

THE LESSER MEALWORM, *ALPHITOBIUS DIAPERINUS* (PANZER), AND ITS ROLE  
IN *SALMONELLA* TRANSMISSION TO POULTRY

by

AUBREE JULIA ROCHE

(Under the Direction of Nancy C. Hinkle)

ABSTRACT

*Alphitobius diaperinus* (Panzer), or the lesser mealworm, is a common insect pest of commercial poultry operations. *Salmonella* is a common foodborne pathogen associated with poultry. Living in litter, lesser mealworms potentially come in contact with human and animal pathogens and are then consumed by foraging broilers. The reservoir competence of *A. diaperinus* for *Salmonella* was evaluated utilizing a nalidixic acid-resistant marker strain *Salmonella* Typhimurium. The persistence was assessed in adults, larvae, and pupae as well as the ability of *Salmonella*-inoculated beetles to colonize broiler chicks. The marker *Salmonella* persisted in adult beetles up to 64 d and 49 d in larvae, and high levels were maintained for up to 30 d post-inoculation. Day-of-hatch broiler chicks inoculated with pools of 4 adults or larvae were colonized with the marker *Salmonella* and able to disseminate the bacteria to other birds in the flock for up to 6 wk.

KEYWORDS: Darkling beetles, Lesser mealworm, broilers, *Salmonella*, *Alphitobius diaperinus*, disease transmission, insects

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B. S. E. H., The University of Georgia, 2005

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial  
Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2007

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December 2007

## DEDICATION

I would like to dedicate my thesis to my family and closest friends who mean everything to me. Deborah, John, and Shawn Roche supported me through every (sometimes crazy) pursuit I have started; I couldn't have done this without you. To Lillie Craton, who showed me that it is possible to survive graduate school in one piece, and to Scott Kelly, for helping me through many long nights of writing. And finally to my friends whose creative endeavors allowed me to maintain my (in)sanity throughout the writing of this thesis.

## ACKNOWLEDGMENTS

The following thesis benefited from the insights and direction of several people. My greatest debt is to Dr. Nancy Hinkle for giving me so many opportunities other students could only have dreamed of and for pushing me to do the things I needed to do, even when I was scared. You have been my greatest ally.

Great appreciation is extended to the members of my advisory committee, whom I could always turn to for help. I couldn't have hoped for better advisors. To Dr. Nelson Cox, I thank you for inviting me into your lab, for a thousand ideas a minute, and for humor when the project was most unpleasant. To Dr. Brian Fairchild, I thank you for not hesitating to save me when I needed help, even if it meant digging for beetles in poultry litter on a Saturday morning,

Dr. Jeff Buhr, I thank you so much for your comments and for many mornings of litter sampling.

Jason Richardson, this page is not long enough to thank you for all the help you have given me throughout this project.

Jeromy Jackson, thank you for long days of streaking plates, drag swab samples, and ceca sampling.

To my lab mate and best friend Jaime Fuest, who shared my ups and downs from beginning to end, and to Floyd Shockley, who was a great source of guidance and weekly lunches.

Thank you to Sigma Xi Scientific Research Society for the grant in aiding my research.

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## CHAPTER 1

### INTRODUCTION

Salmonellae continue to be a leading cause of foodborne bacterial illness due to widespread occurrence in the environment, prevalence in many segments of the global food chain, and bacterial virulence and adaptability (Pegues et al. 2002). Effective commensals and pathogens, Salmonellae cause a variety of diseases in humans and animals (Stern 1987, Pegues et al. 2002). Among foodborne pathogens, *Salmonella* spp. present a significant public health hazard due to the number of bacterial foodborne illnesses in the United States attributed to the organism (Mead et al. 1999). Moreover, in 2002, *Salmonella* Typhimurium and *Salmonella* Newport accounted for 2 of the top 3 most common *Salmonella* serovars isolated from humans (CDC 2004).

The difficulties in controlling *Salmonella* spread in animal husbandry, as well as in production and processing of animal products, have led to raw meats, poultry, and eggs being implicated as significant primary routes of foodborne salmonellosis (D'Aoust et al. 2001). To reduce the prevalence of *Salmonella* on poultry during processing, intervention strategies should be implemented during the other phases of poultry production as well (Russell 2002). During broiler grow-out, chickens eat frequently and grow rapidly to reach market weight and age and are exposed to pathogens like *Salmonella* via a number of reservoirs such as rodent and insect pests (Borland 1975, Russell 2002, Kinde et al. 2005).

*Alphitobius diaperinus*, also known as the darkling beetle or lesser mealworm, has become a serious pest in commercial broiler farms. Their presence in broiler grow-out houses can be costly for poultry integrators as *A. diaperinus* beetles become more resistant to insecticides and production costs related to pest management increase. Lowered feed conversion efficiency (Despins and Axtell 1994, 1995) can reduce profits if young birds prefer to eat the small moving beetles in the litter rather than feed. Broiler consumption of *A. diaperinus* has also been linked to the transmission of a number of pathogens including bacteria, viruses, fungi, and protozoa. A low level of a mild form of avian influenza virus was detected in a pool of adult darkling beetles collected from an infected poultry farm (Wilson et al. 1986).

The average time a broiler spends in a grow-out house is 6 to 8 weeks and the time between flocks, when house maintenance, cleaning, and pesticide application occur, can be from a few days to two weeks. Adults begin emerging from the soil before the end of the flock and continue emerging through the cleanout period between flocks and during the subsequent flock, constituting a major source of broiler house reinfestation (Axtell and Arends 1990, Lambkin et al. 2007).

By identifying the most common points of the dissemination of salmonellae in broiler production, control protocols can be developed to improve the safety of poultry products. The role of darkling beetles in *Salmonella* dissemination through a broiler flock during grow-out was evaluated using a nalidixic acid-resistant marker *Salmonella* Typhimurium. The persistence of *Salmonella* in adult and larval beetles was determined as well as *Salmonella* maintenance through lesser mealworm pupation and

adult emergence. The reservoir competence to day-of-hatch broiler chicks was also evaluated by oral administration of *Salmonella*-inoculated adult and larval *A. diaperinus*.

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## CHAPTER 2

### OBJECTIVES

In the interest of food safety, the objective of this project was to understand the colonization of *Salmonella* in broiler chicks via *A. diaperinus*. To accomplish the objectives the following had to be determined and evaluated:

1. whether *Alphitobius diaperinus* larvae and adults can acquire and sustain infectious doses of *Salmonella* Typhimurium.
2. what level of *Salmonella* Typhimurium inoculum is required to infect *Alphitobius diaperinus* larvae and adults.
3. how long *Salmonella* Typhimurium are maintained in *Alphitobius diaperinus* larvae and adults.
4. the ability of *Salmonella* Typhimurium-contaminated beetle larvae to sustain the infection through pupation and percentage of newly-emerged adults that retained the inoculated *Salmonella* Typhimurium.
5. infect day-of-hatch broiler chicks with known doses of *Salmonella* Typhimurium via contaminated adult or larval *A. diaperinus* beetles; determine whether the chicks become colonized; and evaluate subsequent transfer to other chicks in the flock.



**CHAPTER 3**  
**LITERATURE REVIEW**  
**Broiler Production**

The broiler industry in most of the United States is made up of many components working under the control of a vertically integrated system. Vertical Integration is the ownership and management of two or more successive stages of the marketing system by a single firm (Bell 2002). Vertically integrated broiler production is made up of 6 basic components: breeder rearing farms, parent or laying farms (multiplies flocks for broiler hatching eggs), the hatcheries, the broiler production farms, the processing plants, and the feed mills. In a vertically integrated system, the contract grower provides the housing and everything included in housing, as well as the labor. The integrator (the broiler company) provides the chicks, feed, necessary medications and supervision as well as labor and equipment for hauling the birds to market. The farmers are contracted by the companies (integrators) and are paid per pound. This system reduces transaction costs and provides the company with greater control over quality and uniformity of the product (Dozier et al. 2001). The systems in which *Salmonella* is most often transmitted will be discussed further with *Salmonella* transmission.

The primary breeder companies, though not usually owned in the vertical integration system, maintain the foundation as well as the great-grandparents used in the production of commercial genetic lines of poultry. If a single primary breeding bird is

colonized by *Salmonella*, the infection can become widespread throughout the entire poultry production system (Davies 2005). A primary breeder hatchery usually includes parent stock rearing, where the breeders are raised until sexual maturity, and hatching egg production farms, where the breeders are put on an egg laying cycle (Bell 2002). Hatcheries have been a major contributor to *Salmonella* dissemination in breeder and broiler chicks.

Broiler production farms are where chicks are reared to market weight and age. During grow-out, broiler chickens eat approximately every 4 h (Russell 2002), to gain weight and put on edible muscle rapidly. At the end of grow-out, prior to catching the birds for transport, the feed is removed for approximately 8 to 12 h. As the birds get hungry they forage through the litter and consume potentially contaminated material as well as insects (Russell 2002). This contributes to the level of *Salmonella* on processed carcasses, due to the high levels of *Salmonella* in the birds' crops (Byrd et al. 2001).

At the end of grow-out, the broilers are transported to the processing plant where they will be slaughtered and prepared for market. The transportation to the processing plant is another point where *Salmonella* can be spread. The coops used in transportation of birds are difficult to clean and sanitize. Rinsing the coops without removing dried excreta can cause *Salmonella* to proliferate rather than be removed. The presence of water changes the dry excreta to a more favorable environment for *Salmonella* growth (Russell 2002).

Implementation of sanitary practices along with Hazard Analysis and Critical Control Point (HACCP) programs in 1998 greatly reduced the prevalence of *Salmonella*

on broiler carcasses processed in the U.S. (Wabeck 2002). One great source of *Salmonella* dissemination in a processing plant is the crop removal process. During the removal of a chicken's crop, it can break and leak the contents onto the equipment and then to the inside and outside of the carcass (Hargis et al. 1995). These are some of the reasons why it is important to control the presence of *Salmonella* in all of the components of the poultry production system.

### ***Salmonella***

The genus *Salmonella* consists of rod-shaped, Gram-negative bacteria in the family Enterobacteriaceae (D'Aoust et al. 2001). Highly adaptable to extreme environments, *Salmonella* are facultative anaerobes resistant to freezing and desiccation (D'Aoust 1989). Though salmonellae can grow in refrigerated foods stored at 2-4°C or in temperatures as high as 54°C, the optimum temperature for growth is between 35 and 37°C (Anderson and Ziprin 2001, D'Aoust et al. 2001). The optimal pH for growth is between pH 6.5-7.5 at high water activity and low osmolarity (D'Aoust et al. 2001, Ziprin and Hume 2001, Pegues et al. 2002); however salmonellae do have the ability to grow in pH ranging from 4.0-9.5 (D'Aoust et al. 2001). The ability of *Salmonella* spp. to adapt within broad and sometimes extreme ranges of temperature and pH is why it has become a common foodborne pathogen.

**Taxonomy.** The nomenclature of *Salmonella* is complicated. To unify serotype designation, the CDC adopted the Kauffmann-White Scheme on January 1, 2003 from its previously modified version. The Kauffmann-White Scheme for designation of *Salmonella* serotypes is maintained by the World Health Organization (WHO)

Collaborating Centre for Reference and Research on *Salmonella* at the Institut Pasteur (CDC 2007). The genus *Salmonella* includes two species, *Salmonella enterica* and *Salmonella bongori*. Prior to the most recent update in nomenclature, *Salmonella enterica* was known as *Salmonella choleraesuis* and *Salmonella bongori* was previously *Samonella enterica* subspecies V (Anderson and Ziprin 2001).

*Salmonella enterica* is the type species and has six subspecies. Subspecies I serotypes are named (e.g. *Salmonella enterica enterica*), subspecies II through VI serotypes are identified by antigenic formula (Brenner et al. 2000, CDC 2007). As mentioned previously, *Salmonella bongori* was originally designated *Salmonella enterica* subspecies V, until it was determined to be a separate species. These strains are commonly referred to by antigenic formula (subspecies V) for the purpose of serotype designation (Brenner et al. 2000, CDC 2007). *Salmonella enterica* subspecies IIIa and IIIb were at one time in the genus *Arizonae*, and are still sometimes referred to by this name (CDC 2007). *Salmonella enterica* subspecies *enterica* is then further divided into serotypes (e.g. Enteritidis, Typhimurium).

Under the current system of abbreviation, *Salmonella* serovars in the subspecies *enterica* (I) are given names that are not italicized and begin with a capital letter. For example, *Salmonella enterica* subspecies *enterica* serotype Typhimurium may also be written as *Salmonella* Typhimurium (Anderson and Ziprin 2001).

**Antibiotic resistance and *Salmonella* isolation.** In research, antimicrobial resistant strains are developed from wild-type strains and used to inoculate food products as microbial markers. Antimicrobial-resistant marker strains can be easily

isolated from background microflora using several antibiotics, including nalidixic acid, rifampicin, and streptomycin (Killinger-Mann 2005). Nalidixic acid mutations are performed in a step-wise fashion. To do this, the wild-type strain is exposed to a low concentration of nalidixic acid and is subsequently exposed to higher concentrations until full resistance is achieved (Killinger-Mann 2005).

***Salmonella* Host Specificity.** *Salmonella* serovars have the ability to infect a wide range of hosts. Some serovars may infect one host exclusively while other serovars are capable of infecting multiple hosts from different species. *Salmonella* Typhi and Paratyphi, for example, only colonize humans and have no other known animal reservoirs, and *Salmonella* Typhimurium can colonize both humans and other animals, such as poultry (Lacey 1993). *Salmonella enterica* subsp. *enterica* contains the highest number of serovars, most of which live in the gastrointestinal tract of warm-blooded animals. The other subspecies of *S. enterica*, as well as *S. bongori*, are usually found in the gastrointestinal tract of cold-blooded animals and the environment (Pegues et al. 2002).

Some of the important serovars that cause human salmonellosis are also linked to animal health. *Salmonella* Enteritidis is a pathogen found in both chickens and humans. *Salmonella* Dublin, found in cattle, and *Salmonella* Choleraesuis (in swine) are pathogenic to livestock as well as humans. *Salmonella* Typhimurium has a wide range of hosts such as rodents or humans (Ziprin and Hume 2001, CDC 2004).

