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ORIGINAL ARTICLE

# Transient gut retention and persistence of *Salmonella* through metamorphosis in the lesser mealworm, *Alphitobius diaperinus* (Coleoptera: Tenebrionidae)

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## Abstract

**Aims:** This study was undertaken to determine the retention of *Salmonella* through *Alphitobius diaperinus* metamorphosis and its contribution, through defecation, to external contamination.
**Methods and Results:** Insects were exposed to a tagged *Salmonella enterica* and evaluated for external elimination. (i) Each day for 3 weeks, a filter collected frass from a restrained insect for analysis. (ii) Exposed larvae in a closed container were followed through pupation, and newly emerged adults were examined for their retention of marker bacteria.
**Conclusions:** Exposed adults and larvae produced *Salmonella*-positive frass for an average of 8 days, ranging from 6 to 11 days and 6 to 12 days, respectively. Nineteen per cent of the larvae carried *Salmonella* through metamorphosis and eclosion, with 5% of the pupal exuviae being positive as well.
**Significance and Impact of the Study:** Many sources of foodborne pathogens within the poultry production facilities, including reservoir populations, currently go unrecognized. This diminishes the ability of producers to mitigate the transfer of pathogens between animals, humans and the environment. Poultry management standards accept the reutilization of litter. *Alphitobius diaperinus* survive between flock rotations on the reutilized litter, and it was demonstrated in this study that the *Salmonella* they carry can survive with them.

## Introduction

Salmonellosis in humans is generally contracted through the consumption of contaminated food. The causative organism, *Salmonella*, can enter the production process at any point (i.e. preharvest, postharvest or during food preparation) and pass through the chain from primary production facilities to households or food-service operations. Although these infections often result from a small number of infectious agents unintentionally amplified by the improper handling of the food commodities, a thorough understanding of the on-farm ecology and epidemiology of *Salmonella* from which to mitigate the initial contamination is lacking. Between 2 and 4 million cases of Salmonellosis occur in the United States annually (http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM052519.pdf; accessed June 2011). The World Health Organization estimated 1·4 million nontyphoidal *Salmonella* infections, resulting in 168 000 visits to physicians, 15 000 hospitalizations and 580 deaths occurred in 2005 (http://www.who.int/mediacentre/factsheets/fs139/en/; accessed June 2011).

Poultry are commonly infected with a wide variety of *Salmonella* serovars; however, infections are generally subclinical (Wray *et al.* 1996). Of the broad host spectrum of

*Salmonella* serovars isolated from chickens, *Salm.* Enteritidis and *Salm.* Typhimurium have been the most common in recent years. Mortality from *Salm.* Enteritidis may be high in chicks under 1 week of age, resulting in direct economic losses to the producer (Smith and Tucker 1980; Gast and Beard 1989). Infection in older chicks and adult chickens is largely asymptomatic. Occasionally, adult layers can show clinical symptoms, particularly during times in which the immune system may be stressed, such as transport or moulting (Hopper and Mawer 1988; Lister 1988). Infection in older chicks can result in uneven growth, stunting and rejection at slaughter because of lesions from pericarditis and septicaemia (Lister 1988; O'Brien 1988). While these *Salmonella* strains may cause disease in poultry, of greater concern is their ability to cause foodborne illness in humans via the consumption of contaminated poultry meat or egg products. This concern led to the establishment of federal measures to control the contamination levels in poultry at the production stage. Compliance with these measures is an added cost to producers. If the pathways of *Salmonella* dispersal and contamination within a poultry production facility can be determined and controlled, then the cost benefits to the producer are self-evident.

The lesser mealworm beetle, *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae), is a serious pest in poultry facilities known to carry pathogens affecting both human and animal health (reviewed in: Crippen and Poole 2012). Their mobility, indiscriminate feeding habits and their potential to then be preyed upon by poultry facilitate the dissemination of *Salmonella*. Current farm management practices perpetuate infestations and contribute to the dissemination of beetles and pathogens (Calibeo-Hayes *et al.* 2005). Producers are challenged to reduce pathogen load at the farm level; however, poultry management standards accept the reutilization of litter for successive flocks of broilers. The lesser mealworm can survive between flocks rotated onto the same litter, where they add to the bacterial exposure of the new flock or to the environment when the insect-laden litter is removed for application onto nearby fields (Armitage 1986; Calibeo-Hayes *et al.* 2005).

