (7.9% bifenthrin) showed fairly hopeful results for some populations, causing around 50% mortality (Tomberlin et al. 2008). This product was also the only product tested that showed an increase in darkling beetle mortality after 24 h (Tomberlin et al. 2008). Dagnet SFR (36.8% permethrin) exhibited the least effectiveness out of the four insecticides tested, with five out of six populations exhibiting <10% mortality (Tomberlin et al. 2008).

Currently it is still uncertain what the darkling beetle's resistance condition is to other commonly used pyrethroid and organophosphate products (Stringham 2006). The loss of insecticidal efficacy of such products often results in increased application frequencies and rates, as well as more costly replacements (Georghiou 1986). The rapidity with which resistance can



occur and the fact that some insect pests have developed resistance to almost all products labeled for use against them have led to the instigation of more sustainable control measures along with a more vigilant approach to insecticide application, utilization, and monitoring.

## **Broiler Industry**

### **Economic Impact**

Broilers are male or female chickens that are utilized exclusively for meat production (Constance 2008). The broiler industry, which is considered one of the most dynamic animal industries in the U. S., is an integral part of world agribusiness (Aho 2002a) and is one of the most successful sectors in U. S. agriculture (NCC 2002). Traditionally chicken meat was only a subsidiary of egg production (NCC 2002); however, in the 1920's farmers started raising chicks strictly for meat (NCC 2002), and thus the "broiler" was born. Nevertheless, it was not until the 1950's that the broiler industry experienced its economic boom (NCC 2002). With the advent of vertical integration, which is "the ownership and management of two or more successive stages of the marketing system by a single firm" (Aho 2002b), the broiler industry was able to become more efficient, responsive, and profitable (NCC 2002). By the 1960's vertical integration had taken a strong hold on broiler production, with 90% of broilers produced coming from integrated farms (NCC 2002). Today 99% of broilers are grown under contract integration (Cunningham 2009).

Poultry meat currently represents almost one-third of the meat produced and consumed worldwide (Scanes 2007), and in the U. S. production and consumption of poultry meat has surpassed that of beef and pork (Scanes 2007). The total number of broilers produced in the United States in 2010 was 8.63 billion, up 1% from 2009, while the value of broilers produced in the U. S. was up 9% at \$23.7 billion (USDA 2011).

Broiler production is a vital factor in Georgia's economy. Georgia has ranked number one in the U. S. in value of broiler production for the past fourteen consecutive years and has been the leader in broiler production for the past twenty-five consecutive years (Irvin 2009). In 2008 Georgia produced 1.41 billion birds, which accounted for 16 percent of the total number of birds produced in the United States for that year and 15 percent of the total pounds produced (Irvin 2009). The number of broilers produced in 2010 was around 1.31 billion birds, with total value of commercial broiler production equating to around \$3.3 billion (USDA 2011).

### **Contract Broiler Systems**

In a contract system under vertical integration the integrator generally provides the chicks, feed, and any medication, vaccinations, and supervision necessary (Bernard and Willett 1996). They also commonly own the facilities necessary for each stage of production, such as hatcheries, feed mills, and processing plants (Bernard and Willett 1996). The grower provides the housing, essential equipment for the grow-out period, and pays for inputs such as water, electricity, fuel, litter and labor (Bernard and Willett 1996). Producers are compensated for their efforts based on pounds of live birds produced (Cunningham 2009). One advantage to this system is that a large proportion of the risks associated with production and marketing are placed on the integrator and not solely on the producer (Cunningham 2009). Contract integration has allowed for substantial gains in production efficiency, which has led to more uniform, high-quality broilers, at lower prices (Martinez 2000).

### **Broiler Production and Beetle Livability**

Broiler houses are typically 40-50 ft wide, 400-600 ft long, with side walls 8 ft in height and compact dirt floors (Fairchild 2005). They provide maximum control over the bird's environment (Renck 2001), which is essential for survival and high productivity. Day-old chicks

are placed in the broiler house at the start of a new production cycle. Coinciding with the establishment of this new flock is the emergence of adult darkling beetles, serving as a source of reestablishment in broiler houses (Axtell and Arends 1990, Lambkin et al. 2007). Although unintentional, these beetles tend to be treated as well as or better than the birds, with many aspects of broiler production practices not only supporting bird growth, but also sustaining darkling beetle populations.

Environmental conditions conducive to broiler production are favorable to darkling beetle development and longevity (Axtell 1999). With preferred temperatures in broiler houses ranging from around 32-21°C, depending on age and function of the bird (Fairchild 2002), and relative humidity around 50-60% (Weaver and Meuerhof 1991), *A. diaperinus* populations can thrive (Sarin and Saxena 1973, Rueda and Axtell 1996).

A thick layer of litter consisting of wood-shavings, spilt feed, feces, and (on occasion) dead birds is found on the floor of broiler facilities. The frequency of complete litter clean-outs and replacement with clean shavings varies from grower to grower; however, this process normally occurs only once every two or three years (Stringham et al. 1999). This extended period between clean-outs results in increased litter depth and nutrient composition that provide long-term shelter and food for *A. diaperinus* populations (Axtell 1999, Stringham and Watson 2007).

Another aspect of production that is conducive for thriving *A. diaperinus* populations is the extended production cycle used for heavier birds (Stringham and Watson 2007), which is around 7 to 8 wk (Axtell 1999). During this time, with birds in the house, management strategies for darkling beetle suppression are limited, giving rise to population build-up.

Darkling beetles also utilize the insulation located throughout broiler facilities as a protective pupation substrate (Geden and Axtell 1987), damaging the insulation and causing as much as 67% increase in energy costs (Geden et al. 2001). With costs of fuel and electricity contributing nearly 60% of Georgia broiler production costs (Cunningham et al. 2010), increasing these expenses can be detrimental to producers.

## CHAPTER 3

### MATERIALS AND METHODS

#### Beetle Populations

Darkling beetle management programs usually vary among poultry production systems depending on the integrator. To represent potential differences, beetle populations were collected from 5 different broiler facilities selected to represent various large scale integrators located throughout the state of Georgia. The history of insecticide use was obtained from each broiler farm (See History of Insecticide Use). For comparison, an insecticide-susceptible population of *A. diaperinus* was acquired from P. Kaufman at the University of Florida. Historically this population was obtained from a laboratory colony in Denmark (Saturnia, Bjerring-brovej 48 2610 Rødovre, Denmark), and was used previously in other susceptibility studies conducted by Hamm et al. (2006) and Kaufman et al. (2008).

*A. diaperinus* populations were collected from August 2008 to September 2009. Both adults and larvae were acquired from under feed lines when birds were present and from aggregations in the corners of broiler houses if birds were removed prior to collecting. This was accomplished by collecting litter, which contained the larval and adult stages, using a garden trowel. Collections were then placed in 16 qt. (15 liter) plastic containers (Aero Plastic Inc., Leominster, MA) and transported to the University of Georgia Entomology laboratory. Populations containing all life stages were held in separate 5 gal (18.9 liter) glass tanks, fitted with Styrofoam pupation substrates. Chicken feed and apples were provided as sources of food

and moisture. Populations were held in the laboratory at 25°C ( $\pm$  3°C) under natural photoperiod.

### **History of Insecticide Use**

Farm F was 58.26 km (36.2 miles) from Farm U, the closest test farm, and had the longest production history of over 23 yr. In the ten years prior to beetle collecting, insecticide treatments at this facility had consisted of three different pyrethroids: Exile CS (active ingredient lambda-cyhalothrin), Tempo Ultra (active ingredient beta-cyfluthrin), and Tempo (active ingredient cyfluthrin). They had also utilized two formulations of the neonicotinoid imidacloprid, Dominion (active ingredient imidacloprid) and Credo SC (active ingredient imidacloprid); and the organophosphate Duratrol (active ingredient chlorpyrifos). At the time of the beetle collections they had not reported any apparent loss of efficacy of products being utilized.

Farm P was located 17.7 km (11 miles) from Farm U, and had been in production for 12 yr. Insecticide history for the past ten years had involved the use of three pyrethroids, Tempo (active ingredient cyfluthrin), Tengard (active ingredient permethrin), and Exile CS (active ingredient lambda-cyhalothrin); and two formulations of the organophosphate chlorpyrifos, Duratrol (active ingredient chlorpyrifos) and Durashield (active ingredient chlorpyrifos). Loss of efficacy of Tempo had been observed at this facility.

Farm U was located approximately 17.7 km (11 miles) from Farm P. It had been in production for over 12 yr, however, in the past ten years they had applied only Tempo (active ingredient cyfluthrin), which is in the pyrethroid chemical class. Loss of efficacy of this product had been reported.

Farm S was the most distant farm, at 344.4 km (214 miles) from Farm U. It had been in production for 5 yr, during which time they had applied a broad range of products including the juvenile hormone mimic NyGuard (active ingredient pyriproxyfen); pyrethroids Tempo (active ingredient cyfluthrin), Bifentrin (active ingredient bifenthrin), and Permethrin Pro (active ingredient permethrin); neonicotinoids Delphi (active ingredient imidacloprid) and Dominion (active ingredient imidacloprid); and an organophosphate Durashield (active ingredient chlorpyrifos). The producer had noticed possible resistance occurring to the active ingredient bifenthrin.

Farm H was 31.2 km (19.4 miles) from Farm P and had been in production for only 3 yr, utilizing a single pyrethroid, Tempo Dust (active ingredient cyfluthrin), and had reported no loss in efficacy.