***Salmonella* and Human Illness.** The two major types of disease caused by *Salmonella* are commonly referred to as typhoid salmonellosis and non-typhoid

salmonellosis, also known as gastroenteritis. Typhoid salmonellosis in humans is caused by human specific *S. enterica* serovar Typhi and a similar but less severe syndrome is caused by *S. Paratyphi A*, *S. Paratyphi B*, and *S. Typhi C* (Lacey 1993, Pegues et al. 2002). Typhoid salmonellosis, caused by *S. Typhi* and commonly referred to as typhoid fever or enteric fever, is characterized by invasion of the intestinal mucosa and entry into the gastrointestinal lymphoid tissue. Once inside, it proceeds to a systemic infection invading the spleen, liver and blood stream (Ziprin and Hume 2001). Common symptoms include malaise, headache, non-productive cough, abdominal pain, constipation and fever (Goldberg and Rubin 1988).

The non-typhoid salmonellosis is a localized infection of the intestinal epithelium about 8 to 72 hours after exposure and lasts about 4 to 10 days (D'Aoust 1989). Some infected individuals may become asymptomatic carriers for up to 5 weeks after infection. The time period that *Salmonella* is excreted in the feces may be increased if antimicrobial drugs are used to treat the infection (Bell and Kryriakides 2002, Pegues et al. 2002). People usually experience diarrhea and abdominal pain, though sometimes vomiting and fever occur.

The symptomatology of non-typhoidal salmonellosis is dependent on the means of infection and the infectious agent, as well as preexisting condition of the host. The mechanisms by which salmonellae cause gastroenteritis is not fully elucidated (D'Aoust et al. 2001, Pegues et al. 2002). A number of enterotoxins similar to the toxins produced by *E. coli* and other enteropathogens have been described in *Salmonella*, however they have not been fully characterized biochemically (Pegues et al. 2002). It is

believed that the diarrhea is caused instead by an immune response due to entry of *Salmonella* into enterocytes of the host immune system (Pegues et al. 2002). Human infections with nontyphoid strains can also degenerate into systemic infections and can precipitate chronic conditions. Some serovars can progress toward septicemia more often than others. Preexisting conditions such as immunological disorders leave the host defenses unable to effectively protect the host and fight the pathogen.(D'Aoust 1989)

**Invasion/Colonization.** The ability of *Salmonella* to establish itself in an animal depends on its ability to attach to and invade intestinal epithelial cells and specialized M cells located in the intestinal epithelium that process antigens in the gastrointestinal tract, invading the host's immune system and competing with the host's gut microflora for attachment sites. *Salmonella* is unique from other invasive foodborne pathogens in that replication occurs within the host cell inside vacuoles instead of the host cell's cytoplasm (D'Aoust, 1997).

**Economic Significance.** Salmonellosis is one of the most common foodborne diseases in the United States. During 1998-2002, a total of 6,647 outbreaks of foodborne disease were reported. These outbreaks caused a reported 128,370 persons to become ill. Among 2,167 bacterial pathogens reported, salmonellae caused the largest percentage of outbreaks and the largest percentage of cases. Among those reported bacterial pathogens, *Salmonella* Enteritidis accounted for the largest number of outbreaks and outbreak-related cases (Lynch et al. 2006). In 2005, a total of 16,614 laboratory-confirmed cases of infections in FoodNet surveillance areas were identified.

*Salmonella* had the highest number of cases (6,471 cases) compared to *Campylobacter* (5,655) and *Shigella* (2,078) (MMWR 2006).

Costs of foodborne salmonellosis are divided into two general categories: costs related to human illness and costs incurred by producers due to salmonellae in the flock. In the United States, costs related to human illness are estimated at over \$2 billion annually to pay for medical costs including laboratory diagnosis and treatment of patients as well as lost productivity (Mead et al. 1999). More direct costs include epidemiological incident investigation costs and loss of income by the patient. Direct costs to the producers include *Salmonella* control measures, such as biosecurity practices, pest control programs, vaccination, and testing. Businesses can lose capital due to bad publicity, having to recall a product, or legal settlements for suffering or fatalities.

**Salmonella and Poultry.** The most common sources of human salmonellosis are from animals via food and water supply. Though dairy, beef, and pork products have been associated with outbreaks of salmonellosis, raw poultry and poultry products have been the primary source of non-host specific *Salmonella* transmission to humans (D'Aoust et al. 2001). Although occasionally associated with exposure to pets, or contaminated water, salmonellosis is primarily a foodborne disease. It was stated that 9.5% of the total 2 million cases of salmonellosis reported in 1981 could be linked to poultry (CDC 1983). During 1985-1995, state and territorial health departments reported 582 *S. Enteritidis* outbreaks, which accounted for 24,058 cases of illness, 2290 hospitalizations, and 70 deaths (CDC 1996).



So far, over 2300 serovars of *Salmonella* have been identified, but of these only about 10% have been isolated from poultry (Gast and Beard 1990). Colonization of chicks depends on age of the birds at day of challenge and the level of the inocula. Day-old chicks can be routinely colonized by as few as 10 cells of *Salmonella* (Milner and Shaffer 1952). As chicks age, gut microflora maturation occurs, requiring higher doses of *Salmonella* to colonize the gut (Barnes et al. 1972). *Salmonella* can be introduced into broiler flocks from a number of different sources and by vertical or horizontal transmission.

### Vertical Transmission

Vertical transmission of *Salmonella* contamination in broilers has been linked to its presence in broiler hatching eggs. One theory pertaining to *Salmonella* contamination of hatching eggs is that the bacteria are deposited into the contents of eggs from infected hens as the egg passes down the oviduct, which is considered true vertical transmission (Cox et al. 2000, Davies 2005). Pseudovertical transmission involves the contamination of the egg shell by fecal contamination from the parent hen. *Salmonella* can penetrate the egg shell before the cuticle forms and infect the developing embryo (Stokes et al. 1956, Davies 2005). Once the bacteria have invaded the membrane of hatching eggs, the cell numbers increase with the rising temperature of pre-warming and incubation (Rizk et al. 1966).

## Horizontal Transmission

Once inside the hatchery, contaminated eggs could serve as a source of horizontal transmission. Due to the favorable conditions for microbial growth during incubation, hatcheries can be highly contaminated with salmonellae (Cox et al. 1990a, Cox et al. 1991). While chicks hatch, salmonellae on egg shells and other hatching debris are released into the air and spread by the fans in the hatchers (Bailey et al. 1994). Because newly hatched chicks have an under-developed protective intestinal microflora, they are very susceptible to colonization by *Salmonella*. Day-of-hatch chicks have a *S. Typhimurium* colonization dose-50% (CD<sub>50</sub>) of about 100 cells via oral route and less than 10 cells through the cloaca (Cox et al. 1990b).

Poultry feed is another potential source of horizontal transmission. Schleifer et al. (1984) reported that very low levels of *Salmonella* in feed can cause colonization of the intestinal tract of 1- to 7-day-old chicks. In a study by Cox et al. (1983) samples were collected from U.S. commercial mills, and salmonellae were found in 92% of meat and bone meal samples and in 58% of finished feed samples. *Salmonella* was not found in samples of pelleted feed, however other Enterobacteriaceae with similar heat resistance traits were present, suggesting that pelleting may not totally eliminate *Salmonella* (Cox et al. 1983).

Rodents and wild birds are competent vectors of *S. Enteritidis* in poultry flocks (Borland 1975, Kinde et al. 2005). Henzler and Opitz (1992) found that a single mouse fecal pellet could contain 10<sup>5</sup> *S. Enteritidis* cells. *Salmonella* spp. were also detected in samples of wild bird fecal deposits from the outside of broiler houses (Craven et al.

2000). In a one year survey, Faddoul et al. (1966) reported 12 *Salmonella* outbreaks naturally occurring in wild bird populations of Massachusetts and Rhode Island.

*Salmonella* have been experimentally transmitted by a number of different types of insects including fleas, flies, beetles and cockroaches (Mackerras and Mackerras 1948, Eskey et al. 1949, Greenberg et al. 1963, Hald et al. 1998). Cockroaches have also been reported as mechanical vectors for *Salmonella* spp. (Kopanic et al. 1994). The hairy fungus beetle, *Typhaea stercorea*, was determined to be a potential carrier of *Salmonella* Infantis to poultry (Hald et al. 1998). *Alphitobius diaperinus* (discussed later) may carry *Salmonella* internally and/or externally and spread it throughout poultry houses and into neighboring farms.

### ***Alphitobius diaperinus***

**Taxonomy.** *Alphitobius diaperinus* has commonly been called the lesser mealworm, 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). It belongs in the order Coleoptera and the family Tenebrionidae. The generic name *Alphitobius* was first described by Stephens in 1829, as cited by Spilman (1966). Spilman (1966) went on to explain that in 1797 Panzer had already described the species and assigned it to the genus *Tenebrio*, with the specific epithet *picipes*. In the end, Panzer was credited with the naming of *Alphitobius diaperinus* Panzer in 1797 (Spilman 1966, Poole and Gentili 1996).

**Life Stages.** 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 was reported to be about 36 to 29 days (Rueda and Axtell 1996). At the higher temperature (35°C) the development of darkling beetles is accelerated to 29 days. Decreasing the temperature by five degrees causes the development time to increase by approximately 7 days. At 21°C the *A. diaperinus* developmental time from egg to adult can last as long as 60-85 days (Barké and Davis 1969).

Egg: The lesser mealworm egg is creamy white in color and elliptical shaped. The average length is 1.0 to 1.4 mm and the average width is 0.4-0.5 mm. Female darkling beetles anchor their eggs in cracks and crevices using a clear sticky substance (Wilson and Miner 1969). Depending on temperature, eggs hatch 3-13 d after oviposition between 21.1°C and 37.8°C (Lancaster and Simco 1967, Preiss and Davidson 1971).

Larva: (Figures A.4, A.5) The larval stage lasts from 21 to 133 d and, depending on temperature, larvae go through 6 to 12 instars. A newly hatched larva is about 1.5 mm in length and white in color. As it grows and the cuticle hardens, the larva darkens to a brownish color. The final instars grow to about 10 mm before pupation. Francisco and Prado (2001) used mean head capsule widths to characterize larval instars.

Pupa: The pupal stage lasts about 4 to 17 d, again depending on temperature (Rueda and Axtell 1996). Pupae are exarate (naked) and initially white in color, but darken as they develop. The final larval instar seeks out hiding places in the earth floor

or insulation for protection against predators and cannibalism from adult and larval *A. diaperinus* (Despins et al. 1987). The genitalia develop as pupae get closer to adulthood, and sex can be determined microscopically (Barké and Davis 1967).

Adult: (Figures A.1, A.2, A.3) A newly eclosed adult darkling beetle is reddish brown in color. Over 7 d the cuticle hardens and darkens to the characteristic shiny brown to black color (Wilson and Miner 1969, Preiss and Davidson 1971). Adults are small, 5.1-6.1mm in length (Wilson and Miner 1969) with evenly spaced punctations on the elytra (Fig. A. 1), or the modified, hardened forewings of certain insect orders, notably beetles. The pronotum (Fig. A.1) is deeply emarginated anteriorly (Preiss and Davidson 1970). The sexes can be determined by the shape of the metathoracic tibial spines (Fig. A.3). The male has one straight and one curved metathoracic tibial spine; both spines are straight on a female (Barké and Davis 1967). 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 females have a pre-oviposition period of about 10 to 13 d (Wilson and Miner 1969, Preiss and Davidson 1971). The mean life-span was reported to be greater than 400 d (Preiss and Davidson 1971). Due to the longevity of adult *A. diaperinus* and long oviposition period with female fecundity as high as 3.6 to 7.3 eggs per day (Rueda and Axtell 1996), populations can build to very high numbers.

**General Biology.** *Alphitobius diaperinus* originates from sub-Saharan Africa. Because of its tropical origin, *A. diaperinus* has become well adapted to the broiler house environment. 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). Poultry litter mixed with droppings and feed has become an optimal habitat for the lesser mealworm.

Habitats. In a survey of Coleoptera in poultry manure, *A. diaperinus* was consistently one of the most abundant species in all of the three regions of North Carolina studied: the mountains, piedmont, and coastal plains (Pfeiffer and Axtell 1980). First thought to have originated in bird nests in Africa, darkling beetles have been found living with house sparrows and purple martins in Wisconsin (Thompson 1966) and pigeon houses in Sudan (Yagi and Razig 1972). Darkling beetles live not only with birds, but have been found living with many different animals, such as a piggery in Ireland (O'Connor 1987) and on dairy farms in the United States. The most notable habitat of the lesser mealworm was in the scrotum of a Norway rat, in which 4 adults and 16 larvae were living (Crook et al. 1980).

The distribution pattern of darkling beetles in a typical broiler house changes during the different stages of their life cycle corresponding to the broiler flock phenology. By the end of the broiler growth period and during the cleaning period, penultimate (or next to last instar) larvae, pupae, and adults are found within the top 10 cm layer of litter or insulation if available (Safrit and Axtell 1984, Salin et al. 2000). Adult beetles emerge from the compacted dirt floor soon after a new flock is brought into the house. These newly emerged adults constitute a major source of reinfestation (Axtell and Arends 1990, Lambkin et al. 2007). Females lay eggs after emergence, and the newly hatched

larvae predominantly aggregate under feed pans and, during the warmer seasons, along the house walls (Salin et al. 2000). The peak of the beetle population is when the birds are about 3 weeks of age. By the time the flock is 6 weeks old, the mature larvae begin to burrow into the earth floor to pupate, then adults emerge with the new flock (Lambkin et al. 2007).

### Diet

Darkling beetles have the ability to find nourishment in many places; they can be proficient predators as well as scavengers. Though once thought of as herbivorous, it was noted that larvae bored into the flesh and internal organs of dying and dead chicks (Harding and Bissell 1958, Lancaster and Simco 1967). 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. She also discovered that the digestive system of *A. diaperinus* contains a combination of enzymes found in omnivorous and phytophagous insects, giving this species the ability to digest proteins, fats, starch, sucrose, maltose, lactose, and cellulose (Sarin 1973). Adults and larvae will readily consume manure as well as moribund or dead animals and in conditions of starvation and overcrowding, become cannibalistic (Harding and Bissell 1958, Despins et al. 1988). Newly molted larvae, prior to cuticle hardening, and pupae are particularly susceptible to cannibalism. In a study by Hulley and Pfeleiderer (1988), darkling beetles were reported to feed on house fly (*Musca domestica*) eggs, small live larvae, and adults if the cuticle is damaged (Despins et al. 1988). Hundreds of beetles killed and consumed all but the skin and bones of a ten inch snake (Harris 1966).