To devise effective measures to prevent dissemination, the on-farm ecology and epidemiology of this microbe must be elucidated, including the dynamics of movement of the bacteria between possible reservoir populations during the production phase. It is unlikely that *Salmonella* can ever be completely eliminated from the production system, but awareness of the constituents contributing to *Salmonella* transfer helps producers improve intervention strategies. This study investigated the duration of *Salmonella* contamination by adult and larval *A. diaperinus* defecation subsequent to a single exposure to *Salmonella*, and the persistence of *Salmonella*, acquired during the larval stage, through metamorphosis to newly eclosed adults.

## Materials and methods

### Beetles

The Southern Plains Agricultural Research Center (SPARC) starter colony of *A. diaperinus* was originally isolated from a poultry farm located in Wake County, NC, and maintained by Dr. D. W. Watson (North Carolina State University, Raleigh, NC, USA). The SPARC colony was initiated and has remained in production since 2004. Beetles were reared in 1000 ml wheat bran (Morrison Milling Co., Denton, TX, USA) in plastic containers (15 × 15 × 30 cm) with screen tops and held at 30°C in an 8-h:16-h (light/dark) cycle. Additionally, each cage contained a 6-$cm^2$ sponge moistened with deionized water and a 0·5-cm-thick slice of a medium-sized apple replenished twice per week, and 30 ml of fishmeal (Omega Protein, Inc., Hammond, LA, USA) was added to the wheat bran once per week.

### Experimental design

Three replications (with insects from separate generations) of each experimental exposure protocol were conducted using at least 30 beetles per treatment. The fluorescent marker bacteria, *Salmonella enterica* serovar Typhimurium-green fluorescent protein (ST-GFP), a generous gift from Drs. Roy J. Bongaerts and Jay Hinton, Norwich Research Park, Norwich, UK, were used to track the movement of bacteria through the insect alimentary canal (Hautefort *et al.* 2003). Adults >4 weeks postemergence and larvae of 5–6 weeks of age were used for internal retention, and late-instar larvae, *c.* 1 week prior to pupation, were used for retention through metamorphosis studies.

### Exposure

Insects were selected from the colony and exposed to phosphate-buffered saline (PBS; control) or a lawn ST-GFP (treated) in exposure tubes, as previously described (Crippen *et al.* 2009). Briefly, exposure tubes were produced by placing 7 ml trypticase soy agar (TSA) into a 17 × 100 mm (14 ml) snap cap, polypropylene, round-bottom tube (Fisher Scientific, Pittsburgh, PA, USA) and seeding the agar with the appropriate concentration of bacteria in a 10-$\mu$l volume. The insects were placed into the tubes and allowed to move freely for the entire exposure time at 30°C in the dark. The insects were then

collected and externally disinfected by sequential wash in 95% EtOH, SporGon® (Decon Labs, Inc., Bryn Mawr, PA, USA) and sterile water, as previously described (Crippen and Sheffield 2006). Disinfection was validated by rinsing the insects for 3 min in buffered peptone water (BPW). The BPW rinse was incubated for 24 h at 37°C, then plated on TSA and enumerated after incubation for 24 h at 37°C. Following exposure to the marker *Salmonella* and surface disinfection, three of the study insects were randomly harvested to determine the initial bacterial uptake by enumeration via serial dilution onto TSA plates. In addition, ST-GFP was cultured from a random sample of the exposure tubes to assure that the insects were exposed to viable bacteria.