### **Chemical Products**

Technical grade spinosad (90% [AI], Elanco Animal Health, Greenfield, IN), bifenthrin (94.88% [AI], Dr. Jeff Tomberlin, Texas A&M, College Station, TX), and imidacloprid (95% [AI], Bayer HealthCare LLC, Shawnee, KS) were evaluated against six populations of adult *A. diaperinus*. These are all currently registered as premise treatments for darkling beetle control in broiler facilities. Bifenthrin and imidacloprid must be applied after birds are removed, however, spinosad can be applied while birds are present in the house.

### **Spinosad (Spinosyn)**

Spinosad belongs to the chemical class spinosyns. This unique chemical group is derived through the aerobic fermentation of a soil actinomycete, *Saccharopolyspora spinosa*, which was discovered in the early 1980s by Eli Lilly and Company (Kirst 2010). This chemical class has low environmental effects but shows strong insecticidal activity against a broad range of

important pest species of crops, as well as external parasites of livestock, companion animals, and humans (Kirst 2010). Spinosad is registered under the U.S. EPA Reduced Risk Pesticide Program and is classified as an organic substance by the USDA National Organic Standards Board (Dow 2008). It was first commercialized by DowAgroscience in 1997 (Thompson and Hutchins 1999), specifically for control of caterpillar pests resistant to broad-spectrum insecticides such as pyrethroids (Bret et al. 1997). It is comprised of a mixture of 85% spinosyn A and 15% spinosyn D, two of the most active macrolides isolated from the soil bacterium (Nauen and Bretschneider 2002). Spinosad is a neurotoxin activating insect nicotinic acetylcholine receptors (nAChRs) and prolongs acetylcholine response similar to imidacloprid and other nAChR-based insecticides via a novel binding site (Nauen and Bretschneider 2002). It also has effects on the gamma-amino butyric acid (GABA) receptor function, which may also contribute to its insecticidal activity (Thompson et al. 2009). While the exact mode of action of spinosad has not yet been completely defined (Salgado and Sparks 2005), this biological product is known to cause excitation of the nervous system, leading to involuntary muscle contraction, cessation of feeding, paralysis and eventually death of the insect (Thompson et al. 2000). Spinosad is a rapid contact and ingestion toxicant, which was first introduced into agricultural animal systems for fly control (Stringham and Watson 2007). Labeling of this active ingredient for darkling beetle control in the U. S. occurred in 2002 under the trade name Elector® PSP. Due to its low vertebrate toxicity, this product can be applied while birds are present in the house, giving producers more flexibility in their treatment program (Stringham and Watson 2007). Residual effects of spinosad are limited due to photodegradation and sensitivity to certain disinfectants utilized in broiler houses (Stringham and Watson 2007).



In Australia, Lambkin and Rice (2007) evaluated spinosad efficacy on 13 darkling beetle populations and a cyfluthrin/fenitrothion-resistant population. No preexisting resistance was observed in any of the beetle populations and there was no cross-resistance to spinosad in the cyfluthrin/fenitrothion-resistant population (Lambkin and Rice 2007). However, some resistance to spinosad has been reported in insect populations in Hawaii, Mexico, and Pakistan, along with varying degrees of cross-resistance in a variety of pest species (Kirst 2010). Strong resistance has also been selected for in laboratory evaluations of *Musca domestica* L. (Diptera: Muscidae) (Scott 1998), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) (Zhao et al. 2002), and *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) (Wyss et al. 2003). While some spinosad resistance has developed in insect populations, this biological product has shown overall to have outstanding insecticidal efficacy, resulting in its rapid registration and vast acceptance by agriculturalists worldwide (Thompson et al. 2000). Spinosad is considered a great fit for IPM programs, reducing the likelihood of resistance development through its novel mode of action, its low activity with respect to beneficial insects, and its moderate residual effects (Thompson et al. 2000). The discovery of spinosyns and the subsequent development of spinosad have provided the world with an entirely novel class of chemicals (Thompson et al. 2009).

### **Bifenthrin (Pyrethroid)**

Pyrethroids are synthetic insecticides based on the structure of pyrethrins, which are natural compounds isolated from the *Chrysanthemum* genus of plants (Casida 1980). Studies on the chemistry of this class of insecticides were initiated around 1910 (Katsuda 1999), however, they were not introduced to the market until the late 1970s (Wirtz et al. 2009). They are the third largest class of chemical insecticides, with a market value of \$1.3 billion (Wirtz et al. 2009). Pyrethroids have a broad range of insecticidal applications, including agricultural crop land,

companion animals and livestock, urban landscapes and gardens, urban structures, and areas of public health (Fishel 2005, Spurlock and Lee 2008). They offer desirable traits such as rapid knockdown activity at low rates, relatively low mammalian toxicity, and improved environmental stability (Fishel 2005).

Bifenthrin, which is found in the pyrethroid class of insecticides, is a neurotoxicant acting on the nervous system of insects through the modulation of sodium channels, causing paralysis of the insect (Salgado et al. 1983). It is a fourth-generation pyrethroid, which was discovered and developed by FMC Corporation Pty Ltd (Mukherjee et al. 2010). In 1985 an Experimental Use Permit was issued for use of bifenthrin on cotton in the U. S., and a Conditional Registration was issued in 1988 (Dong 1995). It has been registered for use against *A. diaperinus* in broiler facilities since 2008 as ActiShield™ Liquid Insecticide. Bifenthrin has greater insecticidal activity and photostability compared with earlier pyrethroids (Mokry and Hoagland 1990) and is virtually insoluble in water (Mukherjee et al. 2010). However, it is a restricted used pesticide due to its high toxicity to fish and other aquatic organisms (Fecko 1999) and is classified by the World Health Organization as moderately hazardous (WHO 1998). With both contact and ingestion toxicity, bifenthrin is active against a broad spectrum of arthropod pests (Mukherjee et al. 2010). Active as an insecticide and acaricide, bifenthrin targets pests such as caterpillars, grasshoppers, fleas, ants, cockroaches, moths, beetles, mites, aphids, thrips, scales, termites, mosquitoes, scorpions, wasps, and spiders (WHO 2010). However, numerous arthropods have developed resistance to bifenthrin, including the two-spotted spider mite (*Tetranychus urticae*) (Ay and Gürkan 2005), western flower thrips (*Frankliniella occidentalis*) (Immaraju et al. 1992), the southern chinch bug (*Blissus insularis*) (Cherry and Nagata 2007), and the tarnished plant bug (*Lygus lineolaris*) (Snodgrass 1996), among others.

Efficacy of pyrethroids on both the adult and larval stage of the darkling beetle has been investigated since the 1970s (Saxena and Sarin 1972, Vaughan and Turner 1984, Geden et al. 1987b), with the first report of control failure occurring in the UK in 1996 (Cogan et al. 1996). Since this time, efficacy of commercial formulations of bifenthrin has been evaluated for *A. diaperinus* control by Tomberlin et al. (2008). Their findings indicated that sufficient field control was accomplished in some of the darkling beetle populations examined, however, strong resistance to other pyrethroids, such as cyfluthrin, has been observed both in Australia and in the United States (Hamm et al. 2006, Lambkin and Rice 2006). With rising concerns of pyrethroid resistance it is important to continue evaluating pyrethroid products commonly used by broiler producers.

### **Imidacloprid (Neonicotinoid)**

Imidacloprid is a member of the neonicotinoid class of insecticides, based on compounds produced by tobacco plants (Kim 2006). The discovery of imidacloprid was initiated in 1970 at Purdue University; however, imidacloprid was not synthesized until 1984 by chemists at what is now known as Nihon Bayer (Maienfisch et al. 2001). It was introduced to the market in 1991 by Bayer CropScience, making it the first member of the neonicotinoid class of chemicals to be commercialized (Liu et al. 2005). Imidacloprid, like bifenthrin and spinosad, targets the nervous system of the insect, acting as an agonist to the nicotinic acetylcholine receptor (Liu et al. 2005). This leads to overstimulation of the nervous system causing convulsions, paralysis, and death (Bloomquist 2009). It is the most widely used neonicotinoid (Jeschke and Nauen 2005), accounting for approximately 41.5% of the entire neonicotinoid market (Jeschke et al. 2011). Imidacloprid has a broad spectrum of activity ranging from crop protection, animal health applications, and urban pest management (Liu et al. 2005, Jeschke et al. 2011). It is categorized

as moderately toxic by the EPA, falling under both toxicity class II and class III, which requires the signal word “Warning” or “Caution” on the label (Fishel 2005). Due to their low mammalian toxicity, extensive insecticidal potency, and broad systemic properties (Gangadasu et al. 2009), neonicotinoids are currently the fastest-growing class of insecticides (Jeschke & Nauen, 2005), with estimated worldwide sales of approximately \$1 billion annually (Liu et al. 2005). In 2008 imidacloprid was registered for darkling beetle control under the trade name Credo SC™, which can be applied as a whole house treatment or banded for treatment of target areas. Field trials with Credo SC showed residual efficacy of around 6 wk (Stringham and Watson 2008).

While still a concern, resistance to neonicotinoids has been slow to develop, is localized geographically, and is still manageable (Kim 2006). To date, imidacloprid has not been evaluated for darkling beetle resistance; however, resistance has been found in a number of other significant insect pests including the Colorado potato beetle (*Leptinotarsa decemlineata*), and the whitefly, *Bemisia tabaci* (Nauen and Denholm 2005). With limited products available for darkling beetle control, increased applications of readily available insecticides, like those containing imidacloprid, will set the stage for resistance development in this insect pest.

### **Culture Method**

A culturing system was employed to obtain large batches of adult beetles that were of a similar age, developed under uniform conditions, and had no prior exposure to insecticides (Rice and Lambkin 2009). This allows for limiting variability in test results due to differences in generation, age, and holding conditions of treated beetles (IRAC 2009). The culturing technique used in this study is based on the novel method laid out by Rice and Lambkin (2009), with slight modifications.