McAllister et al. (1995a) observed no midgut caeca or regenerative crypts in either the larvae or adults. Regenerative crypts are usually found in insects with “coarse” diets to replace cells destroyed during digestion which supports the observation of darkling beetles consuming softer foods. They also noted the lack of a crop, indicative of continuous feeding, for the crop primarily functions in food storage (McAllister et al. 1995a). Continuous feeding of darkling beetles makes transmission of pathogens possible even if the pathogens do not necessarily colonize inside the beetle. The constant feeding can increase populations of pathogens inside the beetles without requiring the pathogen to reproduce inside the beetle.

## Environment

In spite of their ability to thrive in poultry houses, darkling beetles do require relatively high moisture, particularly the immature stages. Female adults do not lay eggs below 50% relative humidity (Farkas 1966). Sarin and Saxena (1973) determined the optimum conditions for growth were 30°C at 90% relative humidity. The mean supercooling point for adult darkling beetles (both sexes) was found to be -12.4°C, and chill coma occurs at 5.8°C. The duration and intensity of the cold both play a role in mortality. The temperature considered lethal to darkling beetles is about 6-10°C because activity decreases at these temperatures and mortality occurs due to starvation (Renault et al. 1999).



Preiss (1969) noted that adult darkling beetles could survive an average of 19 days without food or water. Early and mid-instars could survive an average of 10 days without food or water, while late instars can pupate early to survive (Rahman et al. 1991).

**Significance of *A. diaperinus*.** As stored product pests, darkling beetles do not pose a significant economic threat. The grains these beetles feed on are often already damaged. However darkling beetles have become a problem for broiler management.

### Nuisance

In 2006, Georgia produced 1.38 billion broilers (USDA 2007). Annually, a broiler farm produces 1.4 millions of metric tons of litter (Gascho et al. 2001). This litter can be applied to Georgia pastures and crop fields as inexpensive fertilizer. Contained in the litter are large numbers of darkling beetles. The beetles subsequently migrate either back into the poultry house or to nearby farms and residences when the litter is spread. These migrating beetles could potentially contaminate homes and compromise the biosecurity of other farms. In 1998, families living near the egg complex of Buckeye Egg Farms (AgriGeneral) filed a \$25 million class-action lawsuit against the company over an infestation of darkling beetles in their homes (Hinchey 1997). There is substantial potential liability associated with darkling beetle infestations.

### Insulation Damage

Final instars tunnel into insulation for a protective pupation site when unable to find suitable soil. Larvae in houses without soil floors cause about 4-fold as much

damage at higher densities as at lower densities (Geden et al. 1987). Adults are also known to climb the walls and burrow into the insulation when population densities become too high. Damage by darkling beetle larval and adult tunneling can reduce the effectiveness of the insulating material by as much as 20–30% (Vaughan et al. 1984, Despins et al. 1987). In only 3-4 years, darkling beetles can devastate extruded foam insulation (Hinkle and Hickle 1999). Directly, the insulation destruction by darkling beetles increases production costs due to replacement of materials and labor. Indirectly, growers lose money from energy loss, market loss while houses are out of production for repairs, and losses to bird growth efficiency due to less than ideal environmental conditions (Vaughan et al. 1984).

### Bird Performance

In broiler production, feed is the most costly aspect, accounting for as much as 70% of the cost in some instances; efficient feed utilization by a flock can be of considerable economic importance to the broiler grower (Vest 1999). When the humidity is very low and the beetles become desperate for water, they will crawl up the feathers of resting birds and bite the skin around feather follicles for moisture. When molested by biting beetles, birds move to avoid their attacks and only rest briefly. The repetition of resting and wandering wastes the birds' energy, which lowers performance and feed conversion efficiency. The bite sites also can become pink and swollen around the follicles, resembling avian skin leukosis (Savage 1992).

Despins and Axtell (1995) assessed the effects of feeding *A. diaperinus* larvae to chicks. They discovered that chicks would readily consume as much as 33 grams live

weight of larvae per day. Calibeo (2002) included a photo in her master's thesis showing the distention of the crop of a chick that had consumed so many darkling beetles the skin of the crop stretched to the point that the ingested adults and larvae were visible within the crop. Chicks that fed only on larvae for 6 days weighed less than chicks that fed on starter feed. The insect-fed chicks were then returned to starter feed, and on day 9 they still weighed less than chicks fed only starter feed. These chicks experienced a number of negative effects, showing signs of stress, having problems defecating, and produced watery stools (Despins and Axtell 1995). Chickens lack chitinase (the enzyme necessary for digestion of chitin). Whole larval cuticles were present in the chicks' feces, confirming that the larval cuticle was indigestible. The ingestion of adults and larvae causes a decrease in the growth efficiency of broilers and hence a decrease in pay for the growers (Savage 1992).

### Disease Transmission

Previous studies have implicated insects in bacterial transmission. As mentioned earlier, *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). Crumrine et al. (1971) showed that *Salmonella* Montevideo could be carried by a number of stored product pests exposed to wheat contaminated with the pathogen. Their study concluded that the seven species of stored product pests studied, including *Sitophilus oryzae* L., *S. granarius* L., and *Tribolium castaneum* (Hbst.), could transmit *S. Montivideo* to clean wheat from contaminated wheat.

The preferred environment and eating habits of *A. diaperinus* make this species a favorable vector in the horizontal transmission of pathogenic organisms. Darkling beetles have been implicated in the transmission of several pathogens to poultry and humans. From 7 poultry farms, Goodwin and Waltman (1996) collected darkling beetles that were then tested for common avian pathogens including *Clostridium*, *Salmonella*, *Eimeria*, and coronaviruses. The study concluded that darkling beetles do serve as vectors for common avian pathogens, such as *Eimeria* sp., and pose a risk to the health of birds that are exposed to them (Goodwin and Waltman 1996). Alicata (1939) first reported that *A. diaperinus*, as well as five other insects, were naturally infected with encysted larvae of *Subulura brumpti*, a common cecal worm of poultry in Hawaii. Adult darkling beetles were shown to be an intermediate host for the poultry tapeworm, *Choanotaenia infundibulum* (Elowni and Elbihari 1979).

Not all pathogens are transmitted by darkling beetles after colonization. Fowl pox virus does not multiply in lesser mealworms and persists for a maximum of 6 days; however darkling beetles can be a source for contamination through excrement (De las Casas et al. 1976). Recovery of Newcastle disease virus from the lesser mealworm was only achieved from adults fed highly infected chorioallantoic membranes, and colonization was unsuccessful for the virus, which was only recovered for 2 days post inoculation (De Las Casas et al. 1976). It was also concluded that though the lesser mealworm tested positive for reovirus 24 for up to 25 days, the titers were too low for them to be effective carriers (De las Casas et al. 1973). Healthy chickens developed symptoms of infectious bursal disease after being fed beetles collected from a chicken house contaminated with the virus (Snedeker et al. 1966). Their study was later

confirmed by McAllister et al. (1995b) by demonstrating that adult darkling beetles could harbor the virus for at least 14 days after ingestion, and that it could be found on the mouthparts, in the digestive tract, and in the hemolymph of adult beetles 24 hours after ingestion. Marek's disease, caused by avian leukosis virus, was revealed to be transmitted by the lesser mealworm (Eidson et al. 1966). In 1972 a vaccine was produced for Marek's disease and focus on the lesser mealworm diminished (Vaughan et al. 1984). Avian influenza virus was detected in 1 pool of *A. diaperinus* collected from an avian influenza positive poultry farm (Wilson et al. 1986).

Bacteria in the genera *Micrococcus*, *Streptococcus*, *Staphylococcus*, *Serratia*, *Klebsiella*, *Pseudomonas*, and *Salmonella* have been isolated from *A. diaperinus* (De las Casas et al. 1968, De las Casas et al. 1972, Olsen and Hammack 2000). Twenty-six pathogenic serotypes of *Escherichia coli* were isolated from darkling beetles (Harein et al. 1970). McAllister et al. (1996) detected a strain of *E. coli* from adult and larval lesser mealworms externally and internally for up to 12 days.

Darkling beetles were also implicated in the transmission of *Campylobacter* (Bates et al. 2004, Skov et al. 2004). Strother et al. (2005) were able to detect *C. jejuni* from the exterior and feces of larvae for 12 h, the interior for 72 h. They also demonstrated that consumption of one inoculated larva or adult lesser mealworm by a three-day-old chick resulted in *C. jejuni* positive birds.

Following a single 24 h feeding, *Salmonella* Typhimurium was detected in darkling beetle feces for 28 d. 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 lesser mealworms for at least 45 days (De las Casas et al. 1968). One-day-old chicks were colonized with *S. Typhimurium* within 24 hours of ingestion of 1 adult or larval beetle inoculated with  $3 \times 10^8$  cells/ml via chicken feed (McAllister et al. 1994). Davies and Wray (1995) tested the maintenance of *S. Enteritidis* in *A. diaperinus* using lower levels of inocula ( $10^3$ - $10^4$ ) in fish meal. Moist blotting paper, placed on the surface of clean meal, was used during the six day evaluation to detect surface contamination via contact with the beetles. They were unable to detect any presence of *Salmonella* on the paper so they attempted to culture *Salmonella* by surface sterilizing the inoculated beetles. Once again, no presence of *Salmonella* was detected from the surface sterilized beetles and they determined darkling beetles to be resistant to *Salmonella* colonization and a minimal hazard (Davies and Wray 1995).

**Control of *A. diaperinus*.** Though they can be consumed by hungry chickens, darkling beetles have no known natural enemies. In dimly lit broiler houses the nocturnal darkling beetles are active at all times of the day and night. So there are few factors controlling their population. Darkling beetles have become increasingly difficult to control because of habitat preference in broiler houses as well as resistance to insecticides (Lambkin 2001). Discovery that more than half of the population of darkling beetles reside in less than 9% of the litter in the broiler house (Lambkin et al. 2007) means that control methods can be more concentrated and then more likely to be successful.

## Insecticides

Residual application of insecticides has been of limited use to poultry producers. Government agencies restrict the chemicals that can be used to control darkling beetles and when they can be applied. Typically, pesticides are applied between flocks after removal of litter when adults and pupae are hidden in the building insulation or in the soil beneath the litter (Axtell and Arends 1990, Hamm et al. 2006). Application of a topical insecticide during cleanout when the beetles are hidden may not be very effective.

*A. diaperinus* are developing resistance to the already limited number of pesticides registered for their control. In Australia a population of darkling beetles displayed high resistance to the pyrethroid cyfluthrin despite having no known previous exposure to the pesticide (Lambkin and Rice 2006). Cyfluthrin resistance has also been reported from the eastern U.S. (Hamm et al. 2006). Lambkin (2005) demonstrated resistance to the organophosphate fenitrothion (which was used for about 20 years prior to this study) in 23 of 27 populations of darkling beetles tested in Australia. Darkling beetles have seen a similar level of resistance to tetrachlorvinphos, another organophosphate used in the U.S. (Hamm et al. 2006).

Juvenile hormone analogs (JHAs) disrupt the processes of metamorphosis and, in some species, reproduction. Two JHAs, methoprene and fenoxycarb, produced 100% mortality in darkling beetle larvae after 12 weeks exposure to treatment (Edwards and Abraham 1985). Insect growth regulators (IGR) interfere with synthesis of the cuticle and prevent molting. These chemicals have potential for controlling beetles in

the poultry industry due to their low vertebrate toxicity, but because IGRs do not produce immediate mortality of the long-lived and constantly reproducing adults, convincing producers to use them may be a challenge. Combining an IGR (triflumuron) with an adulticide (cyfluthrin) greatly reduced larval and adult darkling beetle numbers during broiler grow-out periods (Salin et al. 2003).

### Bio-control

Nematodes are potential biological control agents for *A. diaperinus*; however the population of nematodes has to coincide with a high population of lesser mealworms to be effective (Geden et al. 1987). Early laboratory studies, by Geden et al. (1985), of 3 nematodes and their infectivity of darkling beetles in different substrates have shown that soil treated with entomogenous nematodes in the genera *Steinernema* and *Heterorhabditis* have some success in controlling coleopteran pests. *Steinernema feltiae* was infective against all stages of *A. diaperinus*, and was most virulent against the larval stage. In a later study, the 3 nematode strains were evaluated for control of *A. diaperinus* in the field. Nematodes persisted in the soil for 5 to 9 weeks. For 3 weeks, following house cleanout, the treated populations of *A. diaperinus* grew slower than the untreated. Unfortunately, the studied nematodes were not effective after 10 to 13 weeks post-treatment and the population of darkling beetles was just as high in treated houses as untreated houses.

Adult and larval darkling beetles have been observed infected with the protozoans *Gregarina alphitobii*, *Farinocystis tribolii*, and *Mattesia alphitobii* (Bala et al. 1990, Steinkraus et al. 1992). *Gregarina alphitobii* and *F. tribolii* are not usually at high



enough levels to cause pathogenicity in darkling beetles. It is possible that these two protozoans could compound the negative effects of stressful conditions, such as overcrowding, starvation, or the effects of another pathogen (Apuya et al. 1994).

*Mattesia alphitobii* 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).

A naturally-occurring fungal pathogen, *Beauveria bassiana*, was effective against darkling beetles in laboratory studies when applied to a starch dust. 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 (Steinkraus et al. 1991).

A predaceous mite was discovered that attacks *A. diaperinus* eggs. *Acarophenax mahunkai* parasitized more than half of the egg masses in a beetle colony in a laboratory study by Steinkraus and Cross (1993). They found that it had many characteristics of an effective parasitic mite for biological control. It is very host specific, has a shorter life cycle than the *A. diaperinus* host, and approximately 30 mite offspring can develop from one host egg.

## Cultural methods

Cleaning the house may decrease the population of *A. diaperinus*. Treating the litter with alum, a chemical used to control ammonia in litter, was also shown to reduce beetle numbers (Worley et al. 2000).

Polyethylene terephthalate (PET), used as a mechanical barrier, prevented larvae from climbing up to insulation as long as it was kept clean of fly spots (Despins et al. 1989). Maintenance of drinker lines, and other means of keeping the litter dry, reduced beetle numbers (Turner 1986).