### Internal retention

To determine the internal retention interval of *Salmonella*, three replications were conducted using *c.* 40 adults or larvae per experiment. The insects were exposed to PBS (control) or a mean of $2{\cdot}58 \times 10^8$ CFU ml$^{-1}$ ST-GFP (treated) for 2 h in exposure tubes and externally disinfected, as described above. The insects were attached, using Super Glue® (Super Glue Corp., Rancho Cucamonga, CA, USA), by the ventral side of the thorax to the top of a sterile nail (no. $17 \times 1''$; Crown Bolt, Aliso Viejo, CA, USA). A piece of sterile filter paper (Whatman Grade 3; 2·3 cm diameter) was skewered onto the nail, beneath the insect to collect frass (faeces) for 24 h. The filter paper was collected and replaced daily, and each insect was sampled individually. The insects were fed twice a day with a drop (*c.* 5 $\mu$l) of liquid feed consisting of 20 ml whole milk, 1·5 g sugar and 1 g fish meal.

Each filter paper collected was placed into a 2·0-ml microcentrifuge tube with 1·0 ml BPW (BVA Scientific, San Antonio, TX, USA), mixed briefly and incubated at 37°C for 24 h to enrich for *Salmonella*. After 24 h, the sample was transferred into 9 ml tetrathionate broth (BVA Scientific) and incubated at 37°C for 24 h, at which time 100 $\mu$l was transferred into 5 ml Rappaport–Vassiliadis medium (BVA Scientific) and incubated at 42°C for 24 h. To determine the presence or absence of *Salmonella*, a 100-$\mu$l aliquot was then plated onto Brilliant Green agar (Biolink Scientific, Austin, TX, USA) containing 1·2% chloramphenicol (BGA-C) and incubated at 37°C for 24 h. Bacterial growth was visually determined, and the presence of ST-GFP was confirmed using fluorescence microscopy. This enrichment sequence was repeated until the filter samples from each insect were negative for *Salmonella* for at least three consecutive days. Subsequently, the negative insects were homogenized in BPW, and the above-described enrichment sequence was conducted to determine whether any *Salmonella* remained internally in the insect.

### Retention through metamorphosis

To determine the retention of *Salmonella* through metamorphosis, three replications were conducted using 40–50 larvae per experiment. The insects were exposed to PBS (control) or a mean of $6{\cdot}94 \times 10^8$ CFU ml$^{-1}$ ST-GFP (treated) for 6 h in exposure tubes and externally disinfected, as described above. Postexposure, three adults or larvae were removed, and initial bacterial uptake was determined. Larvae were placed 20–25 per 237-ml plastic tub and reared as described above. The insects were checked daily for metamorphosis and pupae were collected, surface-disinfected, as described above, and placed individually into $17 \times 100$ mm (14-ml) snap cap polypropylene round-bottom tubes (Fisher Scientific) at 30°C. Upon eclosion, each insect and discarded exuviae from ecdysis was sterilely collected, homogenized separately in 15-ml centrifuge tubes with 1·0 ml BPW, incubated at 37°C for 24 h and enriched for *Salmonella* by the procedure described above. The presence of ST-GFP was confirmed using fluorescence microscopy.

### Data analysis

Data were analysed using commercially available statistical software (Prism ver. 5.01; GraphPad Software Inc., La Jolla, CA, USA). Descriptive statistics were generated using the geometric mean (GM) calculated as the *n*th root of the product of (*n*) numbers and 99 and 95% confidence intervals (CI) presented in table formats and scatter dot plot with mean and range. A Student's *t*-test was performed for comparison of treatment effect on the presence of bacteria and on days to eclosion.

## Results

### Internal retention

Postexposure to ST-GFP, three adults or larvae were randomly collected and the geometric mean of initial bacterial uptake was determined to be $3{\cdot}17 \times 10^5$ CFU ml$^{-1}$ ST-GFP per adult and $7{\cdot}40 \times 10^5$ CFU ml$^{-1}$ ST-GFP per larva. The retention time of *Salmonella* in the alimentary canal was determined by the number of days the insect excreted viable *Salmonella* within its frass (Table 1). The experimental design prevented reinfection of the individual by their own frass, so that retention after a single controlled exposure could be measured. Exposed adults and larvae produced *Salmonella*-positive frass for an average of 8 days. The variation in retention time by adults ranged from 6 to 11 days and for larvae ranged from 6 to 12 days.