One hundred adults from each population were removed from their laboratory holding containers using a U.S. standard 2.0 mm sieve and placed in a 2.25 liter Ziploc® plastic container, which was labeled with the population name and date of culture initiation. The number of adults selected was based on work by Winks (1981) who considered 100 parent beetles the lowest number required to preserve genetic integrity of a coleopteran strain in culture. Culture boxes contained 1000 ml of culture medium, consisting of 76% wheat bran (Mennel Milling Co., Fostoria, OH), 17% chicken feed (University of Georgia Poultry Feed Mill, Athens, GA), and 7% torula yeast (BioServ, Frenchtown, NJ) by volume, which is proportional to culture medium utilized by Rice and Lambkin (2009). This combination of media material is one of the most common culture diets utilized in darkling beetle rearing systems (Barké and Davis 1967, Rueda and Axtell 1996). Two apple halves, which were washed prior to use to ensure that no chemical residue was present, were placed face down on the medium (Rice and Lambkin 2009). Rice and Lambkin (2009) found that apples provided the most suitable source of moisture. A section of the lid (10cm x 10cm) was removed from each culture container and replaced with a piece of insect mesh to allow adequate air flow. Beetles were incubated at 32°C and 55% relative humidity in a Natureform incubator (Natureform Hatchery Systems, Jacksonville, FL). These environmental conditions have been shown to be optimum for darkling beetle development under culturing conditions (Wilson and Miner 1969, Winks 1981, Rice and Lambkin 2009). They were maintained on a 16:8 h (L:D) photoperiod (Steelman 2008) through the use of a Timex® light controller. The light source utilized was a 60 watt compact fluorescent, which produces a light output of 830 lumens. Compact fluorescent lights are often utilized in broiler facilities because of their energy saving abilities.

Adult darkling beetles placed in culture boxes were allowed 4 d to reproduce, giving them sufficient time to recover from any shock imposed by handling that might interfere with reproduction (Rice and Lambkin 2009). They were then removed from the boxes by sieving the medium with a U. S. standard 2.0 mm sieve. Twenty-one days post initiation two fresh apple halves were placed in each box, to ensure adequate moisture and food availability. The medium containing the larval progeny was removed from the boxes on day 35 and pupation substrates consisting of two pieces of extruded polystyrene foam (17 x 4 x 2.5 cm) were placed in each container. This provided a protected pupation site similar to what is exploited by larvae in broiler facilities (Vaughan et al. 1984). One apple half was positioned face down on each pupation substrate. The medium, along with the larvae, was then returned to the boxes and incubation was continued. At 56 d the culture medium and pupation substrates were sieved and broken apart to remove newly emerged adults. Adults were counted and placed in new holding boxes, containing fresh medium (half the original volume) and an apple half, and incubated for 7 d. Insecticide tests were then initiated, with treatment completion within 21 d of progeny removal (Lambkin 2005).

## **Topical Bioassays**

### **Preliminary Bioassays**

Insecticide bioassays were performed on the adult stage of the darkling beetle using a combination of the topical dosing procedure performed by Lambkin (2005) and Lambkin and Rice (2006), with adjustments when necessary. The topical bioassay method provides reliable data that are more linear in response compared to other insecticide testing methods (Lambkin 2001, 2005). Lambkin et al. (2010) demonstrated its repeatability and found it to be overall a reliable method for resistance testing of adult darkling beetles (Lambkin et al. 2010). Bioassays

conducted were limited to the adult stage of *A. diaperinus*, which has been shown to exhibit a higher level of resistance for most products tested, compared to the larval stage (Vaughan and Turner 1984, Hamm et al. 2006). Six dilution series doses of each insecticide were evaluated during preliminary tests to determine the appropriate dose range that would provide around 10-90% mortality in each population (Steelman 2008, IRAC 2009). Preliminary doses consisted of a 12,745 ppm stock solution of insecticide concentration, which consisted of technical grade chemical in 100 ml of AR grade acetone (Fisher Scientific, Fair Lawn, NJ), with serial dilutions of 1274.5 ppm, 127.45 ppm, 12.745 ppm, 1.2745 ppm, and 0.12745 ppm. For each dose 4 replications were conducted, treating a total of 100 beetles (25 beetles per replication) at each dose, including the control group.

Before insecticide treatments, adult darkling beetles from each population were extracted from culture boxes using a U. S. standard 2.0 mm sieve, counted into groups of 25, and placed into corresponding 30 ml plastic cups labeled with the strain and dose level (Lambkin 2005). Batches of beetles were weighed before each treatment to determine mean weight of individual beetles from each population. Once ready to treat, beetles were removed from cups and placed onto a Koolit® freezer block (12 x 15 x 1.4 cm), rendering them torpid, making insecticide application feasible (Lambkin and Rice 2006). After beetles were immobile they were transferred to a glass panel (10 x 15 cm) where 1 µl of each insecticide dilution (technical grade chemical in AR grade acetone) was applied to the ventral side of individual beetles with a hand micro-applicator (Burkard Manufacturing Co. Ltd., Hertfordshire, England). The micro-applicator was equipped with a 1 ml Burkard glass syringe and a hypodermic needle (3/10 x 25 mm). Control beetles were treated with 1 µl of AR grade acetone. Darkling beetles were then returned to their corresponding cups, which contained two pieces of damp sponge (10mm<sup>2</sup>), and

were placed in front of a fan for 5 min to allow rapid evaporation of the acetone (Lambkin 2005). Beetles and sponge were then poured into a 2 ½ oz. Conex® portion container (Dart Container Corp., Mason MI). Containers held one tablespoon of wheat bran and were incubated at 25°C and 55% relative humidity (Lambkin 2005). Mortality was evaluated at 4, 24, 48 and 72 h for spinosad; however, due to insecticidal recovery observed during preliminary trials, beetles treated with imidacloprid and bifenthrin were also evaluated at 96 and 120 h post-treatment. Assessments were conducted by allowing the beetles to walk across a given surface (30 x 20 cm) and observing their movements. Those unable to walk or perform normal muscular functions involved in walking (those with jerky, uncoordinated movements) were classified as dead, while those that executed movement similar to the control group were classified as alive (Lambkin 2005).

### **Dose-Response Bioassay**

Dosing technique, postdosing procedure, mortality assessment, and criterion for mortality was consistent for all bioassays through employment of the methods listed above. One hundred individuals from each population were treated as a control (25 beetles per replication) for each test, and for the field-collected populations a minimum of 252 beetles were treated per dose. This was based on studies conducted by Lambkin (2001, 2005) indicating that 200 beetles is the minimum accepted treatment number for topical bioassays (Lambkin and Rice 2006). Doses evaluated varied between treatments depending on preliminary data, with a minimum of 5 doses tested per population. The sample size and doses evaluated for the Denmark susceptible population also varied between treatments, depending on preliminary response and requirements necessary to obtain an LD<sub>99.9</sub> (Robertson et al. 2007), which is a dose that affects a high percentage of susceptible genotypes in a population but does not affect resistant genotypes in a



resistant population (Roush and Miller 1986). This dose can be utilized for subsequent resistance monitoring and determination of the frequency of resistant individuals in a population (Zettler and Cuperus 1990).

For bifenthrin the number of doses applied and the rates evaluated varied among the darkling beetle populations. Doses utilized bracketed the preliminary doses, providing a range in mortality with a lower limit of >0-10% and upper limit of 90-100%. The stock solution for bifenthrin was 12,734 ppm, which consisted of 1,000,000 µg of active ingredient in 100 ml of acetone. For Farms U and H doses used were as follows: 12.745 ppm, 127.45 ppm, 637.25 ppm, 1274.5 ppm, and 6372.5 ppm, with only Farm U receiving the 6,372.5 ppm rate. Farm S was treated with the same doses as Farm U, with the addition of 63.725 ppm. Farm P was treated with a dose range of 127.45 ppm, 318.625 ppm, 636.7 ppm, 892.15 ppm, 1274.5 ppm, 2549 ppm, and 6372.5 ppm. For Farm F doses started at 318.625 ppm and comprised the same dose range as Farm P with the exception of the 892.15 ppm rate, which was excluded. A total of 2,880 beetles from the Denmark population (320 beetles per dose) were treated with 9 doses, which consisted of 31.8625 ppm, 63.725 ppm, 95.5875 ppm, 127.45 ppm, 318.625 ppm, 637.25 ppm, 955.875 ppm, 1593.125 ppm and 3186.25 ppm.

Preliminary treatment with spinosad indicated relative uniformity in dose-response between populations, so doses evaluated were the same for each population. The stock solution consisted of 1,000,000 µg of active ingredient in 100 ml of acetone, and the doses evaluated ranged from 318.625 ppm to 6,372.5 ppm, with intermediate doses consisting of 637.25 ppm, 955.875 ppm, and 1274.5 ppm. Doses for Farm S did not exceed 1274.5 ppm. The insecticide-susceptible population (Denmark strain) was treated with 8 doses. Doses were 127.45 ppm, 318.625 ppm, 637.25 ppm, 955.875 ppm, 1274.5 ppm, 1911.75 ppm, 3186.25 ppm, and 6372.5

ppm. Four hundred adults were treated per dose, giving a total of 3,200 individuals treated from the Denmark population.

For imidacloprid, preliminary bioassays indicated variations in response between populations, with relatively low mortality occurring at the original stock solution of 12,745 ppm (1,000,000  $\mu\text{g}$  of active ingredient in 100 ml of acetone) compared with the other chemical treatments. Therefore, Farms F and P, which exhibited the lowest levels of mortality, were treated with doses of 127.45 ppm, 1274.5 ppm, 12,745 ppm, 25,490 ppm, and 63,725 ppm, with only Farm F receiving the 127.45 ppm rate. Farms H, S, and U, which showed slightly more sensitivity, were treated with 12.745 ppm, 127.45 ppm, 1274.5 ppm, 12,745 ppm, 25,490 ppm, and 63,725 ppm of the insecticide solution. For the susceptible Denmark population a total of 2,800 individuals were treated (400 beetles per dose) with a series of doses consisting of 3.8235 ppm, 7.647 ppm, 15.294 ppm, 38.235 ppm, 76.47 ppm, 127.45 ppm, and 318.625 ppm.