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## CHAPTER 4

### PERSISTENCE OF INOCULATED *SALMONELLA* TYPHIMURIUM IN ADULT, LARVAL, AND PUPAL LESSER MEALWORMS, *ALPHITOBIUS DIAPERINUS* (COLEOPTERA: TENEBRIONIDAE)<sup>1</sup>

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## Abstract

The lesser mealworm, *Alphitobius diaperinus* (Panzer), is a common litter pest in broiler houses. Both adults and larvae are regularly ingested by broilers and could serve as vectors of *Salmonella* to the current and subsequent broiler flocks. The objectives of this study were to evaluate the persistence of a marker *S. Typhimurium* strain in adult and larval darkling beetles. Adult and larval beetles were deprived of feed for 24 h and then provided non-medicated broiler feed for 24 h that had been sprayed with a nalidixic acid-resistant marker strain of *Salmonella Typhimurium* at either a high ( $10^9$  CFU/ml), medium ( $10^5$  CFU/ml), or low ( $10^3$  CFU/ml) inoculation level. The beetles were then moved to a sterile container with non-inoculated feed. Individual (n=10) and pooled samples (n=1) of the adult and larval beetles, along with the feed, were sampled up to 63 d for adults and up to 36 d for larvae. After each sampling time, beetles were moved into a new container and fresh feed was added. In study 2, larvae were inoculated and allowed to pupate, then sampled. In study 1, persistence lasted up to 64 d (20% positive) at the high inoculum, 27 d at the medium inoculum (pooled positive), and 9 d at the low inoculum (10% positive). In the beetle larvae, the *Salmonella* persisted for up to 49 d (end of sampling) in the high (pool) and medium (30%) inocula, and 35 d in the low inoculum (30%). From study 2, 19% of the larvae were positive for the marker *Salmonella* following pupation. These data suggest that *Salmonella* can persist in adult and larval darkling beetles for sufficient time to colonize subsequent broiler flocks and can be maintained through pupation.

Keywords: *Alphitobius diaperinus*, *Salmonella Typhimurium*, lesser mealworm, darkling beetle, poultry

## Introduction

The lesser mealworm, *Alphitobius diaperinus* (Panzer), is one of the most problematic pests in the poultry industry, particularly during the grow-out period for broilers. Most broiler grow-out houses in the United States are approximately 12 to 15 m wide by 120 to 180 m long (40 to 50 ft x 500 to 600 ft) and have compacted earth floors covered by a bedding material such as wood shavings. The litter (bedding plus chicken manure) and the earthen floors are where *A. diaperinus* are found in large populations (Harding and Bissell 1958, Axtell and Arends 1990, Steelman 1996, Lambkin et al. 2007). The time a broiler spends in a grow-out house depends on the target weight and can vary from 6 to 8 wk. The time between flocks, when house maintenance, cleaning, and pesticide application occur, can be from a few days to more than two weeks. Lambkin and Cupitt (2002) found that the life cycle of *A. diaperinus* can be completed within the time of a single broiler grow-out flock. Late-stage larvae burrow predominantly into the earth floor to pupate. Lesser mealworm populations in the floors consist mainly of late instars, pupae, and newly-emerged adults (Salin et al. 2000, Lambkin and Cupitt 2002, Lambkin et al. 2007). Adult *A. diaperinus*, commonly referred to as darkling beetles, begin emerging from the soil before the end of the flock grow-out period and continue emerging through the cleanup period between flocks and during the subsequent flock grow-out period, thereby constituting a major source of broiler house reinfestation (Axtell and Arends 1990, Lambkin et al. 2007).

Darkling beetles readily feed on dead or moribund chicks, chicken feed, and feces (Despins et al. 1994). In one study nearly 6,000 beetles were observed inside and on a broiler carcass (Harris 1966). Darkling beetles have been found to be

effective predators of house fly larvae, and in a situation of high population density and low food availability darkling beetles can become cannibalistic (Harding and Bissell 1958, Despins et al. 1988). Darkling beetles live in broiler litter and feed on potentially contaminated materials. Because of the environment darkling beetles live in, they are in constant contact with pathogenic bacteria. If offered both chicken starter feed and darkling beetle larvae, a 2- to 3-d-old broiler chick will readily consume 33 g of larvae (average of 389 larvae) in 1 d (Despins and Axtell 1995). At 2- to 3-d of age, the gut microflora of a chick has not yet matured, so it is more susceptible to colonization by bacteria (Barnes et al. 1972). If chicks are consuming darkling beetles from the litter, before they have acquired sufficient immunity, darkling beetles may be a source of *Salmonella* colonization for broilers during grow-out.

Darkling beetles have been implicated in the spread of avian and human pathogens and parasites. In a prior study, it was reported that if fed dog food inoculated with 5 to 6 million cells per ml of *Salmonella* Typhimurium, darkling beetles remained positive for up to 24 d (De las Casas et al. 1968). McAllister et al. (1994) found that adult beetles could void *S. Typhimurium* in their feces for at least 28 d after one 24 h exposure to inoculated chicken feed (McAllister et al. 1994). In that same study, they discovered that a day-of-hatch chick could test positive for *Salmonella* 24 h after being fed a single darkling beetle adult or larva. A marker strain was not used, and a later study found the protocol used for external sterilization of the beetles to be an incomplete sterilization procedure (Crippen and Sheffield 2006), bringing into question the validity of the results from McAllister et al. (1994).

The objective of this study was to determine the length of time *A. diaperinus* can remain contaminated with *Salmonella* when given various doses of inoculated feed utilizing a marker-strain of *Salmonella* Typhimurium.

## **Materials and Methods**

***Alphitobius diaperinus* collection.** The darkling beetles used in this study were collected from two broiler farms in Georgia. Litter and beetles were collected using a garden trowel, then sieved through U.S. standard 2 and 3.35 mm sieves into plastic pans (12 in diameter). The 2 mm sieves were primarily used to extract the larval stages. The resulting litter and beetles were again sieved into 29.3 liter Rubbermaid® “Latchables” plastic storage containers. The beetles were then transported to the UGA entomology laboratory where they were housed in Sterilite® drawer carts. The adults and larvae were housed together, given styrofoam for pupation, as well as a combination of chicken feed and produce for food and moisture. The room temperature was maintained at approximately 27°C with natural daylight hours.

***Salmonella* culture.** A nalidixic acid-resistant strain of *S. Typhimurium* was utilized for all tests. Inocula were grown overnight on Brilliant Green Sulfa Agar (Acumedia, Baltimore, Maryland) with the addition of 200 ppm of nalidixic acid (Sigma, St. Lois, MO) (BGS+NAL). Cells were suspended in 0.85% NaCl; the optical density was measured with a spectrophotometer (Spec 20, Milton Roy, Rochester, NY) at 540 nm. Using a standard curve, the number of cells for the inoculum was determined. Inoculum level was confirmed by serial dilution and plating onto BGS +NAL.

**Beetle Contamination.** For each of the inoculation levels and life stages, beetles (n=1000) were placed in a confinement container and starved for 24 h. However, at day 15 a sponge moistened with water in a petri dish was added to each of the larva confinement containers as a source of moisture. The beetles were given 60 g of broiler starter mash that had been spray-inoculated with a high ( $10^9$ ), medium ( $10^5$ ), or low ( $10^3$ ) level of nalidixic acid-resistant *Salmonella* Typhimurium and were allowed to feed for 24 h. After 24 h on the inoculated feed, the beetles were removed and the inoculated feed weighed and compared to the initial amount of feed to determine the approximate per beetle consumption of inoculated feed. The level of inocula in the feed and per beetle consumption are shown in Tables 1.1 and 1.2.

**Sampling and Isolation.** Samples were taken 24 h post-inoculation, then at varying intervals based on the number of positives in the previous sample. The beetles were removed from the inoculated feed and placed in a separate confinement container with non-medicated, non-inoculated feed after each sampling.

Individual beetles (n=10) were aseptically collected utilizing a sterile forceps for each beetle and placed in 10 individual 2.2 ml sterile centrifuge tubes. Sampled beetles were macerated with a sterile wood applicator and 1 ml of 1% buffered peptone was added to each sample. In addition, a pooled sample was taken where ten beetles were aseptically collected and placed into a sterile 15 ml centrifuge tube. Pooled beetles were macerated and 10 ml of 1% buffered peptone was added to the sample and incubated at 37°C overnight.

Following incubation, 0.1 ml of the enrichment broth was then streaked onto BGS plates containing 200 ppm of nalidixic acid and incubated at 37°C overnight.

**Persistence through pupation.** One thousand late instar (close to pupation) *A. diaperinus* were contaminated utilizing the same methods as previously mentioned; however the larvae were exposed to the contaminated feed continuously until pupation. styrofoam was included in the confinement container for a pupation site. Weekly, the styrofoam was removed from the contaminated confinement container and placed into a sterile container. When adults emerged, they were tested for the presence of the marker *S. Typhimurium* utilizing the aforementioned methods.

## Results and Discussion

For the high ( $10^6$ ) inoculum of adult beetles (Table 1.1 and Fig. 1.1) all sampled beetles were positive for the first 3 d post-inoculation in the first study and for the first 24 h in the second study. Positive samples were obtained for up to 64 d from the high inoculation group in both studies. In the McAllister et al. (1994) study, following a single 24 h feeding, *Salmonella Typhimurium* was detected in adult darkling beetle feces for up to 28 d. Surface swabs and whole body homogenates were positive for *S. Typhimurium* 16 d post-exposure. Figures 1.3 and 1.2 show the level of persistence through 57 d of sampling in both studies. Day 64 is not on either graph, for only the pooled samples were positive at that time.

For the first 24 h, all of the larvae sampled were positive for the marker *S. Typhimurium*. The number of positive samples remained high through 36 d post-inoculation. Larvae exposed to the high inoculation remained positive for up to 49 d



post-inoculation in both trials (Table 1.2, Figs.1.3 and 1.4). This is considerably longer than the 13 d *S. Typhimurium* was detected in larval beetle feces and the 16 d of external rinses after a 24 h exposure to  $3 \times 10^8$  cells/ml of *S. Typhimurium* by McAllister et al. (1994).

Due to insufficient numbers of larvae to sample, these larval tests had to be terminated prematurely before all of the samples were negative for the marker *Salmonella*. If the sampling period for the larvae had been longer it is likely that the marker *Salmonella* would have been maintained as long as or longer than in the adults. There were a number of possible reasons that the larval colonies were more difficult to maintain than the adult colonies.

Larvae are known to become cannibalistic, especially on newly molted larvae before the cuticle hardens, as well as on pupae (Despins et al. 1988). Therefore, consumption of their conspecifics may have played a large role in decline of larval numbers. Since this study utilized late instars, and the larval stage can be as short as 21 d (Rueda and Axtell 1997), it is possible that some may still have been contaminated with the marker *Salmonella* but were pupating without finding adequate hiding places and hence succumbing to cannibalism.

Larvae also have higher moisture requirements than the adults and are more susceptible to desiccation when not provided adequate water (Preiss 1969). At day 15 a sponge moistened with water in a petri dish was added to each of the confinement containers as a source of moisture. Unfortunately, because of the more delicate biology

of the larval stage than the adult stage, we were unable to keep sampling the contaminated larvae for any longer than 49 d.

The adult beetles exposed to the medium level of inoculum remained positive for only 7 d (Table 1.1) in the first study and up to 27 d at the medium inoculum in the second trial (pooled positive). De las Casas et al. (1968) found positive adult darkling beetles for up to 24 d after exposing them to dog food inoculated with approximately  $10^6$  cells/ml of *Salmonella* Typhimurium. The presence of the marker *Salmonella* in the pooled samples from this study after 27 d agrees with the results found by De las Casas (1968). The marker *Salmonella* persisted for up to 49 d on (or in) the larvae exposed to the medium inoculum ( $10^5$ ) feed. The average length of time a grow-out house remains empty is 7-10 d, therefore *Salmonella*-positive beetles from a previous flock have the potential to contaminate subsequent flocks, which indicates that larvae could serve as a potential *Salmonella* reservoir for the next broiler flock.

The larval stages inoculated with medium and low levels of *S. Typhimurium* remained positive much longer than the adults tested at the same inoculation level. The longer persistence of *Salmonella* in the larval stage at these levels suggests that the larvae would most likely remain positive longer than the adults had there been a sufficient number of larvae to sample. The natural level of *Salmonella* exposure to beetles in a broiler grow-out house is not fully elucidated. However, the exposure level in a commercial broiler house will vary greatly depending on the source of contamination the beetles have access to, whether it be contaminated litter, which will contain very low levels of *Salmonella*, or fresh contaminated feces, that will contain very high levels of *Salmonella*, could greatly affect the length of time the beetles will harbor *Salmonella*. In

a study of *Salmonella* population estimations in commercial turkey farms, the level ranged from  $<1$  to  $\geq 5.3$  log MPN/g for litter samples and from  $<1$  to 4.9 log MPN/g for fecal dropping samples. Litter collected from farms housing the younger birds had significantly higher mean *Salmonella* populations compared with older birds (Santos et al. 2005). The fact that *Salmonella* was able to persist as long as 27 to 49 d after exposure to low and medium levels of *Salmonella* gives an idea of how long it may last on a beetle in a real world situation.

Of the adults exposed to the feed inoculated with a low level of *S. Typhimurium* ( $1.03 \times 10^3$ ), all samples were consistently negative (Table 1.1), so the bacteria were either not consumed or not able to adhere to the beetles at this level. In the second study the level of inoculum was higher ( $2.8 \times 10^3$ ), and samples were positive up to 9 d. The ability of beetles to consume or of *Salmonella* to adhere in the second trial, and not the first, may be due to the higher level of *Salmonella* inoculum in the feed in the second trial. Even though variation was seen between trials, it still showed that a low level of inoculation does not persist as long in adult beetles as compared to larvae.

Though the tables give the impression that *Salmonella* persisted longer on adults when exposed to the high inocula, larvae had consistently higher numbers of positives throughout the sampling periods. The larvae exposed to the low inoculum in the second trial remained positive for 35 d post-inoculation (Table 1.2). Ten out of ten beetles sampled were positive for the marker *S. Typhimurium* 24 h post-inoculation. By the 35<sup>th</sup> day of sampling the number of beetles, due to sampling and some mortality, had declined to 3. Of those last three tested, one was positive.

Of the total 21 adults to emerge from pupation, 4 were positive for the marker *S. Typhimurium*. The relatively few adults to emerge may be attributed to variation in the duration of pupation for each beetle. It also may be due to the larvae being able to find and consume the pupae due to the small amount of space in the pupation material given. Because of the smaller scaled population used in this experiment compared to a commercial broiler flock, detection of *Salmonella* in even a few adults could be much more significant on a larger scale.

Prepupae, larval beetles that will soon pupate, search for a hiding place in the insulation as well as deep below the litter in the earth floor, to escape predation. Removal of litter from broiler houses did not noticeably reduce the number of beetles found in the subsequent flock (Lambkin et al. 2007). Pesticides may not be able to reach larvae and pupae hiding in the insulation or the compacted earth floor, and the litter removal may not be successful in eliminating the beetles permanently. The resulting adult emergence is a source of reinfestation when the next flock of birds is brought into the house. The results show that some of the larvae will remain positive through pupation and emerge as *Salmonella*-positive adults. They may then contaminate the subsequent litter, beetles, or flock of birds that were previously free of *Salmonella*.