**Table 1** The longevity (days) of *Salmonella* in the gut of *Alphitobius diaperinus* was determined by the capture and enrichment of frass produced after a single 2-h exposure to marker *Salmonella* (average of $2{\cdot}58 \times 10^8$ CFU ml$^{-1}$)

| | Confidence interval | | | | Range | | |
|---|---|---|---|---|---|---|---|
| | Lower | | Upper | | Time | | |
| | GM* (days) | 95% | 99% | 95% | 99% | Shortest | Longest |
| Adult | 7·88 | 6·87 | 5·42 | 8·97 | 10·37 | 6 | 11 |
| Larvae | 7·89 | 7·42 | 6·78 | 8·37 | 8·98 | 6 | 12 |

*Geometric mean (GM) calculated as the *n*th root of the product of (*n*) numbers.

A mean of 97% of the larvae and 79% of the adults were shedding *Salmonella* within the first 24 h (Fig. 1). The percentage of insects defecating *Salmonella* into their surroundings fluctuated between 69 and 97% of the total insects (adults and larvae) over the first 7 days. That percentage steadily declined over the course of the subsequent 9 days. On day 8, 46% of adults were still shedding *Salmonella*, which dropped to 19% on day 9 and <10% thereafter. On days 8 and 9, 33% of larvae were still shedding *Salmonella*, which dropped to <10% thereafter. A total of 28% of the adults shed *Salmonella* on a daily basis; the remaining insects intermittently shed contaminated frass (data not shown). In contrast, 58% of the larvae shed *Salmonella* daily. In all but one case, *Salmonella* was shed on 50% or more of the total days; one larva excreted *Salmonella* only three of 7 days (43%).

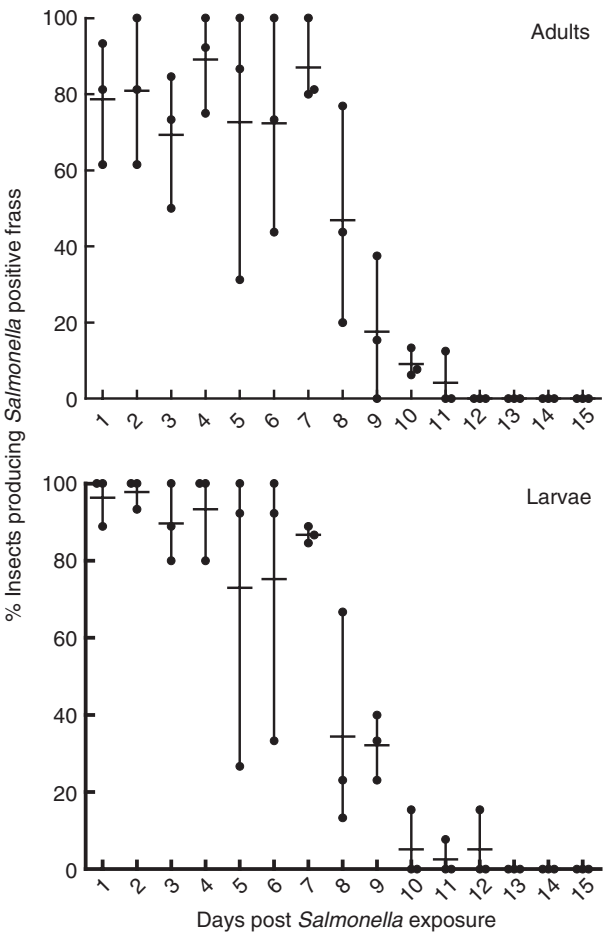


**Figure 1** *Alphitobius diaperinus* adults and larvae shedding *Salmonella* on a daily basis, determined by the capture and enrichment of frass produced after a 2-h exposure to a marker *Salmonella* ($2{\cdot}58 \times 10^8$ CFU ml$^{-1}$). The mean and range of triplicate experiments are presented in a scatter dot plot. Data were collected daily over 21 days; however, no *Salmonella* was recovered after 12 days (figure depicts the first 15 days).