## **Statistical Procedure**

### **Logit Analysis**

Logit analysis is a technique used to analyze the binomial response in biological assays based on logistic distribution. It was first created in the 19<sup>th</sup> century to analyze the growth of populations and the course of chain reactions (Cramer 2003). It was introduced as an alternative to probit for analyzing bioassays by Joseph Berkson in 1944, however, it was not until 1960 that the logit transformation was widely adopted (Cramer 2003). While outputs from this type of analysis are similar to results from probit, the distributions of the two differ. Probit assumes a normal distribution and logit uses a logistic distribution (Robertson et al. 2007). Logit has a wide use in statistical theory and applications. It is used in the social sciences and in educational research, particularly higher education (Peng et al. 2002).

Logit analysis is commonly used in binary dose-response bioassays with one explanatory variable to assess the relationship between the level of a stimulant and the probability of response (Robertson et al. 2007). To perform this type of logit analysis the response must be binomial (e.g. dead/alive). Multinomial quantal response can also be assessed using the multinomial logit model (Robertson et al. 2007). Toxicologists frequently use logit analysis to determine the toxicity of substances to biological organisms (Hayes 1989). This is accomplished by observing the response of groups of organisms to various concentrations of a substance and determining the concentrations at which a response is obtained (Hayes 1989). The results of logit analysis can then be utilized to compare toxicities of different amounts of the substance to the organism.

This specialized regression model is also used by entomologists to determine the susceptibility of a population of insects to certain insecticides (Robertson et al. 2007). This is accomplished by observing the population's response to various doses of the insecticide and comparing their response to the response of a "susceptible" population. A sigmoid relationship is displayed between the response and the various doses, which is transformed to linear by logit analysis, and a regression on the relationship is performed (Finney 1971). By utilizing logit analysis, a dose-response regression can be fitted to the mortality and LD<sub>50</sub> values (dose at which 50% of the population responds) can be estimated (Robertson et al. 2007). The resistance factor is then obtained by comparing the LD<sub>50</sub> of a field population to the LD<sub>50</sub> of the "susceptible" population (Robertson et al. 2007).

For the purpose of this study dose-response regressions were fitted to mortality data for all populations using logit analysis in SAS Version 9.2 (SAS Institute Inc., Cary, NC, USA). Mortality in all control groups was  $\leq 2\%$ , therefore no correction for control mortality was made

(Sosa-Gómez et al. 2009, Kamgang et al. 2011). The LD<sub>50</sub> values (with 95% fiducial limits) for each population and the LD<sub>99.9</sub> value (with 95% fiducial limits) for the Denmark population were estimated from the regression equations (Finney 1971). A comparison between the LD<sub>50</sub> value for each broiler farm population and the LD<sub>50</sub> value of the susceptible Denmark population was made to determine resistance ratios (Lambkin and Rice 2006). Based on results obtained from the bioassays, a discriminating dose was set to approximate the LD<sub>99.9</sub> of the Denmark population (Lambkin and Rice 2006).

The mean individual weights of beetles from each population were determined by weighing multiple batches of 30-100 beetles just before testing. The ANOVA procedure using SAS Version 9.2 (SAS Institute Inc., Cary, NC, USA) was performed to determine if there was any significant difference in beetle weights between populations.

## CHAPTER 4

### RESULTS AND DISCUSSION

#### Results

Susceptibility data for *Alphitobius diaperinus* populations exposed to bifenthrin, imidacloprid, and spinosad are presented in Table 1. Of these three insecticides evaluated, *A. diaperinus* populations showed the most variability in response to imidacloprid, with less variability to bifenthrin and spinosad (Table 1). The Denmark reference strain was the most susceptible to all three insecticides tested.

#### **Bifenthrin**

Dose-response regression estimates for bifenthrin are shown in Table 1. Dose-response curves (Fig. 1) for the 5 field populations evaluated clustered into two distinct groups whose fiducial limits at the LD<sub>50</sub> did not overlap. Farm H, Farm U, and Farm S exhibited the most sensitivity to bifenthrin with LD<sub>50</sub>s of 0.01995, 0.02806, and 0.03486% bifenthrin respectively. Farm P and Farm F exhibited decreased sensitivity, with LD<sub>50</sub>s ranging from 0.13914 to 0.18675% bifenthrin respectively. All populations tested had significantly higher LD<sub>50</sub>s compared to the susceptible Denmark population, based on lack of overlap of fiducial limits. Resistance ratios for the five farm populations indicated varying levels of resistance occurring to bifenthrin, with resistance ratios for the most sensitive populations (Farm H, U, and S) ranging from 2.71 to 4.74, while Farms P and F exhibited significantly higher resistance ratios of 18.90 and 25.37 respectively. The steepest slope was 6.65, obtained from the susceptible Denmark population, while the other slopes were all flatter, ranging from 3.19 to 4.67. For future

bifenthrin susceptibility tests a discriminating dose of 0.08%, which is approximately the LD<sub>99.9</sub> obtained from the Denmark susceptible population, is proposed.

### **Imidacloprid**

Extremes in response to imidacloprid were exhibited by the 5 field populations tested with no overlap in fiducial limits at the LD<sub>50</sub> (Table 2). Farm H exhibited the most susceptibility to imidacloprid (LD<sub>50</sub> = 0.01498) with a resistance ratio of 5.7 when compared to the Denmark population (LD<sub>50</sub> = 0.00263). The remaining populations exhibited drastic levels of resistance to imidacloprid, ranging from 44- to 3,605-fold, with the lowest LD<sub>50</sub> of the resistant populations obtained from Farm U (LD<sub>50</sub> = 0.11500) and the highest LD<sub>50</sub> from Farm F (LD<sub>50</sub> = 9.48035). The Denmark population again exhibited the steepest slope (5.12), with the flattest slope exhibited by Farm U (0.82). All other slopes ranged from 0.94 to 1.92 (Fig. 2). A discriminating dose of 0.06% is proposed for future susceptibility tests, which approximated the LD<sub>99.9</sub> of the most susceptible population (Denmark).

### **Spinosad**

In contrast to dose-response regressions obtained with imidacloprid treatments, relatively consistent levels of sensitivity were obtained for all five populations to spinosad (Table 3). Resistance ratios ranged from 1.37 to 2.88, with the highest level of sensitivity obtained from Farm U and the lowest from Farm F. The LD<sub>50</sub> value for the Denmark population was significantly different from the other populations based on nonoverlapping fiducial limits (Table 1). No history of spinosad use had been reported from the five farm populations. The slopes obtained from all 5 farm populations treated with spinosad were relatively similar, with the steepest slope obtained from Farm H (6.90) and the flattest slope from Farm F (6.16) (Fig. 3). A

discriminating dose of 0.43% is proposed for future studies, which approximated the LD<sub>99.9</sub> of the Denmark population.

### **Beetle Weights**

The average weights, in grams, of beetles from the 6 populations were compared to determine if there was any significant difference between populations that could play a role in insecticide response. Average individual weights ranged from 0.0134 g to 0.0168 g (Table 4) with analysis indicating no significant difference in beetle weights between populations ( $R^2 = 5.86\%$ ,  $P = 0.0521$ ).

### **Discussion**

Insecticide resistance has been found in numerous populations of *Alphitobius diaperinus* worldwide. Topical bioassays have been conducted to determine susceptibility of *A. diaperinus* populations both in the United States and in Australia. Vaughan and Turner (1984) utilized the topical dosing method to determine toxicity of various insecticides to both the larval and adult stages of the darkling beetle. In Australia Lambkin (2005) performed topical bioassays, finding up to 79-fold resistance to fenitrothion in *A. diaperinus* populations collected from long-established broiler houses, leading to the discontinuation of fenitrothion for darkling beetle control in Australia. Using the topical application method, resistance to cyfluthrin in field populations of *A. diaperinus* has also been evaluated by Lambkin and Rice (2006). They detected resistance in almost all populations of darkling beetles assessed, with up to 22-fold resistance occurring in some populations. More recently, Lambkin et al. (2010) utilized this testing method to evaluate cyfluthrin-resistant and susceptible darkling beetle populations to determine if cross-resistance could be conferred to  $\gamma$ -cyhalothrin. Their finding indicated that

cyfluthrin can confer cross-resistance to  $\gamma$ -cyhalothrin, however, the extent of cross-resistance conferred is unpredictable.

While multiple methods have been utilized for susceptibility testing, Lambkin et al. (2010) demonstrated the reliability and repeatability of the topical testing method by performing repeated tests and regression analyses of darkling beetle populations to  $\gamma$ -cyhalothrin. They found no significant difference between the slopes and intercept values in repeated dose-response assays for each population tested, and the dose-response curve from a single treatment fitted the data from each bioassay within populations.