Skewes and Monroe (1991) could not show a relationship between darkling beetle population levels and bird mortality, feed conversion, condemnation rate, or production costs in commercial broiler flocks. With the use of a marker *Salmonella* strain, we have shown that darkling beetles can be a significant source of *Salmonella* dissemination in a broiler grow-out house, confirming the results of McAllister et al.

(1994). These insects should be considered when developing broiler management protocols, and pest management should incorporate methods of controlling darkling beetles that target inaccessible stages such as prepupae and pupae secluded in refugia.

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## Tables

Table 1.1 Persistence time for *Salmonella* Typhimurium in adult darkling beetles, based on levels of inocula

Challenge	Inoculum Level/gram of feed	Inoculum level per beetle	Persistence Time (d)	Percent Positive
High	2.03 x 10 <sup>9</sup>	3.72 x 10 <sup>7</sup>	64	20
High	6.42 x 10 <sup>8</sup>	3.02 x 10 <sup>6</sup>	64	pool
Medium	1.22 x 10 <sup>4</sup>	123.22	7	30
Medium	4.67 x 10 <sup>4</sup>	474.47	27	pool
Low	124.89	.0608	0	0
Low	466.67	2.52	9	10

Table 1.2 Persistence time for *Salmonella* Typhimurium in larval and pupal darkling beetles, based on levels of inocula

Challenge	Inoculum Level	Inoculum level per beetle	Persistence Time (d)	Percent Positive
High	$7.5 \times 10^8$	$2.96 \times 10^6$	36	70
High	$1.17 \times 10^9$	$5.89 \times 10^6$	49	Pool
Medium	$1.17 \times 10^5$	$6.42 \times 10^2$	49	Pool
Medium	$1.17 \times 10^5$	$5.99 \times 10^2$	49	30
Low	$1.17 \times 10^3$	7.02	43	30
Low	$1.17 \times 10^3$	7.01	35	33
Pupa	$1.17 \times 10^9$	--	Through Pupation	19

### Figures

Fig. 1.1 Persistence of *Salmonella* Typhimurium in adult darkling beetles post-inoculation (High inoculum: trial 1)

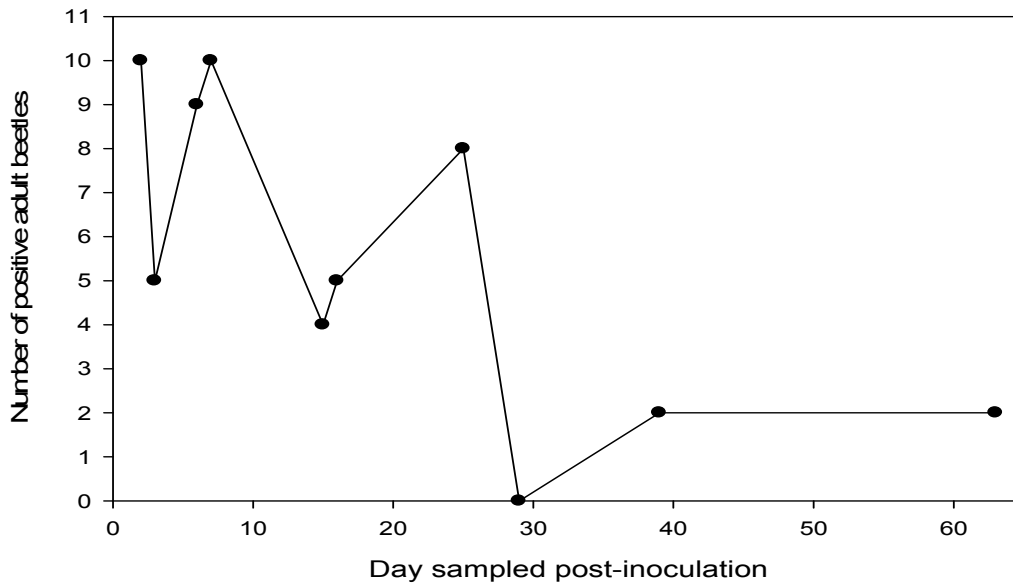




Fig. 1.2 Persistence of *Salmonella* Typhimurium in adult darkling beetles post-inoculation (High inoculum: trial 2)

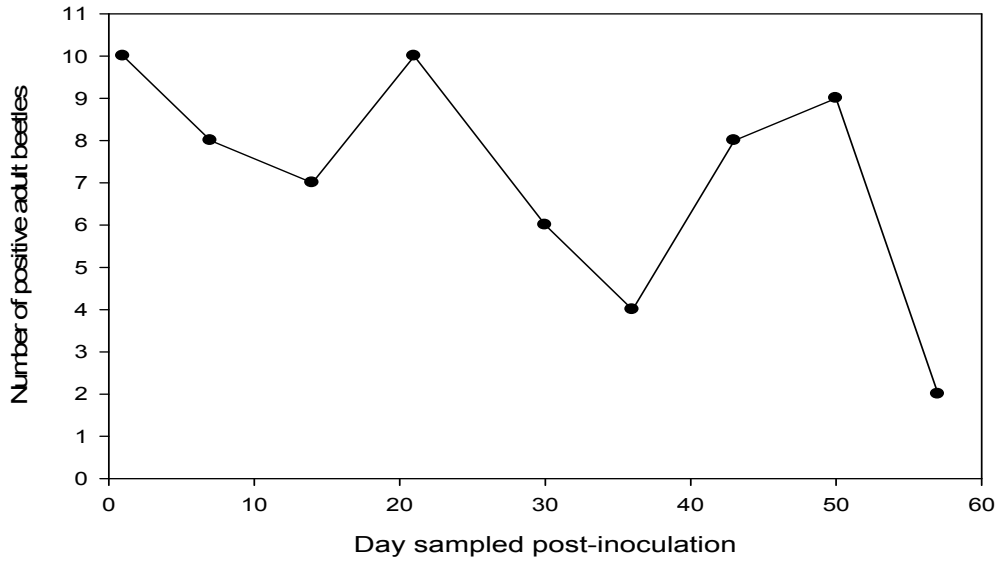


Fig. 1.3 Persistence of *Salmonella* Typhimurium in larval darkling beetles post-inoculation (High inoculum: trial 1)

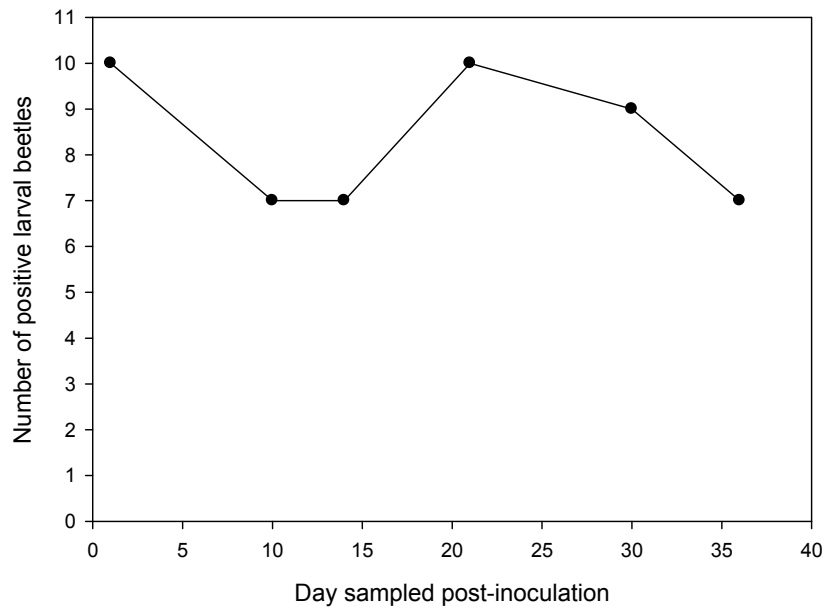
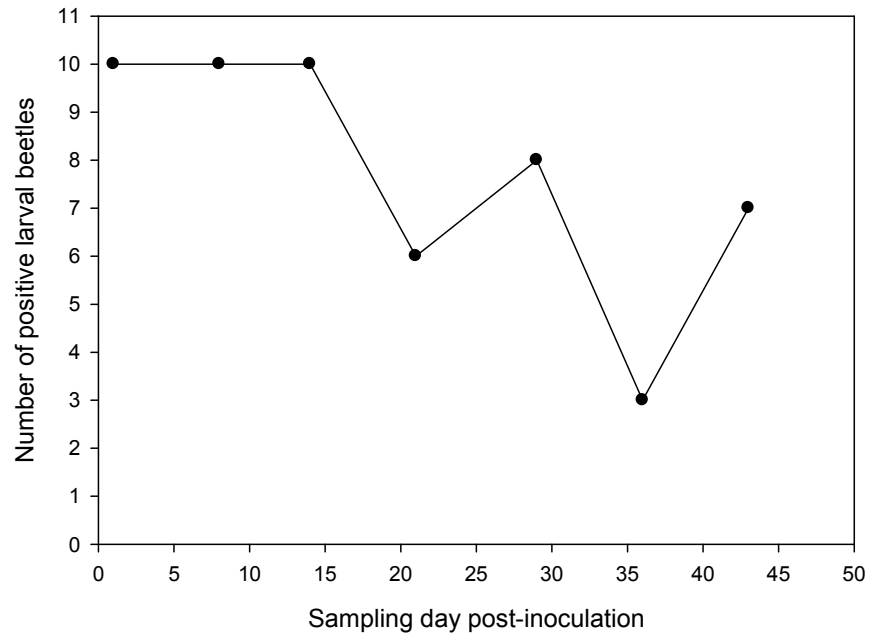


Fig. 1.4 Persistence of *Salmonella* Typhimurium in larval darkling beetles post-inoculation (High inoculum: trial 2)



## CHAPTER 5

### ENUMERATION OF A MARKER *SALMONELLA* TYPHIMURIUM FROM INOCULATED ADULT AND LARVAL LESSER MEALWORMS, *ALPHITOBIUS* *DIAPERINUS* (COLEOPTERA: TENEBRIONIDAE)<sup>1</sup>

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<sup>1</sup> A.J. Roche, N.A. Cox, L.J. Richardson, R.J. Buhr, J. A. Cason, B. D. Fairchild, N.C. Hinkle. To be submitted to *Journal of Medical Entomology*.

## Abstract

The persistence and level of inoculated *Salmonella* Typhimurium in adult and larval lesser mealworms, *Alphitobius diaperinus* (Panzer), were evaluated over time. In the preliminary trial 2, 4, 8, and 16 beetles were inoculated then enumerated to determine level of inoculation after 24 h. The level of inoculation in pooled samples (n=4) was also evaluated for 30 d in the larvae and 64 d in the adults. Beetles (n=1000) were exposed to feed contaminated with a nalidixic acid-resistant marker strain of *Salmonella* Typhimurium for 24 h. Pooled samples of 4 beetles were cultured for the marker *Salmonella* weekly using a modified three-swab method of bacterial enumeration. The averages of the weekly pooled larval samples maintained *S.* Typhimurium levels at logs 3.86 to 6.4 throughout the 30 d sampling period. For adult beetles, the average level of *S.* Typhimurium from the 10 pooled samples each week maintained logs 3.86 to 5.15 for the first 30 d before numbers of positive pools decreased. Adult and larval lesser mealworms can maintain *S.* Typhimurium for over 30 d post-inoculation.

Keywords: *Alphitobius diaperinus*, *Salmonella* Typhimurium, lesser mealworm, darkling beetle, poultry

## Introduction

The lesser mealworm, *Alphitobius diaperinus* (Panzer), is a common insect pest of commercial poultry operations where all life stages can be found. The litter (bedding plus chicken manure) and the earthen floors are where large populations of *A. diaperinus* are found (Harding and Bissell 1958, Axtell and Arends 1990, Steelman 1996, Lambkin et al. 2007). The adults, commonly referred to as darkling beetles, and larvae are known to feed on poultry manure, feed, dead or moribund birds, and other insects such as house fly larvae or other lesser mealworms (Harding and Bissell 1958, Despins et al. 1988, Rueda and Axtell 1997). Concern about the lesser mealworm's constant contact with potentially pathogenic microorganisms in its litter habitat, as well as its tendency to consume potentially contaminated materials in the litter, has stimulated investigations into the reservoir competence of the lesser mealworm for avian and human pathogens.

Mealworms have been shown to harbor numerous poultry and human pathogens. The fowl pox virus was retained by birds fed darkling beetles and excreted through the feces for up to 6 d (De las Casas et al. 1976). Avian leukosis virus was transmitted to healthy chicks by feeding inoculated darkling beetles or injecting extracts of contaminated darkling beetles (Eidson et al. 1966) and both routes of entry produced tumors in chicks. *Choanotaenia infundibulum*, a poultry cecal worm, was found in naturally-occurring and experimentally-infected adult *A. diaperinus* (Elowni and Elbihari 1979). Skov et al. (2004) demonstrated that *Salmonella* and *Campylobacter* could be recovered from beetles sampled from broiler houses during production.

Five serovars of *Salmonella* were isolated from surface-sterilized adult *A. diaperinus* collected from poultry brooder houses in Minnesota (Harein et al. 1970). De las Casas et al. (1968) found that live darkling beetles could serve as reservoirs of *Salmonella* Typhimurium and *Escherichia coli* for up to 24 d, and that mixing cultures of *S. Typhimurium* and *E. coli* did not affect the growth of either bacterium on the feed or in the beetles. The results indicated that dead beetles could maintain *Salmonella* contamination for up to 45 d.

McAllister et al. (1994) fed lesser mealworms chicken starter feed inoculated with  $10^8$  CFU/ml of *S. Typhimurium* and by testing beetle feces were able to determine persistence time in individual beetles. All of the larvae voided the bacteria in their feces for the first 13 d, and adults for the first 12 d. Overall *Salmonella* was detected in the feces for 28 d post-inoculation (McAllister et al. 1994). They were also able to inoculate day-old chicks by feeding them each one adult or larva inoculated with  $10^8$  CFU/ml *Salmonella* Typhimurium. Cox et al. (1990) determined that the  $CD_{50}$ , or colonization dose for 50% of the population, of *S. Typhimurium* for 1-d-old chicks was  $10^{2.4}$  if challenged orally, which is significantly lower than the  $CD_{50}$  for 3-d-old chicks that required  $10^{4.5}$  cells of *S. Typhimurium* by oral route.