### Retention through metamorphosis

The insects were negative for *Salmonella* before exposure of larvae to ST-GFP. After eclosion, the quantity of adults and discarded pupal exuviae that harboured *Salmonella* was determined (Table 2). The number of *ST-GFP*-positive adults from the treated group was significantly different from the PBS control group ($P < 0{\cdot}001$). Exposure to *Salmonella* did not significantly ($P = 0{\cdot}6562$) affect the time to eclosion between the treated and control groups, 4·54 and 4·45 days, respectively. Of the 102 treated larvae that underwent metamorphosis, 18·6%

**Table 2** The retention (days) of *Salmonella* through pupal metamorphosis and eclosion of *Alphitobius diaperinus* were determined by the enrichment of the discarded pupal case and the eclosed adult beetle after a single 2-h exposure of the late-instar larvae to a marker *Salmonella* followed by surface disinfection

| | Number of specimens analysed | Inoculum (CFU ml$^{-1}$) | Initial bacterial uptake (CFU ml$^{-1}$) | Time to eclosion (days) | Positive adults* | Positive pupal case† |
|---|---|---|---|---|---|---|
| *Salmonella* | 102 (34)‡ | $6{\cdot}97 \times 10^8$ | $1{\cdot}14 \times 10^6$ | 4·54 | 19 (6·33) | 6 (1·73) |
| PBS | 137 (46) | 0 | 0 | 4·45 | 0 | 0 |

*Adult insects from which marker *Salmonella* could be cultured after eclosion.

†Pupal cases from which marker *Salmonella* could be cultured after eclosion.

‡Total number of specimens analysed in three replicate experiments, followed by the mean of the three experiments, in parentheses.

harboured viable ST-GFP, and from those, 4·9% of the residual pupal exuviae also retained viable ST-GFP.

## Discussion

The fate of ingested foods, foreign bacteria or endogenous bacteria in insects has been minimally studied, but the capacity of insects to pass materials from their digestive system into the environment has been demonstrated. Movement of foodstuffs through the alimentary canal of insects occurs fairly rapidly. In green bottle fly maggots, *Lucilia sericata* Meigen (Diptera: Calliphoridae), gut transit occurs in 60–90 min (Mumcuoglu *et al.* 2001), and in the housefly, *Musca domestica* Linnaeus (Diptera: Muscidae), in about 100 min (Espinoza-Fuentes and Terra 1987). The gut transit of bacteria, however, is more complex. Hurst and Jackson (2002) established that a marker bacterium, *Serratia entomophilia*, could be recovered from grass grub, *Costelytra zealandica* White (Coleoptera: Scarabaeidae), 14 days after exposure and found the bacteria in the luminal fluid, adhering to the crop lining and associated with food particles. Previous results from this laboratory established an average gut transit time of 2–3 h for *Salmonella* in *A. diaperinus* (Zheng *et al.* 2011). Yet, in the current study, results demonstrate that *Salmonella* continues to be present and excreted long past 3 h, even when the insect is initially exposed for only a short period of time (2 h). It appears that *Salmonella* can give rise to transient colonization of the gut of *A. diaperinus*.

A delay of at least 10–14 days between flock rotations in a broiler production facility is recommended to reduce the infection of new chicks by organisms surviving from the previous flock (Khan 2004). However, many producers rotate flocks on intervals as short as 7 days. Our results demonstrate that *A. diaperinus* is capable of shedding *Salmonella* for up to 12 days following a single 2-h exposure. While in the poultry house, these beetles are constantly exposed to birds, litter and feed from the floor environment, all of which can harbour pathogenic bacteria (Kilonzo-Nthenge *et al.* 2008). The effect of continuous exposure of the beetles to *Salmonella* could likely increase the shedding time. The present study demonstrates that any beetle harbouring *Salmonella* in their gut serves as a bacterial reservoir that can feasibly span the time between flock rotations. Therefore, even if treatments are conducted to control bacteria in the litter, viable *A. diaperinus* represent a residual reservoir of pathogen dispersal whose control should be incorporated into a biosecurity management plan.