Resistance and tolerance are two terms that have been under considerable discussion throughout the literature and are often mistakenly used interchangeably. Roush (1980) defined resistance as "... a genetic change, a response to pesticide selection". In contrast, tolerance is referred to by Roush (1980) as "... a natural ability, where no selection has been exerted, to contrast with resistance". However, the standard for use of the term resistant versus tolerant seems to vary throughout the literature. Valles et al. (1997) suggested that insects should not be considered resistant to a chemical until resistance ratios exceed 10. A similar standard was utilized by Ahmad et al. (2008) in determining resistance of *Spodoptera litura* to newer insecticides such as spinosad, indoxacarb, fipronil, and abamectin using a standard leaf disc bioassay. They classified resistance ratios of 1 as susceptibility, tolerance to low resistance at levels of 2 to 10, moderate resistance at 11-30, high resistance at 31-100, and greater than 100 was considered very high resistance. Shelton et al. (1993) evaluated insecticide resistance of the diamondback moth to methomyl, permethrin, and methamidophos using leaf-dip bioassays, stating that though no standards had been defined to determine problematic resistance levels, resistance ratios greater than 10 would probably lead to control problems in the field. However,



Byford et al. (1999) suggested that when pyrethroid resistance is examined in the horn fly, a resistance ratio of 2- to 7-fold with no overlap of fiducial limits indicates insecticide tolerance, while ratios greater than 7-fold indicate resistance. In the same study significant resistance ratios of around 1.5- to 3-fold indicated tolerance to diazinon and ivermectin, with ratios greater than 3 indicating resistance in horn fly populations. When evaluating insecticide resistance in field populations of *Spodoptera litura* using leaf dip bioassays, Ahmad et al. (2007) generally considered resistance factors less than 10-fold as very low resistance, which did not translate to control failure in the field, 11- to 20-fold was considered low resistance, with some control failures observed, 21- to 50-fold was moderate resistance, 51- to 100-fold was high, and greater than 100-fold was very high resistance. Moderate to very high resistance levels observed in the laboratory bioassays did result in field failure of the insecticide evaluated. Cochran (1996) examined the relevance of resistance ratios found for chlorpyrifos, acephate, cyfluthrin, and cypermethrin to operational control in the German cockroach using the lethal-time method. He found that resistance ratios greater than or equal to 2.8 resulted in less than 90% mortality in operational control. Other investigators have used the dose-mortality regression to determine resistance in the German cockroach to chlorpyrifos, finding ratios of greater than 10-fold to result in decreased efficacy in the field (Rust and Reiersen 1991). However, in most cases resistance ratios obtained by dose-mortality regression are higher than those resulting from time-mortality tests (Cochran 1996).

For the purpose of this study a combination of factors was acknowledged when resistance or tolerance to a product was presumed. These factors include the resistance ratios obtained from the topical bioassay tests, historical use of insecticidal products at collection sites, and observational reports of control failure to products applied in facilities. Surrounding land use

was also determined in considering indirect insecticide exposure. However, no information was obtained pertaining to chemical use in those areas.

## **Bifenthrin**

Bifenthrin has been registered for darkling beetle control since 2008. Since this time there have been no published data on *A. diaperinus* susceptibility to bifenthrin using topical bioassays; however, commercial formulations of bifenthrin were evaluated in broiler facilities by Tomberlin et al. (2008) who detected some resistance to these products. Current research has revealed that resistance and cross-resistance in darkling beetle populations have occurred to other pyrethroids such as cyfluthrin, permethrin,  $\beta$ -cyfluthrin, and  $\gamma$ -cyhalothrin (Cogan et al. 1996, Hamm et al. 2006, Lambkin and Rice 2006, Tomberlin et al. 2008, Lambkin et al. 2010). Bifenthrin resistance has also been observed in other arthropod pests. Field populations of western flower thrips (*Frankliniella occidentalis*) exhibited as much as 138-fold resistance to bifenthrin (Immaraju et al. 1992), while southern chinch bugs (*Blissus insularis*) collected from St. Augustine grass throughout Florida exhibited resistance ratios as high as 736.3 (Cherry and Nagata 2005). Cross-resistance to bifenthrin has also been conferred by other classes of chemicals. As much as 7-fold cross-resistance to bifenthrin was observed in an imidacloprid-resistant strain of silverleaf whitefly, *Bemisia argentifolii* (Prabhaker et al. 1997).

Results from the topical bioassay combined with observational efficacy from field applied products indicate that some resistance is being exhibited by *A. diaperinus* populations to bifenthrin. Bifenthrin has not been labeled for darkling beetle control for very long, however, varying levels of susceptibility were observed in *A. diaperinus* populations. Resistance ratios ranged from 2.7 to 25.4 for the five populations collected, and of these populations only Farm S had reported prior use of bifenthrin in control programs. While the other 4 populations had not

had any known exposure to bifenthrin, various pyrethroids had been exploited at all locations for control of *A. diaperinus*. Of the five beetle populations examined, the steepest slope was 4.67 (Farm P) and the flattest was 3.19 (Farm S); all were flatter than the Denmark susceptible laboratory population (6.65).

Farm S had been in production for only 5 yr, in which time they had utilized numerous insecticidal products including various pyrethroids, neonicotinoids, and organophosphates. While a complete loss of control with bifenthrin had not been reported, they had reported an observational increase in beetle numbers with bifenthrin in facility applications, indicating that the 4.7-fold reduction in sensitivity exhibited in the topical bioassay test was possibly translating to lower levels of efficacy in the field.

Farm H had been in production the shortest period of time (3 yr) and reported using only cyfluthrin in beetle control programs. No observational field failure of cyfluthrin had been reported, suggesting that the 2.7-fold increase in tolerance exhibited by Farm H did not correlate to control failure in the field at that time. However, with only one chemical being exploited decreased sensitivity could rapidly emerge (Georghiou 1986, Byford et al. 1999, Kranthi et al. 2001, Romero et al. 2007) and cross-resistance to chemicals in this same chemical class (pyrethroid) such as bifenthrin could establish (Lambkin and Furlong 2011). Prabhaker et al. (1998) compared resistance levels of *Bemisia argentifolii* subjected to various insecticide regimes in greenhouses. They found that sequential use of a single chemical alone conferred more rapid resistance at higher levels compared to populations exposed to chemicals in a rotational scheme.

Farm U exhibited a 3.8-fold reduction in sensitivity to bifenthrin compared to the Denmark population. They had also reported the use of only cyfluthrin, however, they had been

utilizing this product for at least the past 10 yr and control failure to cyfluthrin had been reported at this location. While it is uncertain at this point whether this will translate to control failure with bifenthrin in facility applications, it is evident that with prior loss of control due to heavy selection with a single pyrethroid, failure with bifenthrin could be inevitable (Byford et al. 1985). This situation was observed in a greenhouse population of citrus thrips that were selected for using fluvalinate (pyrethroid) for 10 mo. With extensive selection pressure with a single pyrethroid, 128-fold cross-resistance was conferred to bifenthrin (Immaraju and Morse 1990).

Farm F and Farm P exhibited the highest loss of sensitivity, with 29.4- and 18.9-fold resistance respectively, and the highest slopes, indicating that populations in these broiler houses had become less heterogeneous and more resistant in frequency and intensity to bifenthrin (Immaraju et al. 1989). Both farms had a long history of pyrethroid use including cyfluthrin, permethrin,  $\lambda$ -cyhalothrin, and  $\beta$ -cyfluthrin, however, only Farm P reported loss in efficacy to cyfluthrin. With these high resistance ratios, control failure in field applications of bifenthrin would be expected.

Many insects resistant to a single pyrethroid exhibit a broad cross-resistance to other pyrethroids (Byford et al. 1985). High levels of bifenthrin resistance (ranging from 138-fold to 1,279-fold) were observed in western flower thrips (*Frankliniella occidentalis*) collected from greenhouses in coastal California where no prior use of bifenthrin had been reported (Immaraju et al. 1992). This was believed to be due to strong cross-resistance among pyrethroids since both pyrethrum and fluvalinate had been utilized at collection sites. Pyrethroid cross-resistance has been previously reported in darkling beetle populations. Lambkin et al. (2010) evaluated cyfluthrin-resistant and susceptible darkling beetle populations to determine if cross-resistance could be conferred to  $\gamma$ -cyhalothrin. Their finding indicated that cyfluthrin can confer cross-

resistance to  $\gamma$ -cyhalothrin, however, the extent of cross-resistance conferred is unpredictable. Byford et al. (1985) evaluated two populations of *Haematobia irritans*, one resistant to permethrin and the other to fenvalerate, and found them to exhibit cross-resistance to three other pyrethroids examined (cypermethrin, flucythrinate, and deltamethrin).

### **Imidacloprid**

While imidacloprid has had a long history of use for control of agronomic crop pests, it has been registered for darkling beetle control only since 2008. Extreme variability in response to imidacloprid was observed for all five farm populations evaluated, with resistance ratios ranging from 5.7- to >3,000-fold.

Only two farms had reported using imidacloprid for darkling beetle control, Farm F, which exhibited the highest rate of resistance, and Farm S which was 124-fold resistant. Neither farm reported observational failure of imidacloprid for darkling beetle control. Farm H, which had been in production for only the past 3 yr, had the lowest resistance ratio of 5.7 and has not reported any prior use of imidacloprid. Farm U exhibited reduced sensitivity by as much as 43.7-fold. This location has been in production for over 12 yr, exclusively utilizing cyfluthrin in control programs. The second highest loss of sensitivity was observed in Farm P, with a resistance ratio of 508-fold. Farm P has been in production for 12 yr and has had no previous use of imidacloprid, however, they have utilized numerous pyrethroids and organophosphates. The shallow slopes and high resistance ratios obtained from these five populations may indicate that populations are possibly in transition to higher intensities of resistance (Immaraju et al. 1992).

With the more recent registration of this product and paucity of use in most facilities evaluated, it was surprising to find such high levels of resistance occurring. Resistance to

imidacloprid has occurred fairly rapidly in other insect pests such as the Colorado potato beetle, which exhibited over 100-fold resistance after only 3 yr of commercial use (Zhao et al. 2000). This may partially account for the resistance levels observed in Farm S (RR = 124) where various commercial products containing imidacloprid have been utilized, however, this would not account for resistance in areas with no previous use and is not likely to be the only factor causing the extremely high levels of resistance found in Farm F (>3,000).