The time a broiler spends in a grow-out house ranges from 6 to 8 wk and the time between flocks, when house maintenance, cleaning, and pesticide application occur, can be from a few days to 2 wk. Adults begin emerging from the soil before the end of the flock grow-out and continue emerging through the clean-out period between flocks and during the subsequent flock grow-out, therefore constituting a source of broiler house reinfestation (Axtell and Arends 1990, Lambkin et al. 2007).

If given a choice between starter feed and darkling beetle larvae, 2- to 3-d-old broiler chicks consumed an average 389 larvae per bird per day (Despins and Axtell 1995). Because broilers will readily consume *A. diaperinus*, it is conceivable that the lesser mealworm serves as a reservoir of *Salmonella* to broilers during the grow-out period and could be a source of recontamination of broiler grow-out houses and equipment, as well as a source for colonization of subsequent broiler flocks.

The objectives of this study were to 1) quantify *Salmonella* in beetle pools, varying in number from 1 to 16, 2) determine variation in *Salmonella* numbers between beetle samples, and 3) evaluate the level of *Salmonella* in pooled beetle (larvae and adults) samples over time.

### **Materials and Methods**

In experiment 1, adult (n=50) and larval (n=50) beetles were extracted from the UGA colony, placed in separate confinement containers and starved for 24 h. The beetles were then exposed to 25 g of chicken feed spray-inoculated with 5 ml of a solution containing  $9.7 \times 10^9$  CFU/ml of nalidixic acid-resistant *Salmonella* Typhimurium. After 24 h of exposure, samples of 1, 2, 4, 8, and 16 beetles were taken for analysis.

In experiment 2, adults (n=1000) and larvae (n=1000) were utilized. The beetles were exposed for 24 h to 50 to 60 g of chicken feed spray-inoculated with 10 ml of a  $4.5 \times 10^9$  CFU/ml suspension of the marker organism. The inoculum level of *Salmonella* in the feed for the adults was  $6.42 \times 10^8$  CFU/g of feed, and each beetle consumed an estimated  $3.02 \times 10^6$  CFU of the marker *Salmonella*. The larvae were exposed to  $1.17 \times 10^9$  CFU/g and consumed an estimated  $5.89 \times 10^6$  CFU. The exposed beetles were then sampled at days 1, 3, 7, 14, 21, and 30. After each sampling, the remaining

beetles were removed from the feed and placed on non-inoculated non-medicated broiler feed.

**Swab-plate method.** The number of *S. Typhimurium* cells was estimated using a modification of the swab-plate method (Blanchfield et al. 1984) developed by Bailey et al. (1988). Beetles were macerated and 3 ml of buffered peptone water (BP) was added to the tubes. After the tubes were vortexed, a cotton-tipped swab was dipped and rotated in each tube for 5 s. A Brilliant Green Sulfa agar plate (Acumedia, Blatimore, Maryland) containing 200 ppm nalidixic acid (BGS+NAL) was surface spread plated with the swab (plate A). The swab was then broken off into a 10 ml BP dilution tube, which was vigorously shaken for 10 s. A second BGS+NAL plate was then inoculated by dipping a fresh swab into the dilution suspension and spreading as described for plate A (to yield plate B). The plates were then incubated overnight at 37°C. To enrich the sample, the original sample and the contents of the 10 ml BP dilution tube were combined and incubated overnight. The swab procedure does not detect fewer than 100 *Salmonella*/g. If no colonies grew on plate A, the enriched suspension was streaked onto BGS+NAL plates and incubated overnight (Blanchfield et al. 1984, Bailey et al. 1988).

**Estimation of count.** Blanchfield et al. (1984) quantitated inocula on the plates by measuring the weight of the swab before and after loading with material. Using this method they determined that swabs dipped and rotated to coat with sample broth took up 0.15 g of broth (wet weight) and deposited 0.055 g onto plate A. The amount put on plate A was 18-fold less than 1 g. So results were multiplied by 18 to convert all samples to a 1-g equivalence (Blanchfield et al. 1984). Bailey et al. used a 3-fold



(volume:weight) dilution so the total factor for each estimated count was 54 (18 x 3). Plate B was a 1 to 100 dilution so the multiplier was 5400. This accounts for the amount of broth transferred by the swabs and the dilution.

Log counts were determined from plates A and B. If *Salmonella* was detected from enriched broth but not direct plating, it was given a log count of 1.5. A log count of 0 was given to samples in which *Salmonella* was not detected in either the direct or enriched plates (Bailey et al. 1988). The average log counts from the 10 samples were calculated for every day sampled.

### **Results and Discussion**

In the first experiment, the objective was to quantify the level of *Salmonella* in pooled beetle samples containing varying numbers of larvae or adults. In the adult experiment, the sample containing 1 adult had a log 4. The 2-adult sample was a log 3. The 4-adult sample had a log 4, 8 beetles had a log 6, and the sample of 16 beetles had a log 4. De las Casas (1972) found wide variation in the number of bacteria among individual insects, and so had to use averages and trends as well. The larval samples were more consistent than the adults. One and two larvae had log 3 of *Salmonella*. The sample of four larvae had a log 4, the sample of 8 larvae had a log 5, and the 16 larvae sample had a log 6. There is a positive correlation in number of *Salmonella* cells with increasing numbers of larvae in the sample, a trend not observed in the adults. Considerable variation was seen in the adult samples, but it was determined that four adult or four larval beetles could convey an inoculum of  $10^4$ .

In experiment 2, the level of *Salmonella* in beetles (n=4) was monitored in both larval and adult beetles for up to 64 d. For adults, the averages over the sampling

period are illustrated in Fig. 2.1, and the results from the individual samples are shown in Table 2.1. Twenty-four hours post-inoculation, the adults averaged  $10^5$  *Salmonella* per sample. The averages remained at or near  $10^4$  for up to 30 d in both adult and larval beetles. This suggests that the *Salmonella* was able to colonize the beetles due to the fact that high levels could be observed through the 30 d sampling interval for both. Had the beetles not been colonized it is possible that there would have been a sharp decrease in the level of *Salmonella* after the first week of sampling, since the feed was removed after each sampling. The average *Salmonella* load decreased slightly on d 36, but by the end of the sampling period (64 d) the 10 samples averaged a log 3. Days 50 through 64 had averages lower than log 4, but each of those days contained at least one individual sample above  $10^4$  and could cause colonization of a day-of-hatch chick. At the end of the 64 d study, adult darkling beetles sampled maintained a bacterial load high enough to inoculate young broiler chicks. In a study by Cox et al. (1990) they found that levels of log 2 or greater were sufficient to colonize day of hatch chicks by oral inoculation. Therefore, even though variations in the levels of *Salmonella* were observed in the adult samples, the sample averages remained high enough for colonization to occur in day-old chicks for at least 64 d after inoculation.

The level of *Salmonella* colonization in larvae was monitored for 30 d, at which time the supply of larvae for sampling was exhausted. The counts from the individual larval samples are illustrated in Table 2.2, while Fig. 2.2 illustrates the progression of *Salmonella* maintenance through the sampling period. Twenty-four hours post-inoculation, the average level of *S. Typhimurium* was log 5. A week later (day 7), the average still remained high and on the final day of sampling (day 30) the average was

still high enough to potentially inoculate a day-of-hatch chick. The *Salmonella* in the larvae remained at a high level throughout the entire 30 d of sampling and might have continued longer had sampling continued. The larval *Salmonella* counts were not significantly declining, indicating the level of *Salmonella* on the larvae was not significantly decreasing. This suggests that the larvae could maintain the bacteria for longer than the sampling period.

De las Casas et al. (1972) found the bacterial level in the beetles declined within 2-3 d after collection when beetles were maintained in brooder house litter. They also noted that starvation decreased the number of bacterial colonies per insect, and assumed food in the alimentary tract at the time of analysis may have an effect. The beetles in the current study were not starved after the initial 24 h, but were provided non-inoculated feed that did not result in *Salmonella* decline. In another study by De las Casas et al. (1968), 2 mealworms on sampling day 4 had a bacterial load of  $10^6$  to  $10^7$ . As mentioned previously, when given a choice between starter feed and darkling beetle larvae, 2- to 3-d-old broiler chicks consumed an average of 389 larvae per bird per day (Despins and Axtell 1995), so four beetles is a gross underestimation of the number that could be ingested by a chick in a commercial broiler house. The results show that darkling beetles are a potential vector for *Salmonella* transmission to broilers if even a low number of beetles are ingested.

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### Tables

Table 2.1 Enumeration of *Salmonella* in individual samples of four adult darkling beetles and the average log of the 10 samples taken each sampling day (expressed in log form)

Sample	Sampling Day										
	1	7	10	14	22	30	36	44	50	57	64
1	7.7	3.7	6.7	2.7	5.7	1.5	1.5	3.7	1.5	0	4.7
2	4.7	3.7	5.7	3.7	4.7	4.7	4.7	5.7	3.7	5.7	4.7
3	6.7	5.7	4.7	4.7	4.7	4.7	4.7	3.7	2.7	0	4.7
4	3.7	1.5	5.7	4.7	5.7	4.7	1.5	4.7	2.7	1.5	0
5	3.7	1.5	4.7	4.7	3.7	4.7	4.7	4.7	1.5	0	2.7
6	4.7	4.7	4.7	4.7	5.7	1.5	0	4.7	0	1.7	3.7
7	4.7	3.7	1.5	4.7	1.5	5.7	0	5.7	4.7	1.5	3.7
8	4.7	4.7	5.7	4.7	1.5	2.7	4.7	2.7	2.7	0	0
9	5.7	3.7	6.7	4.7	4.7	4.7	1.7	4.7	4.7	1.5	0
10	3.7	5.7	5.7	5.7	5.7	4.7	4.7	2.7	3.7	0	3.7
<b>Avg.</b>	5	3.86	5.18	4.5	4.36	3.96	2.82	4.3	2.79	1.19	2.79
<b>SD</b>	1.33	1.46	1.48	0.78	1.64	1.49	2.06	1.07	1.49	1.76	2.02

Table 2.2 Enumeration of *Salmonella* in individual samples of four larval darkling beetles and the average of the 10 samples taken each sampling day (expressed in log form)

		Sampling day					
Sample	1	7	10	14	22	30	
1	6.7	4.7	5.7	5.7	5.7	3.7	
2	6.7	4.7	1.5	6.7	7.7	4.7	
3	6.7	4.7	5.7	6.7	5.7	5.7	
4	5.7	5.7	6.7	5.7	5.7	2.7	
5	4.7	6.7	3.7	6.7	6.7	1.5	
6	6.7	4.7	3.7	6.7	6.7	5.7	
7	4.7	4.7	4.7	4.7	7.7	1.5	
8	4.7	5.7	4.7	7.7	6.7	3.7	
9	4.7	3.7	1.7	6.7	5.7	4.7	
10	4.7	5.7	5.7	2.7	5.7	4.7	
<b>Avg.</b>	5.6	5.1	4.38	6	6.4	3.86	
<b>SD</b>	0.99	0.84	1.73	1.41	0.82	1.54	

### Figures

Fig. 2.1 Average log and standard deviation of *Salmonella* Typhimurium in pools of four adult darkling beetles

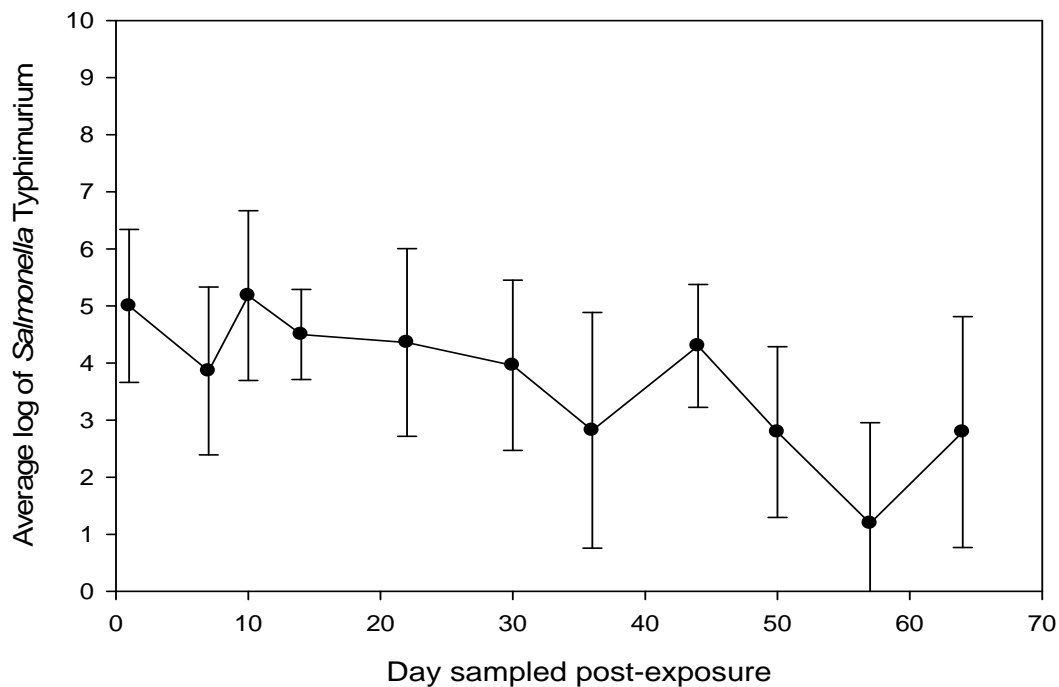
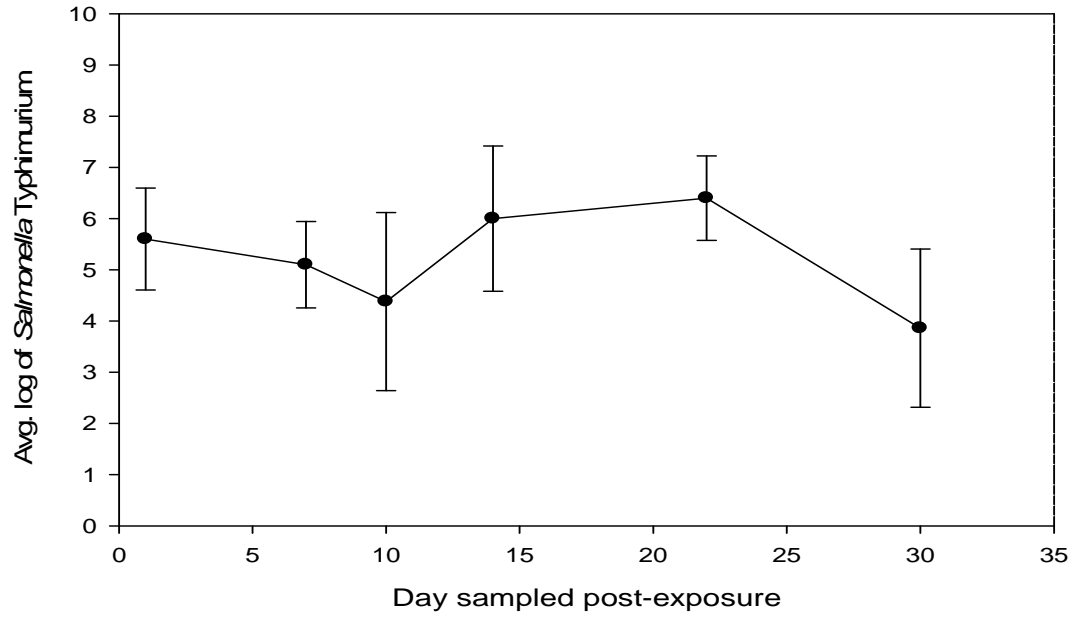