The capacity of bacteria to persist through the metamorphic changes has been studied in a variety of organisms. In fact, the immature stages of some fly species are actually helped by the persistence of specific bacteria during development (Lysyk *et al.* 1999; Ahmad *et al.* 2006; Romero *et al.* 2006). In *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae), gut bacteria can survive pupation and colonize the newly emerged adult (Zurek *et al.* 2000; Ahmad *et al.* 2006). Rochon *et al.* (2005) measured the retention of *Escherichia coli* by houseflies and stable flies from third instars and found that 98–99% of pupae retained *E. coli* after pupation. After emergence, 100% of pupal cases, 78% of adult houseflies and 28% of adult stable flies were infected. The persistence of pathogenic bacteria from larvae into the emergent adult can differ with insect species, and titres can increase, as well as decrease depending on the metamorphic stage (Radvan 1960a,b,c; Hung *et al.* 2004; Gabre 2009).

There are likely threshold levels influencing the survival of bacterial species during metamorphic processes suggesting a natural resistance or competitive exclusion to infection (Radvan 1960a,b; Greenberg *et al.* 1970). In our study, <20% of eclosed adults were infected, demonstrating variability in individual bacterial retention by the beetles. Results also indicated a decrease over time in *Salmonella* load; however, owing to the use of enrichment procedures to ensure the capture of infectivity, we did not enumerate the bacterial load. These results may be concentration dependent, as a dose–response curve has yet to be explored, and certainly the pre-existing enteric bacterial community structure would have some influence on colonization success.

Greenberg (1959) quantitatively studied the persistence of bacteria in *M. domestica*. Analysis indicated that sloughing of the cuticular lining of the fore- and hindgut during pupation displaces the majority of the bacteria to the inner surface of the puparium and exuviae, which become the repository for bacteria (Greenberg 1959). Radvan (1960c) found that *Salmonella schottmuller* was primarily located on the pupal exuviae and the surface of the adult *M. domestica* and *Protophormia terrae-novae* after eclosion of infected pupae and theorized that the nutrient content of exuvial fluid supported the bacterial replication. Both flies and beetles undergo complete metamorphosis; however, the moulting process in the beetle does not completely sterilize the gut (Greenberg 1959). In our study, only 18% of the newly emerged adults were infected and 25% of the resulting exuviae, indicating that the moulting process does not completely expunge all bacteria (Greenberg 1959). Residual *Salmonella* present in the midgut could become a source of continued infection.

If bacterial movement during metamorphosis of *A. diaperinus* mimics that of fly species, then bacteria may be present on the body surface of the newly emerged adult as a result of its displacement into the exuvial fluid. Survival of bacteria on the body surface is theorized to be

markedly shorter than survival within the alimentary canal (Radvan 1960c). However, while conditions in the insect gut may be more accommodating to *Salmonella* survivability, in a poultry house the moist, nutrient-rich environment is generally conducive to its survival. Therefore, both internal and external routes of bacterial transmission via *A. diaperinus* have epidemiological impact within a poultry production facility.

Our understanding of the on-farm ecology and epidemiology of *Salmonella* is important for the development of biosecurity best management practices. We do not currently recognize all the sources and reservoir populations of foodborne pathogens during the poultry production process. This lack of data diminishes our ability to mitigate the transfer of pathogens between animals, humans and the environment. The USDA Food Safety and Inspection Service (FSIS) has reported the annual level of contamination and found a 20% baseline prevalence of *Salmonella* in broiler products prior to 2008 (FSIS 2009). Prevention of infection necessitates proper facilities management and flock biosecurity, including effective manure disposal and pest control (http://www.apd.rdg.ac.uk/AgEcon/livestockdisease/poultry/poultrysalm3.htm; accessed June 2011). Lesser mealworms are prevalent in many poultry production operations but are a difficult pest to control. Further, poultry management standards accept the reutilization of litter for successive flocks of broilers. The lesser mealworm can survive between flock rotations. It is evident from this study that any *Salmonella* they are carrying can survive with them and expose the next flock or the environment when litter is removed onto fields as fertilizer. Evaluation of current management practices should consider reduction in insect infestation levels a factor to help eliminate the unintentional propagation and dissemination of bacterial pathogens.

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