Another factor that could contribute to loss of sensitivity to imidacloprid is cross-resistance, which has been observed to occur with organophosphate, pyrethroid and carbamate resistant populations. Up to 100-fold variation in response to imidacloprid was observed in lygus bug (*Lygus hesperus*) populations collected from Arizona in 1995, with the highest tolerance exhibited by strains also resistant to various organophosphates (Dennehy and Russell 1996). Olson et al. (2000) evaluated the susceptibility of 134 geographically discrete populations of Colorado potato beetles to imidacloprid, finding significant variation in response to imidacloprid before its use in the field. Resistance ratios obtained from populations from Long Island ranged up to 29-fold over the 4 yr study, with much of the tolerance present prior to use of imidacloprid. They attributed the initial tolerance observed in the Long Island populations to broad-spectrum cross-resistance due to heavy insecticide use in this area, which is comparable to our study in that resistance levels were highest in populations where heavy insecticide use has occurred. Multiple studies suggest that mechanisms conferring resistance to chemical classes such as organophosphates, carbamates, and pyrethroids cause a reduction in sensitivity to imidacloprid (Sone et al. 1995, Zhao et al. 1995, Prabhaker et al. 1997), which may be demonstrating the potential for broad-spectrum detoxification systems encompassing

neonicotinoids as well as carbamates, organophosphates, and/or pyrethroids (Nauen and Denholm 2005).

Non-target exposure through insecticide drift could also be a contributing factor in the resistance levels observed. Less than 0.1% of pesticide applications reach their target pest, meaning that more than 99.9% of pesticides move into the environment (Pimentel 1995). Salyani and Cromwell (1992) evaluated drift from typical spray applications on citrus in Florida, finding measurable drift for all applications up to 195 m downwind. Indications of indirect exposure resulting in loss of efficacy to imidacloprid in darkling beetle populations have been found through observational assessments, with more frequent reports of lack of imidacloprid efficacy occurring from broiler houses located near agronomic row crops compared to houses in mountainous regions not located near these areas (J. Arends, personal communication, April 15, 2011). Lack of imidacloprid efficacy has been reported with the first application of this product in broiler houses near agricultural land (J. Arends, personal communication, April 15, 2011). This could be due to the long-term and broad-spectrum use of imidacloprid in agronomic settings. Imidacloprid was launched in 1991 by Bayer CropScience and today is the most widely used neonicotinoid (Jeschke and Nauen 2005), accounting for approximately 41.5% of the entire neonicotinoid market (Jeschke et al. 2011). Spray formulations of imidacloprid allow for possible off-site movement through drift (Fossen 2006). Evidence that imidacloprid residues can drift off-site on plant debris was demonstrated by Greatti et al. (2006) who detected imidacloprid residues on plants growing adjacent to a field sown with seed-treated corn. There is not much in the literature pertaining to insecticide drift and non-target resistance development; however, this has been evaluated in numerous vector species, which have been found to exhibit resistance to insecticides utilized in agricultural treatments (Mouchet 1988). Wilson and Cain (1997)

postulated that the substantial resistance exhibited by *Drosophila melanogaster* to an assortment of insecticides not directed for control of this insect could be due to selection with agricultural chemicals that have been in long-term widespread use.

### **Spinosad**

Spinosad has been registered in the U. S. for darkling beetle control since 2002. Topical bioassays have been conducted to determine *A. diaperinus* susceptibility to spinosad in Southern Australia (Lambkin and Rice 2007). Lambkin and Rice (2007) also examined if cyfluthrin/fenitrothion resistance in darkling beetle populations could confer cross-resistance to spinosad. They found no resistance to spinosad in darkling beetle populations and no cross-resistance to spinosad was conferred.

Topical bioassays with spinosad indicated relative uniformity in response between populations, with resistance ratios ranging from 1.4 to 2.9. Steep slopes were exhibited by all six beetle populations tested ranging from 6.16 to 6.90; therefore a high degree of homogeneity is exhibited within each population. There was no report of spinosad use in any of the facilities where beetle populations were collected. Therefore, the low levels of decreased sensitivity observed to spinosad are not due to insecticide selection, but more than likely due to natural tolerance. Levot et al. (2002) evaluated a spinosad naïve population of sheep blowfly to determine baseline susceptibility. While they observed as much as a 16-fold difference in  $LC_{50}$  (concentration that kills 50% of the population) values between the least and most susceptible field strains evaluated, they attribute this to natural variation among susceptible field populations and determined that spinosad would be extremely useful in control strategies. Variation in response to spinosad was also observed in house flies collected from dairy farms before spinosad was used, with percent survival ranging from 16% to 21% at the  $LD_{99}$  of the susceptible strain



(Deacutis et al. 2006). Scott et al. (2000) also evaluated fly populations to determine the levels of natural variation that existed before commercial use of spinosad. Variations among populations were observed, with populations from one facility exhibiting greater than 60% survival at the LC<sub>99</sub>. However, they concluded that spinosad still appeared to be a promising new product for control of house flies.

Since the introduction of spinosad in 1997, resistance and cross-resistance have been found in several insect pests. Spinosad resistance has been selected for in laboratory colonies of *Musca domestica* L. (Diptera: Muscidae) (Scott 1998) and *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) (Wyss et al. 2003). However, the most remarkable laboratory selected species was *Bactrocera dorsalis* Hendel (Diptera: Tephritidae), which attained a 408-fold resistance to spinosad after only eight generations of selection (Zhao et al. 2002). Zhao et al. (2002) found strong resistance to spinosad in approximately 50% of field populations of diamondback moth, *Plutella xylostella*, collected from crucifers in Hawaii after only 2.5 years of field use. Low levels of cross-resistance to spinosad in permethrin-, naled-, and malathion-resistant populations of the diamondback moth have also been observed; however, this was found in only a few of the resistant populations tested (Shelton et al. 2000, Hsu and Feng 2006). With little to no cross-resistance conferred to spinosad by other products due to its novel mode of action (Levot et al. 2002, Shono and Scott 2003, Wang et al. 2005) and its lack of use for *A. diaperinus* control, it appears that the low levels of tolerance exhibited by these five populations would not translate to control failure in the field, indicating that spinosad is still a promising chemical for *A. diaperinus* control. However, with resistance rapidly developing in other insect populations, continued monitoring and the induction of integrated management approaches are important aspects in reducing the likely development of spinosad resistance in *A. diaperinus*.

## CHAPTER 5

### CONCLUSIONS

Insecticide resistance is a worldwide phenomenon that became a widespread problem in the 1940s (Georghiou 1986). Insects were actually the first pest to develop resistance to modern pesticides and have repeatedly demonstrated their ability to overcome practically all pesticides developed (Elzen and Hardee 2003). Resistance is often associated with reduced sensitivity of the target site or enzymatic detoxification of an insecticide (Brown 1987). Rapid development of resistance in just a few or a single season can drastically affect control strategies and production systems (National Resource Council 1986). With decreasing insecticidal resources and increasing cases of resistance, strategies must be put into practice for the prevention and management of this problem (Georghiou 1994).

Topical bioassays conducted indicated that varying levels of resistance and tolerance are occurring in *A. diaperinus* populations treated with newer available insecticides. However, it appears that spinosad implementation in beetle management programs would give good overall control. Future studies are needed to elucidate the loss of sensitivity that is being observed in *A. diaperinus* populations to the three products examined and to determine if this loss of sensitivity is correlating to loss of efficacy in field applications. First, resistance mechanisms need to be evaluated (Brown 1987). While Lambkin and Furlong (2011) determined that metabolic mechanisms were partially responsible for pyrethroid resistance, this alone did not completely explain pyrethroid resistance in *A. diaperinus* populations. Therefore, further studies need to be conducted to determine other mechanisms involved in pyrethroid resistance and to determine

mechanisms conferring resistance to imidacloprid and spinosad in darkling beetle populations. Secondly, biochemical studies need to be performed to determine if preexisting resistance mechanisms are conferring cross-resistance to insecticides, both within a single chemical class as well as broad-spectrum cross-resistance between chemical classes. Determining if broad-spectrum detoxification systems are present in resistant *A. diaperinus* populations would help explain the high levels of resistance being observed, particularly to imidacloprid. Thirdly, a detailed evaluation of nearby land use and techniques for monitoring drift of agrochemicals would have to be conducted to determine if indirect contact of insecticides is a factor in resistance development.

Literature reporting reliability of topical laboratory bioassays and correlation to field control is variable. Insecticide susceptibility tests utilizing topical bioassay of 3<sup>rd</sup> instar western corn rootworm (*Diabrotica virgifera virgifera*) using technical grade insecticide provided similar results to a field study (Wright et al. 2000). Topical bioassays have also been found to be appropriate for organophosphate and pyrethroid resistance determination in *Blattella germanica* (Scott et al. 1986, Milo et al. 1987, Zhai and Robinson 1991). However, others have shown a lack of field correlation with results obtained by topical laboratory test methods (Arthur and Zettler 1991, Robertson and Preisler 1992). Due to these variabilities, future laboratory tests should compare mortalities between individuals receiving topical contact versus treated surface contact (Tomberlin et al. 2008). Since suppression of *A. diaperinus* in the field is achieved through direct contact of insecticide with the beetles or through residual contact of insecticides on treated surfaces (Tomberlin et al. 2008), evaluating multiple bioassay techniques can help determine the most sensitive method suitable for detection of resistance (Prabhaker et al. 1996).

Also, use of methods that better simulate exposure in the field applications make bioassays more realistic (Robertson and Preisler 1992).