Fig. 2.2 Average log and standard deviation of *Salmonella* Typhimurium in pools of four larval darkling beetles





## CHAPTER 6

### CONTAMINATED LARVAL AND ADULT LESSER MEALWORMS, *ALPHITOBIUS DIAPERINUS* (COLEOPTERA: TENEBRIONIDAE) CAN TRANSMIT *SALMONELLA* TYPHIMURIUM IN A BROILER FLOCK<sup>1</sup>

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<sup>1</sup>A.J. Roche, R.J. Buhr, L.J. Richardson, N.A. Cox, B.D. Fairchild, J.A. Cason, and N.C. Hinkle. To be submitted to *Journal of Medical Entomology*

## Abstract

The ability of the lesser mealworm, *Alphitobius diaperinus* (Panzer), or darkling beetle as it is commonly known, to transmit a marker-strain-*Salmonella* Typhimurium to day-of-hatch broiler chicks was evaluated as well as the spread of the marker organism to adjacent pen mates. In experiment 1, day-of-hatch chicks were orally gavaged with 4 adult darkling beetles or 4 larval darkling beetles that had been exposed to inoculated feed for a period of 24 h. Preliminary studies found that this gave an approximate log 4 inoculation level of the marker organisms. In addition, chicks were orally inoculated with log 4 of the marker organism to serve as a positive control. The chicks were then placed into pens to serve as challenge birds. In experiment 2, chicks were gavaged beetles that had been exposed to inoculated feed for 24 h then removed from the inoculated feed for a period of 7 d. For cecal samples, at 3 wk of age the challenge birds and 5-10 pen mates were sampled for the presence of the marker organism, and at 6 wk of age the remaining birds' ceca were sampled. To monitor the environmental presence of the marker organism within pens, drag swab samples were taken weekly. Drag swab samples were positive from 1 through 6 wk after introduction of challenge birds gavaged with the *Salmonella*-inoculated adult or larval darkling beetles, and the control (peptone-inoculated) birds were positive for 3 to 6 wk. For the control (peptone-challenge) pens, 29% of the challenge broilers and 10-55% of the sampled pen mates were positive at 3 wk of age and 2-6% at 6 wk. For the adult beetle challenge pens, 0-57% of the challenge broilers and 15-40% of sampled pen mates were positive at 3 wk and 7-26% at 6 wk. The larva-challenged pens had the highest number of *Salmonella*-positive birds; 33% of the challenge broilers and 45% of sampled pen mates were

positive at 3 wk and 11% at 6 wk. In this study, challenge chicks became colonized with *Salmonella* by oral gavage of either adult or larval beetles and spread *Salmonella* to pen mates. These results demonstrate that beetles colonized with *Salmonella* could be a significant vector for the transmission of *Salmonella* to chicks when ingested and readily spread *Salmonella* throughout a broiler flock.

Keywords: *Alphitobius diaperinus*, *Salmonella* Typhimurium, lesser mealworm, darkling beetle, poultry

## Introduction

The lesser mealworm, *Alphitobius diaperinus* (Panzer), is a common pest of layer and broiler facilities. It was first known as a cosmopolitan and secondary stored product pest of grain and cereal products, however it is gaining notoriety as a reservoir for poultry and human pathogens (Skov 2004). Commercial broiler farms suffer financial losses due to high population density of the darkling beetle (Steelman 1996). In addition to structural damage, pest control, and decreased feed conversion efficiency, many losses center on the ability of darkling beetles to transmit poultry pathogens as well as the economic impact of human illness (McAllister et al. 1995).

Numerous studies have shown that bacteria and viruses are present in beetles. In one study, 26 pathogenic serotypes of *Escherichia coli* were isolated from 151 adult darkling beetles collected from 5 Minnesota turkey brooder houses (Harein et al. 1970). In addition, avian leukosis virus (Marek's disease) can be transmitted to broilers that ingest beetles contaminated with the virus (Eidson et al. 1966, Lancaster and Simco 1967). It has also been shown that darkling beetles can transmit immunosuppressive

viruses (Goodwin and Waltman 1996) that may contribute to the infection of broilers by other pathogens.

De las Casas et al. (1972) were able to isolate *E. coli*, *Salmonella*, and other potentially human pathogenic bacteria from larval and adult lesser mealworms collected from poultry brooder houses. Harein et al. (1970) collected 1000 adult beetles from turkey brooder houses and found a bacterial contamination rate of 2.2%, with 5 different *Salmonella* serotypes isolated from the positive beetles sampled. Adult beetles killed and stored in a sterile environment were positive for *S. Typhimurium* for up to 45 d (De las Casas et al. 1968). Despins and Axtell (1995) discovered that, when given a choice between starter feed and larval darkling beetles, the average consumption of a 2- to 3-d-old chick was 389 beetle larvae per day. McAllister et al. (1994) orally gavaged day-of-hatch chicks 1 adult or larval beetle contaminated with *S. Typhimurium* and tested for colonization the infection by cloacal swabs 24 and 48 h after consumption. The authors found that 9/10 chicks orally gavaged one larva, and 7/10 chicks orally gavaged 1 adult, had positive cloacal swabs within 24 h of consumption. The 3 chicks orally gavaged adult beetles that tested negative 24 h after inoculation were positive after 48 h.

Among the foodborne bacterial pathogens reported, Salmonellae caused the largest percentage of outbreaks in human cases (Lynch et al. 2006). Implementation of sanitary practices along with Hazard Analysis and Critical Control Point (HACCP) programs in 1998 greatly reduced the prevalence of *Salmonella* on broiler carcasses processed in the U.S., which has decreased the incidence of foodborne salmonellosis associated with poultry products (Russell 2002). To develop better microbial control

methods, all modes of transmission must be identified and interventions implemented at the farm level.

The objective of this study was to determine whether consumption of adult and larval beetles exposed to a marker *S. Typhimurium* could cause colonization in chicks and then spread pen mates.

### **Materials and methods**

In a prior study in the previous chapter, enumeration of *S. Typhimurium* was determined from inoculated adult and larval beetles. From this study, it was estimated that a group of 4 beetles would have an average level of  $10^4$  cells of *S. Typhimurium*.

Trial One: Non-medicated broiler starter feed was inoculated with a nalidixic acid-resistant *S. Typhimurium* at a level of  $10^9$  CFU per beetle. Two groups of beetles, 1 group of 50 adults and another group of 50 larvae, were placed in the treated feed for 72 h. Each group of beetles was then removed from the feed and placed into a 15 ml polypropylene conical tube. Four adult or larval beetles were orally gavaged to day-of-hatch broiler chicks (challenge) with a 1 ml modified syringe to obtain an inoculation level of  $10^4$  CFU of *S. Typhimurium*. As a positive control or standard comparison, other challenge birds were inoculated with a peptone solution containing  $\log 4.7$  CFU/ml of the marker *S. Typhimurium*. The challenge birds were then wing-banded for future identification.

Trial Two: Non-medicated broiler starter feed was inoculated with a nalidixic acid-resistant *S. Typhimurium* at a level of  $10^9$ . Two groups of beetles, one group of 100 adults and another group of 100 larvae, were placed in the feed for 7 d. After the 7 d

period, the above-mentioned procedures in trial 1 were conducted. The peptone solution contained  $3.8 \times 10^4$  CFU/ml of the marker *S. Typhimurium*

**Experimental Rooms.** All tests were conducted in a single building with 6 pens in 6 identical rooms (Appendix B. 1, B. 2). Each pen was 1.06 m wide by 2.29 m long (3 ft 6 in x 7 ft 6 in), on cement floors with wood shavings. Each pen contained one tube feeder and nipple drinker lines, both hung from the ceiling. Feed and water were provided *ad libitum*. Broilers were managed according to the broiler breeder recommendations.

In the first trial, half of the pens had 1 challenge while the others had 2 challenge chicks for each challenge type placed into pens containing non-inoculated chicks. In the second trial, 2 challenge chicks were placed into pens containing non-inoculated chicks. Each pen contained a total of 40 chicks at a density of  $650.3 \text{ cm}^2/\text{bird}$  ( $0.7 \text{ ft}^2/\text{bird}$ ). All procedures and animal care were approved by the University of Georgia's Institutional Animal Care and Use Committee. challenge birds inoculated with each mode of challenge were placed in the 2 end pens in 2 of the 6 rooms (4 pens total for each challenge)

**Drag Swabs.** To detect the presence of the marker *Salmonella* in the pens, weekly the stepped-on drag swab procedure was performed (Buhr et al. 2007). Two drag swabs (DS-001, Solar Biologicals Inc., Ogdensburg, NY) per pen were presoaked in skim milk, unwound, and dragged in such a way to create a figure eight design in the pen. The swabs were stepped on 10 times throughout being dragged around the

perimeter of the pens to transfer *Salmonella* from the litter to the swabs. The swabs were then placed in bags marked by pen.

To recover the marker *Salmonella* from the drag swabs, 100 ml of 1% buffered peptone water was added to each drag swab bag. The bags were then shaken and incubated overnight at 37°C. One loopful of the enriched broth was plated onto Brilliant-Green Sulfa agar (Acumedia, Baltimore, Maryland) with the addition of 100 mg/liter of 200 ppm of nalidixic acid (Sigma, St. Lois, MO) (BGS+NAL) using 3 mm plastic loops. Nalidixic acid added to the agar was used to eliminate background bacteria from the samples and ensure growth of only the marker *Salmonella* resistant to this level of nalidixic acid. The plates were then incubated overnight at 37°C.

**Ceca Samples.** Ceca samples were taken when the birds were 3 wk and 6 wk of age. In the first trial, at 3 wk of age, the wing-banded challenge birds and 5 pen mates were euthanized and their ceca aseptically removed and placed into sterile plastic sampling bags, labeled accordingly and transported back to the laboratory on ice. In the second trial, the ceca of the wing-banded challenge birds and 10 pen mates were removed using the same methods as above. At 6 wk of age the ceca from all of the remaining (non-challenged) birds in each pen were removed using the methods described.

**Isolation.** Each of the ceca sample bags was weighed, contents were macerated with a rubber mallet, and buffered peptone water was added at 3 times the volume to weight of the sample. The bags were stomached for 30 s and placed in a 37°C incubator for pre-enrichment overnight. After pre-enrichment, 0.1 ml of each

sample was streaked onto BGS+NAL agar plates and incubated overnight at 37°C. Following incubation the colony forming units (CFU) on the plates were counted and values recorded.

## Results and Discussion

**Drag Swab Samples** (Figs. 3.1 and 3.2). Drag swab samples were taken every wk during broiler grow-out. All pens containing one and two challenge birds challenged with inoculated adult and larval beetles tested positive for *S. Typhimurium* from 1 to 6 wk. The pens containing control birds inoculated with peptone had more variation; pens remained positive for 4-6 wk in the first trial and 3-6 wk in the second trial. Unexpectedly, some of the peptone-positive controls were outlasted by the experimental treatments. Drag swab sample results indicated that within the first week of grow-out the marker *S. Typhimurium* had contaminated the pens. The continued positive samples for 3 to 6 wk implied that either the bacteria persisted in the litter or the birds continued to shed bacteria over the 6 wk period. This demonstrated that both adult and larval *A. diaperinus* can acquire *Salmonella* from a contaminated environment and convey an infective dose if consumed by day-of-hatch chicks. Sufficient *Salmonella* is subsequently shed in feces to colonize other chicks in proximity to the challenge birds.

**3 and 6 Week Ceca Samples** (Tables 3.1-3.6). All chicks challenged with adult beetles in the first trial had ceca that tested negative for the presence of the marker *S. Typhimurium* 3 wk after inoculation. The absence of the marker *Salmonella* from all of



the challenge birds given adult beetles would suggest that the *Salmonella* persisted in the chicks for less than 3 wk post-inoculation, during which time the *Salmonella* cells may have been sloughed from the ceca or outcompeted by natural flora and excreted with feces. Thirty-three percent of the larva-challenged birds and 29% of the control birds (challenged with peptone) were positive after 3 wk (Table 3.5).

In the second trial, more than half of the adult-challenged birds and pen mates were positive for the marker and continued to excrete *Salmonella* for 3 to 6 wk. Twenty-five percent of the chicks challenged with larval beetles and 29% of the control (peptone-challenged) birds tested positive for the presence of *S. Typhimurium* 3 wk after inoculation. The *Salmonella*-inoculated larvae were able to cause colonization of *Salmonella* in the chicks, with persistence up to 3 wk after ingestion of the beetles. These results agree with the McAllister et al. (1994) study in which day-old chicks were gavaged with 1 adult or larval beetle inoculated with *S. Typhimurium*, and the cloacal swab samples were positive for 24 to 48 h after inoculation. Darkling beetles can be a source of *Salmonella* colonization of day-old broiler chicks.