In order to better manage darkling beetle populations, a correlation between laboratory data and percentage of field mortality following chemical applications needs to be established (Hamm et al. 2006). This will allow resistance levels obtained in bioassays to be correlated to expected field performance (Immaraju et al. 1989). Therefore, field trials utilizing data obtained from topical bioassays tests need to be conducted, with subsequent monitoring of beetle populations utilizing the discriminating dose obtained from the susceptible population. Information pertaining to frequency and rates of field applications also needs to be obtained to correlate operational factors with rates of resistance (Mason et al. 1989).

Resistance of *A. diaperinus* populations to registered insecticides is a major concern for the poultry industry worldwide. While predicting resistance in darkling beetle populations is difficult, the first step toward detection of changes in beetle sensitivity is the development of baseline susceptibility data (Siegfried et al. 2000). Baseline response data provide a reference for tracking changes in insecticide susceptibility over time (Olson et al. 2000, Smirle et al. 2002) and information necessary to provide practical pest management guidance (Castle et al. 1996). The implementation of IPM practices such as rotation among chemicals with different modes of action, continued monitoring for insecticide resistance, and the exploitation of biological and cultural control practices are all integral parts to creating a more effective control program.

Table 1. Results of dose-response assays with bifenthrin

Populations	Slope ( $\pm$ SE)	LD <sub>50</sub> (% AI) (95% FL)	LD <sub>99.9</sub> (% AI) (95% FL)	RR (at LD <sub>50</sub> )	X <sup>2</sup>	df
Denmark	6.65 ( $\pm$ 0.33)	0.00736 (0.0070-0.0071)	0.080 (0.0653- 0.1032)	-	5.17	7
Farm H	3.38 ( $\pm$ 0.20)	0.01995 (0.0174-0.0227)	-	2.71	3.80	2
Farm U	3.36 ( $\pm$ 0.29)	0.02806 (0.0225-0.0344)	-	3.81	7.46	3
Farm S	3.19 ( $\pm$ 0.20)	0.03486 (0.0295-0.0412)	-	4.74	8.21	4
Farm P	4.67 ( $\pm$ 0.32)	0.13914 (0.1262-0.1544)	-	18.90	37.67	5
Farm F	4.63 ( $\pm$ 0.58)	0.18675 (0.1512-0.2352)	-	25.37	51.74	3

Table 2. Results of dose-response assays with imidacloprid

Populations	Slope (± SE)	LD <sub>50</sub> (% AI) (95% FL)	LD <sub>99.9</sub> (% AI) (95% FL)	RR (at LD <sub>50</sub> )	X <sup>2</sup>	df
Denmark	5.12 (±0.22)	0.00263 (0.0024-0.0028)	0.059 (0.0456-0.0796)	-	14.80	5
Farm H	0.94 (±0.07)	0.01498 (0.0086-0.0241)	-	5.70	28.69	4
Farm U	0.82 (±0.07)	0.11500 (0.0690-0.1857)	-	43.73	16.74	4
Farm S	0.94 (±0.05)	0.32670 (0.2452-0.4352)	-	124.22	4.99	4
Farm P	1.92 (±0.26)	1.33565 (1.1367-1.6631)	-	507.85	4.84	2
Farm F	1.53 (±0.22)	9.48035 (5.8796-20.3721)	-	3604.7	21.56	3

Table 3. Results of dose-response assays with spinosad

Populations	Slope ( $\pm$ SE)	LD <sub>50</sub> (% AI) (95% FL)	LD <sub>99,9</sub> (% AI) (95% FL)	RR (at LD <sub>50</sub> )	X <sup>2</sup>	df
Denmark	6.49 ( $\pm$ 0.25)	0.03671 (0.0349-0.0386)	0.426 (0.3619- 0.5147)	-	1.66	6
Farm U	6.71 ( $\pm$ 0.41)	0.05015 (0.0470-0.0532)	-	1.37	0.17	3
Farm S	6.82 ( $\pm$ 0.41)	0.05379 (0.0506-0.0569)	-	1.47	0.72	2
Farm H	6.90 ( $\pm$ 0.41)	0.05664 (0.0534-0.0598)	-	1.54	5.06	3
Farm F	6.25 ( $\pm$ 0.70)	0.08786 (0.0810-0.0956)	-	2.39	16.51	3
Farm P	6.16 ( $\pm$ 0.56)	0.10566 (0.0968-0.1176)	-	2.88	14.13	3

Table 4. Mean weights of *A. diaperinus* populations

Population	N	AvgWt	
		Mean	Std Dev
Farm F	38	0.0154	0.00515081
Farm H	30	0.0139	0.00338113
Farm P	38	0.0137	0.00378576
Farm S	31	0.0141	0.00464884
Farm U	34	0.0168	0.00687600
Denmark	15	0.0134	0.00345929



### Dose-Response Bifenthrin

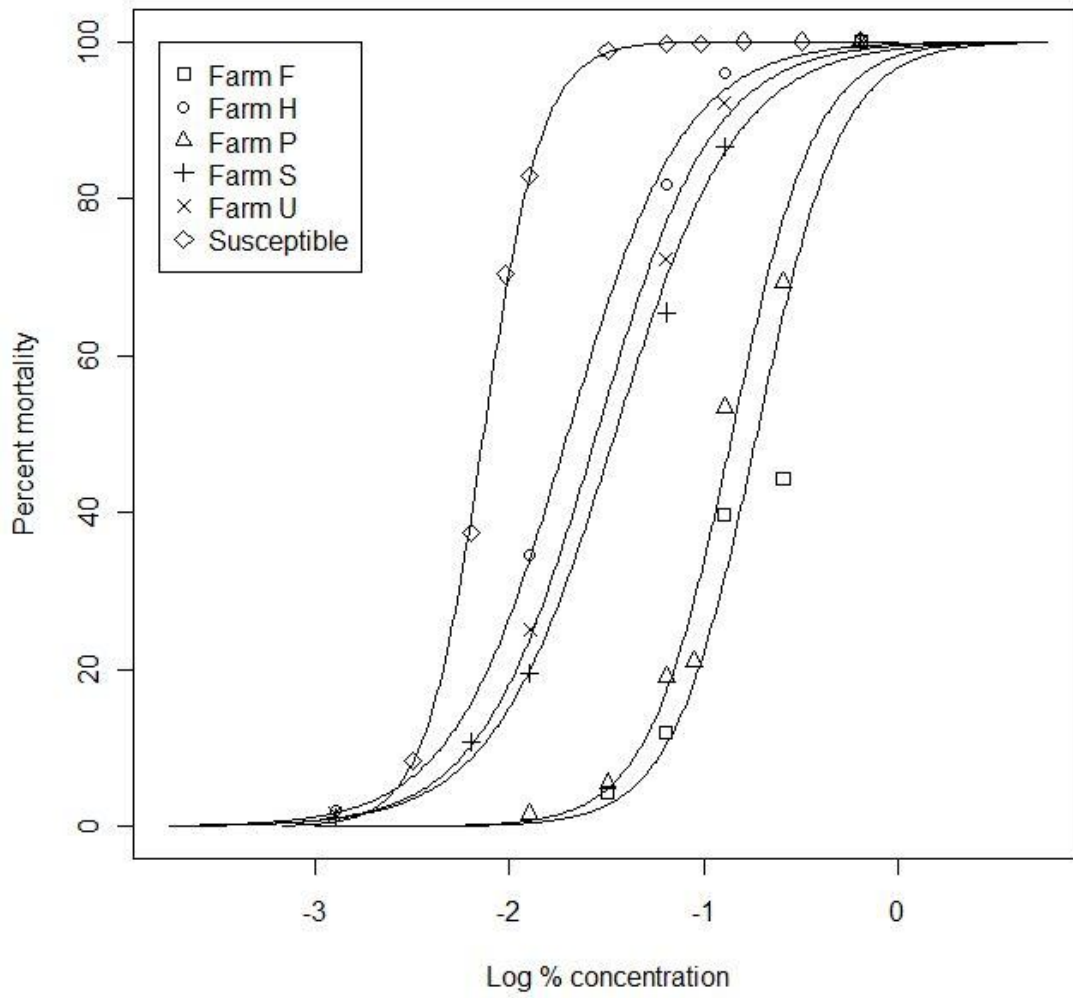


Figure 1. Dose-response curves for all populations topically treated with bifenthrin.

### Dose-Response Imidacloprid

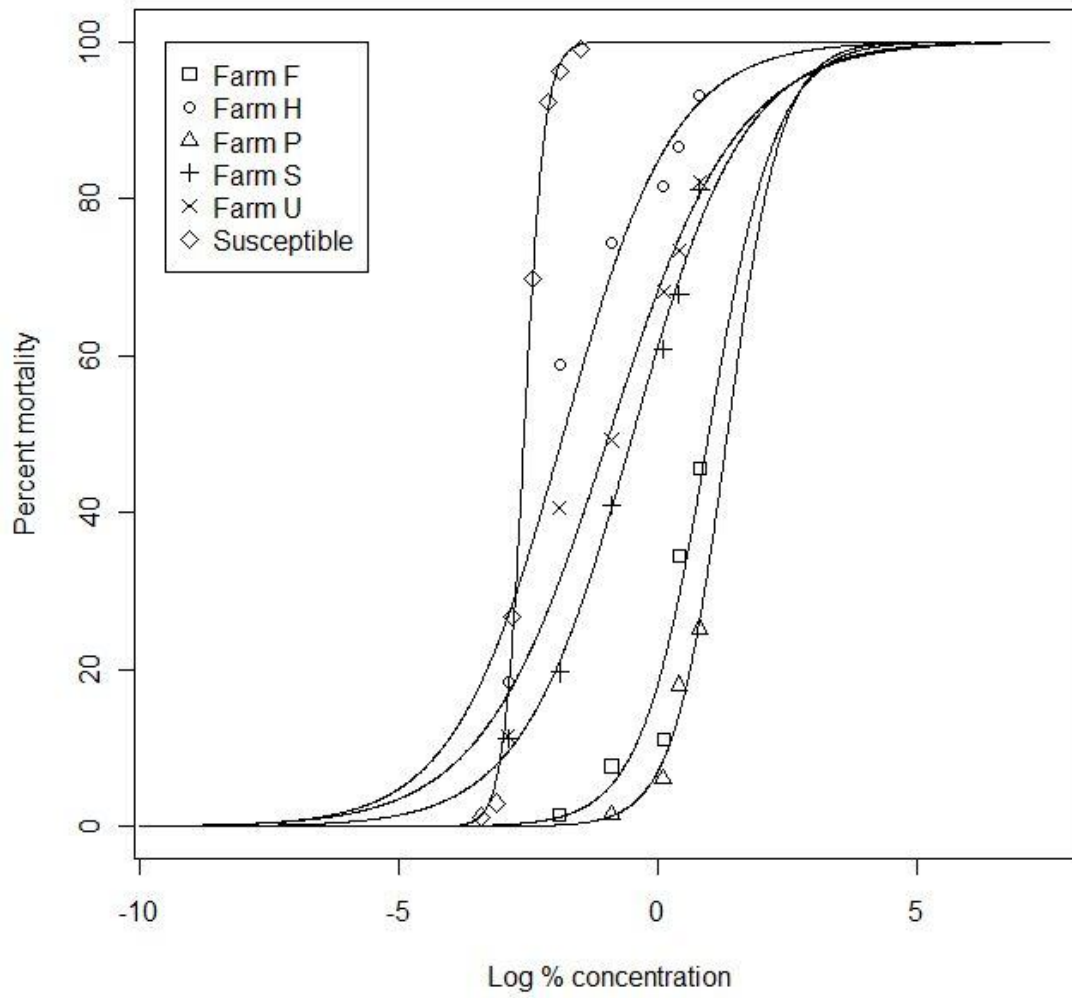


Figure 2. Dose-response curves for all populations topically treated with imidacloprid.

### Dose-Response Spinosad

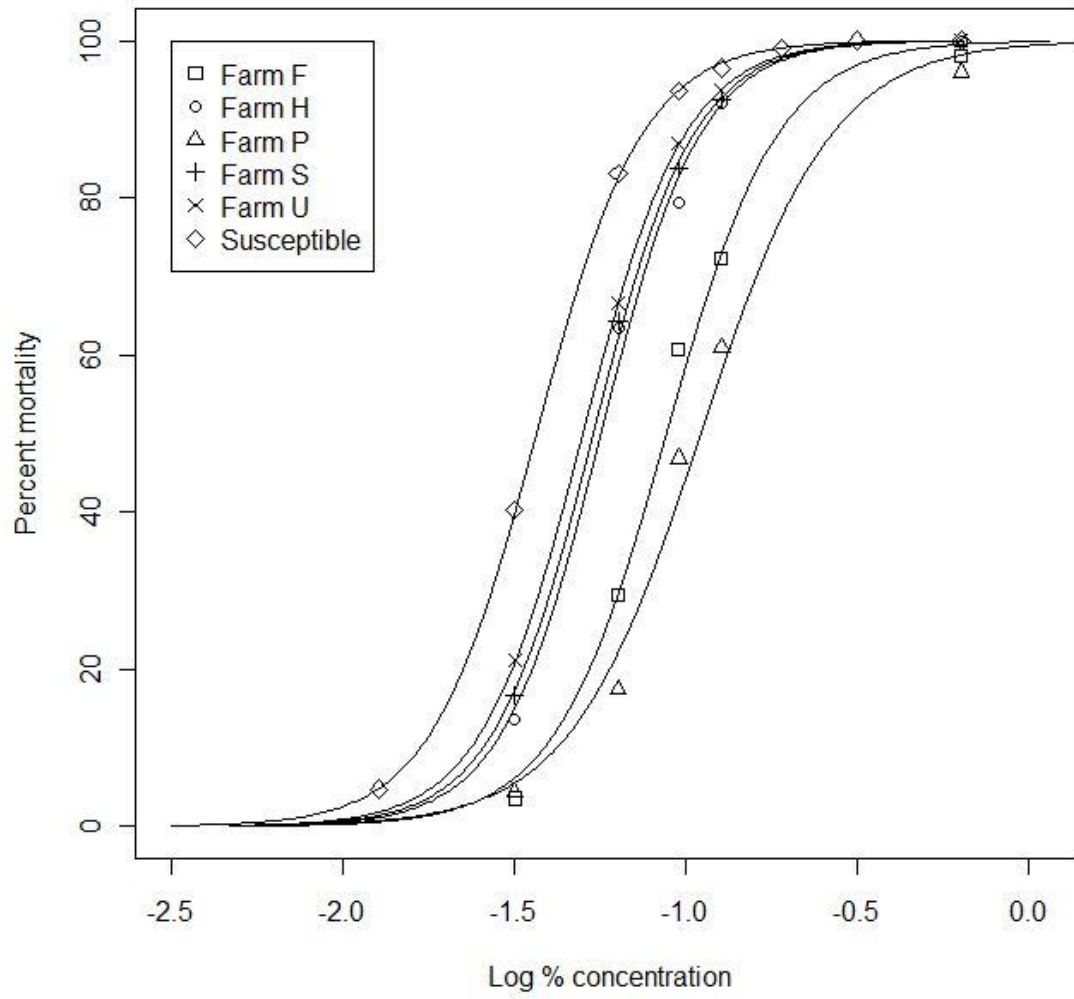


Figure 3. Dose-response curves for all populations topically treated with spinosad.



Figure 4. Microscopic image of dorsal view of adult *A. diaperinus*



Figure 5. Microscopic image of eclosed *A. diaperinus* egg



Figure 6. Microscopic image of dorsal view of an *A. diaperinus* larva



Figure 7. Microscopic image of ventral view of an *A. diaperinus* larva



Figure 8. Microscopic image of *A. diaperinus* pupa



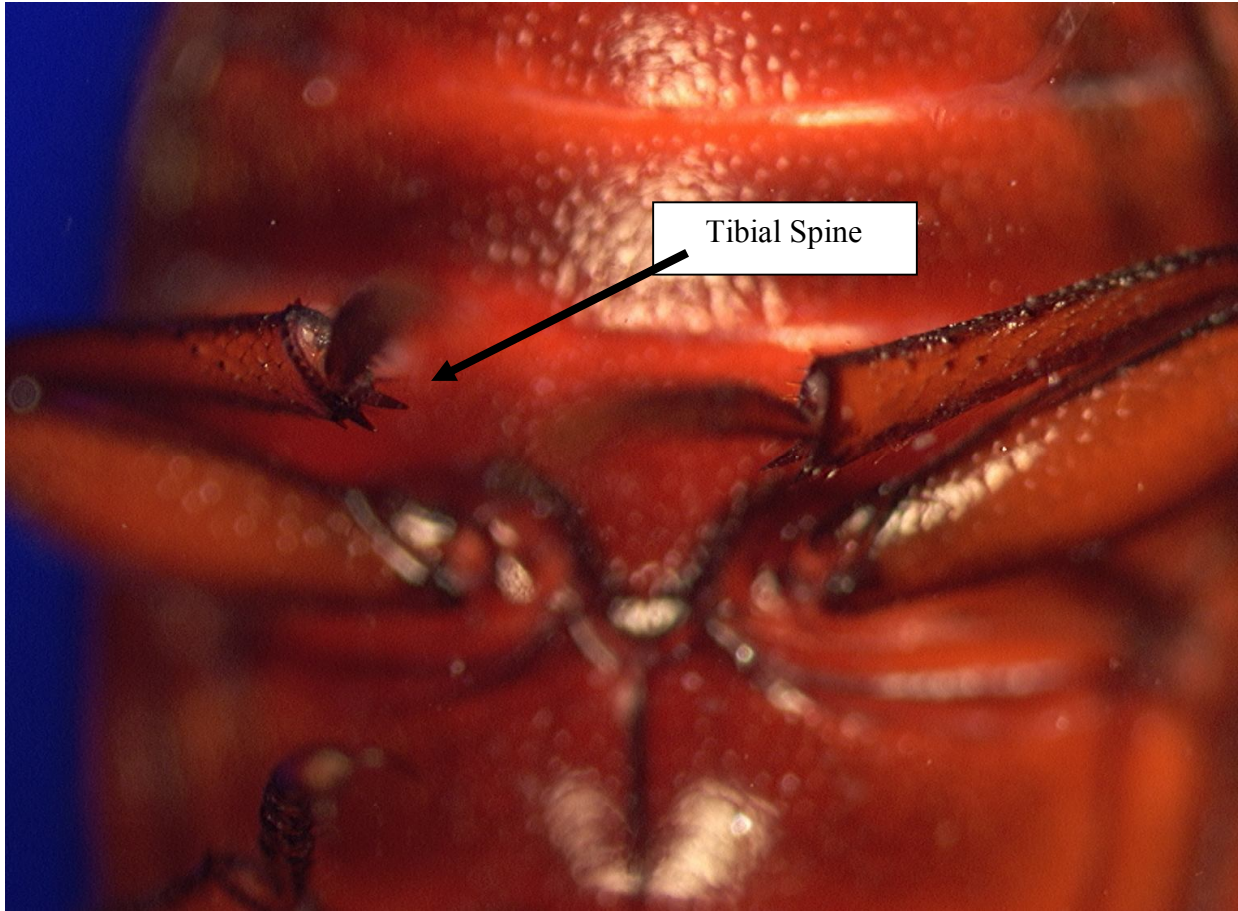


Figure 9. Microscopic image of *A. diaperinus* metathoracic tibial spine

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