More pen mates of larva-challenged challenge birds were positive 6 wk after introduction of the challenge birds than were pen mates of adult- or control (peptone-challenged) birds. The number of positive pen mates of the control (peptone-challenged) birds and the adult-challenged birds were not different. The positive cecal samples of the pen mates at 3 wk and 6 wk indicate that the bacteria did colonize the birds via the beetles and were then transmitted to other birds in the same pen. The pen mates of the birds inoculated with beetle larvae appeared to be more likely to be positive than the pen mates of control (peptone-inoculated) birds. Despins and Axtell

(1995) reported that darkling beetle larvae were made up of about 20% crude lipid and 67% crude protein. Lipid levels in meat have been documented to influence the bacterial heat resistance (Hansen and Riemann 1963, Ahmed et al. 1995), and higher lipid levels in beef result in increased heat and acid resistance of *Salmonella* Typhimurium DT104 (Juneja and Eblen 2000). So the lipid content of the larvae could have been affecting the ability of *Salmonella* to colonize the challenge birds and then the spread to pen mates. The content of adult darkling beetles has not yet been determined, so the role that lipid content played in the success of the larval- versus adult-challenged birds requires further evaluation.

At the end of broiler grow-out, prior to catching the birds for transport, the feed is removed for approximately 8 to 12 h. Hungry broilers forage through the litter and consume *Salmonella*-contaminated beetles, which contributes to the high levels of *Salmonella* in the birds' crops (Byrd et al. 2001), (Russell 2002). During processing, the crop can break and leak its contents, contaminating the carcass and equipment (Hargis et al. 1995). The presence of *Salmonella* in 6-wk-old broilers resulting from exposure to pen mates inoculated by 4 *Salmonella*-colonized beetles demonstrates the reservoir competence of *A. diaperinus* for *S. Typhimurium* in a broiler flock and the potential for subsequent spread during processing.

An important aspect to remember about this study is that only the challenge birds were exposed to beetles, and those birds were only ingesting gavaged beetles. There were no darkling beetles in the experimental pens to further spread *Salmonella*. If the pens had included darkling beetles at population densities comparable to those often seen in commercial broiler houses, there likely would have been greater spread of

*Salmonella* from continued consumption of darkling beetles living in the contaminated litter.

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## Tables

Table 3.1 Trial 1: Three-week ceca samples positive for marker *Salmonella* Typhimurium

Pen #	Challenge	Challenge birds	Pen mates	Total ceca samples	% Positive (not including challenge birds)
1	Adults	0/1	2	5	40
6	Adults	0/2	0	5	0
19	Adults	0/2	1	5	20
24	Adults	0/1	1	5	20
13	Control*	1/1	0	5	0
18	Control*	0/2	2	5	40
31	Control*	0/2	0	5	0
36	Control*	1/1	0	5	0
25	Larvae	0/1	1	5	20
30	Larvae	1/2	3	5	60
7	Larvae	0/2	2	5	40
12	Larvae	1/1	3	5	60

\*Peptone

Table 3.2 Trial 1: Six-week ceca samples positive for marker *Salmonella* Typhimurium

Pen #	Challenge	# positive	Total ceca samples	% positive
1	Adults	5	40	13
6	Adults	2	40	5
19	Adults	2	40	5
24	Adults	2	33	6
13	Control*	0	37	0
18	Control*	3	40	8
31	Control*	0	38	0
36	Control*	0	40	0
25	Larvae	4	38	11
30	Larvae	4	36	11
7	Larvae	4	40	10
12	Larvae	3	40	8

\*Peptone

Table 3.3 Trial 2: Three-week ceca samples positive for marker *Salmonella* Typhimurium

<b>Pen #</b>	<b>Challenge</b>	<b>Challenge birds</b>	<b>Pen Mates</b>	<b>Total ceca samples</b>	<b>% Positive (not including challenge birds)</b>
1	Control*	0/2	7	10	70
6	Control*	1/1	4	10	40
13	Control*	1/2	4	10	40
18	Control*	0/2	7	10	70
7	Larvae	0/2	5	10	50
12	Larvae	0/2	4	10	40
25	Larvae	0/2	4	10	40
30	Larvae	2/2	10	10	100
19	Adults	0/1	8	10	80
24	Adults	2/2	5	10	50
31	Adults	1/2	3	10	30
36	Adults	1/2	0	10	0

\*Peptone

Table 3.4. Trial 2: Six-week ceca samples positive for marker *Salmonella* Typhimurium

Pen #	Challenge	# positive	Total ceca samples	% Positive
1	Control*	3	30	10
6	Control*	0	33	0
13	Control*	4	26	15
18	Control*	0	25	0
7	Larvae	2	28	7
12	Larvae	11	35	31
25	Larvae	14	33	42
30	Larvae	8	35	23
19	Adults	3	38	8
24	Adults	1	35	3
31	Adults	0	34	0
36	Adults	2	34	6

\*Peptone

Table 3.5 Trial 1: Percentage of cecal samples positive for *Salmonella* from peptone, larval, and adult beetle challenge at 3 and 6 wk of age

Challenge	3 wk (challenge birds)	3 wk (pen mates)	6 wk (pen mates)
Control*	29%	10%	2%
Adult Beetles	0%	15%	7%
Larval Beetles	33%	45%	11%

\*Peptone

Table 3.6 Trial 2: Percentage of cecal samples positive for *Salmonella* from peptone, larval, and adult beetle challenge at 3 and 6 wk of age

Challenge	3 wk (challenge birds)	3 wk (pen mates)	6 wk (pen mates)
Control*	29%	55%	6%
Adult Beetles	57%	40%	4%
Larval Beetles	25%	58%	27%

\*Peptone



## Figures

Fig 3.1 Trial 1: *Salmonella* Typhimurium positive drag swab samples by challenge and pen

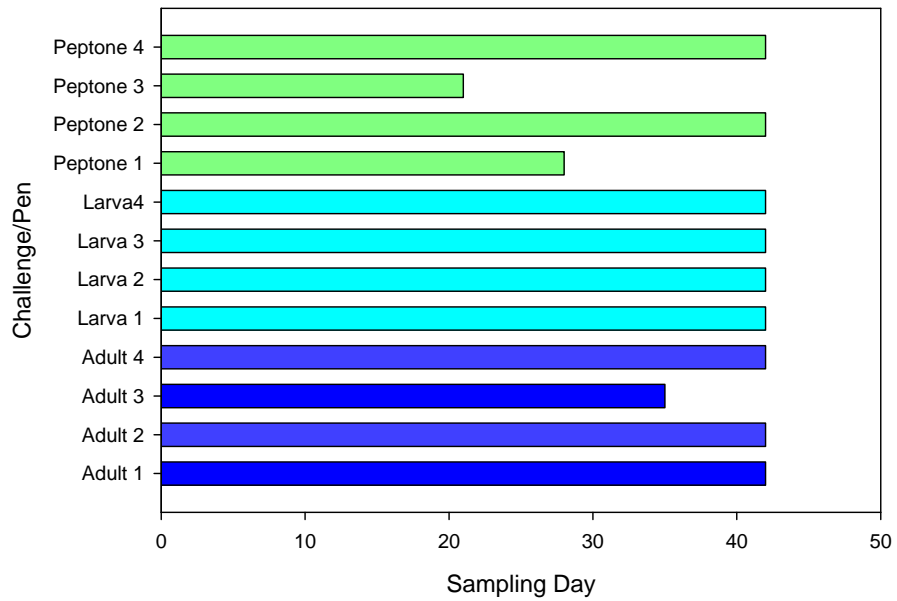
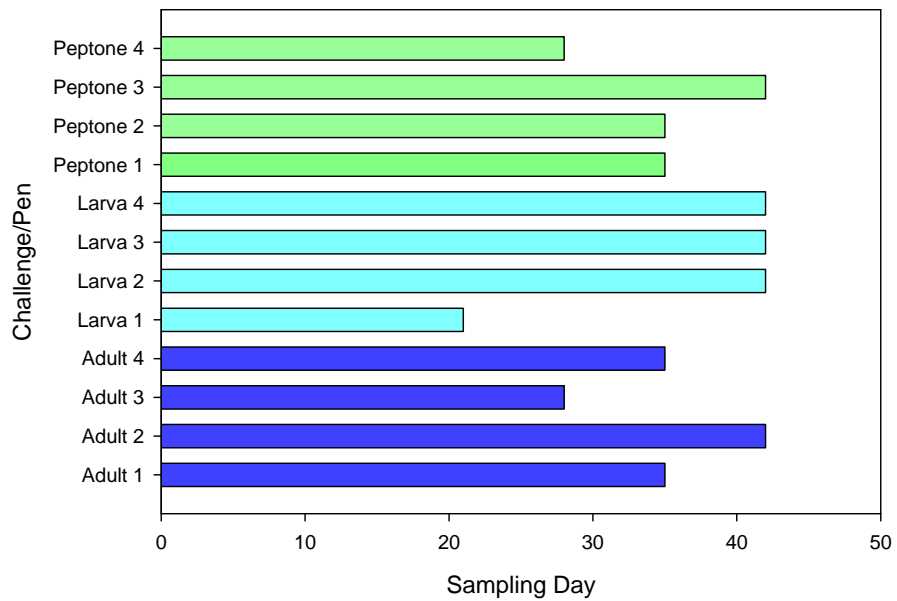


Fig 3.2 Trial 2: *Salmonella* Typhimurium positive drag swab samples by challenge and pen



## CHAPTER 7

### CONCLUSION

These experiments showed that *Salmonella* Typhimurium could persist in three of the life stages of *Alphitobius diaperinus*, and that groups of *A. diaperinus* could maintain levels of *S. Typhimurium* high enough for subsequent transmission for intervals longer than the typical time between flocks that can vary from a few days to three weeks. Chapter 6 demonstrated that broiler chicks can become colonized with *S. Typhimurium* if they consume as few as 4 adult or larval darkling beetles with a combined concentration of  $10^4$  *Salmonella*, which can then spread to other birds in a flock.

The dense populations of darkling beetles in a boiler house can be a liability to the grower as a nuisance, a problem in broiler efficiency or grower operations, and a vector of human and animal pathogens. Before testing the transmission potential of darkling beetles to broilers, the persistence time and level of inoculum of *Salmonella* in *A. diaperinus* was first determined.

*Salmonella* was found to persist on adult darkling beetles for over two months when exposed to high levels of *S. Typhimurium* ( $10^9$ ), for nearly a month when given a relatively medium inoculum ( $10^5$ ), and up to nine days at a low inoculum ( $10^3$ ).

*Salmonella* was able to persist on larval darkling beetles longer at lower doses than on

adults. High ( $10^9$ ) and medium ( $10^5$ ) inocula lasted for up to 49 d, when the larval sampling had to be terminated.

Additionally, this study confirmed the results of McAllister et al. (1994) that the pupal stage was able to maintain *Salmonella* through pupation and adult emergence. So the bacteria can persist in three of the *A. diaperinus* life stages, longer than the period between flocks of most commercial broiler operations. Darkling beetles can thus serve as an important source of *Salmonella* contamination in a broiler house.

When it was determined that four adult and larval darkling beetles inoculated with *S. Typhimurium* could reach an inoculation level of about  $10^4$ , which would be sufficient to colonize day-of-hatch broiler chicks, it was necessary to find out how long the beetles could maintain this level of contamination. Adult beetles contained *Salmonella* for up to 30 d, with the ability to colonize broiler chicks if only four beetles were consumed. Groups of four larvae could maintain the ability to colonize broiler chicks if consumed for at least 30 d (and possibly longer) as well.

Day-of-hatch broiler chicks given four adult or larval darkling beetles were able to excrete enough *Salmonella* in their feces to result in pen mates being colonized. The feces in the pens were positive for the excreted *Salmonella* for up to 6 wk after introduction of challenged birds.

There still exist uncertainties concerning darkling beetles and their relationship to *Salmonellae*. The level of *Salmonellae* naturally occurring in poultry houses has yet to be determined, nor has the level of *Salmonella* in individual adult and larval beetles from

commercial poultry houses been ascertained. Other pests in poultry houses, such as mice and rats, prey on darkling beetles. If these animals consume beetles colonized with *Salmonella*, will they then become colonized? How much of the pathogen would be excreted in their feces?

Where are the bacteria adhering to darkling beetles? Are the bacteria on the outside of these beetles or the inside? While McAllister et al. (1994) claimed that their study confirmed that *A. diaperinus* maintained a viable infective internal *Salmonella* population following surface disinfection, Crippen and Sheffield (2006) disputed whether or not the method used successfully eliminated external contaminants from the beetles.

If the bacteria are indeed colonizing the inside of the beetles, where then are the *Salmonella*? Is the alimentary tract of the beetle being colonized similarly to those of mammals or poultry, or are the bacteria moving to the hemolymph? A histological examination of beetle tissues for *Salmonella* may answer some of these questions, or extraction of beetle digestive tracts at different time periods after consumption of the marker *S. Typhimurium* may permit localization of bacteria in the beetle reservoir.

The main objective of this project was to further reiterate the importance of darkling beetles as pests in poultry operations and to stimulate interest in methods for controlling them. This beetle has been demonstrated to harbor bacteria, viruses, parasites and many other microorganisms that are pathogenic to humans and animals. As these insects become increasingly resistant to the insecticides available, darkling beetles are more difficult to control. Their long reproductive life allows for high population density even if the larval stages are being controlled. An ovicide may stop

the population from growing by breaking the cycle at the egg stage, but adult beetles may persist for a year or more (Preiss and Davidson 1971).

The majority of foodborne *Salmonella* infections have been associated with animal products, poultry meat and eggs in particular. By identifying the modes of *Salmonella* transmission in a broiler operation, control measures can be developed and instituted to improve food safety. This study has illustrated the role darkling beetles play in *Salmonella* persistence and transmission, highlighting beetle suppression as a component of on-farm food-safety security programs.

### References

**Preiss, F. J., and J. A. Davidson. 1971.** Adult longevity, pre-oviposition period and fecundity of *Alphitobius diaperinus* in the laboratory (Coleoptera: Tenebrionidae). *Journal of the Georgia Entomological Society* 6: 105-109.

## APPENDIX A

### ADULT AND LARVAL *ALPHITOBIOUS DIAPERINUS*

**Figure A. 1** Microptic image of the dorsal side of an adult *Alphitobius diaperinus*.



**Figure A. 2** Microptic image of the side view of an adult *Alphitobius diaperinus*



**Figure A. 3** Microptic image of metathoracic tibial spines on the leg of an adult *Alphitobius diaperinus*.





**Figure A. 4** Microptic image of the dorsal side of an *Alphitobius diaperinus* larva



Figure A. 5 Microptic image of the ventral side of an *Alphitobius diaperinus* larva



Figure A. 6 Microptic image of the side view of an *Alphitobius diaperinus* larva



**APPENDIX B** Transmission of *Salmonella* from beetles to broilers

Fig. B. 1. Image 1 of pens in an experimental arena



Fig. B. 2 Image 2 of pens in experimental arena

