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Transstadial transmission of larval hemocoelic infection negatively affects development and adult female longevity in the mosquito *Anopheles gambiae*

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ABSTRACT

During all life stages, mosquitoes are exposed to pathogens, and employ an immune system to resist or limit infection. Although much attention has been paid to how adult mosquitoes fight infection, little is known about how an infection during the larval stage affects the biology of the resultant adult. In this study, we investigated whether a bacterial infection in the hemocoel of the African malaria mosquito, *Anopheles gambiae*, is transstadially transmitted from larvae to adults (both females and males), and whether immune stimulation in the hemocoel as a larva alters development or biological traits of the adult. Specifically, larvae were injected in the hemocoel with either fluorescent microspheres or *Escherichia coli*, and the following traits were examined: transstadial transmission, larval development to adulthood, adult survival, and adult body size. Our results show that transstadial transmission of hemocoel contents occurs from larvae to pupae and from pupae to adults, but that bacterial prevalence and intensity varies with age. Injury, immune stimulation or infection decreases the proportion of larvae that undergo pupation and eclosion, infection decreases the longevity of adult females, and treatment has complex effects on the body size of the resultant adults. The present study adds larval hemocoelic infection to the known non-genetic factors that reduce overall fitness by negatively affecting development and adult biological traits that influence mosquito vector competence.

1. Introduction

The holometabolous life cycle of mosquitoes includes a larval stage that inhabits aquatic environments and an adult stage that inhabits terrestrial and aerial environments, with only the adult stage directly responsible for transmitting disease-causing pathogens to vertebrate animals, including humans. Multiple stress-based experiments have shown that larval environmental factors such as temperature, larval density and food quantity can have carryover effects on adult life history traits, including effects on their susceptibility to infection (Alto, 2011; Araújo et al., 2012; Breaux et al., 2014; Briegel, 1990; Dominic Amalraj and Das, 1996; Dominic Amalraj et al., 2005; Grimstad and Walker, 1991; Kang et al., 2017; Lefèvre et al., 2013; Merritt et al., 1992; Moller-Jacobs et al., 2014; Mourya et al., 2004; Muturi et al., 2011; Roux et al., 2015; Shapiro et al., 2016; Takken et al., 1998; Tun-Lin et al., 2000; Vantaux et al., 2016; Wallace and Merritt, 1999; Yadav et al., 2005). For example, alterations in larval temperature affect the susceptibility of adults to Chikungunya, Dengue and Sindbis viruses; larval competition decreases adult survival; and larval nutritional stress influences developmental timelines, adult body size, fecundity, adult survival, and the rate of *Plasmodium* parasite development. Additionally, exposure of larvae to sub-lethal doses of bacteria augments the immune responses of the resultant adult. For example, a larval infection with *Bacillus* sp. has a negative effect on the development of filarial nematodes and *Plasmodium* parasites that are acquired after eclosion (Kala and Gunasekaran, 1999; Mahapatra et al., 1999; Paily et al., 2012), and inhabiting a larval environment containing *Escherichia coli* enhances the antimicrobial responses of adults (Moreno-García et al., 2015). The mechanisms responsible for the effect that the larval experience has on subsequent infections as adults are unknown, but for culicine mosquitoes it has been suggested that transstadial transmission (passage across molts) of a pathogen could increase immune alertness (Paily et al., 2012).

In mosquitoes, several viruses are both vertically and transstadially transmitted (Becker et al., 2003). It has also been reported that gut bacteria – including bacterial endotoxins – are transstadially passaged (Chavshin et al., 2015; Jadin et al., 1966; Paily et al., 2012; Pumpuni et al., 1996), although others have argued that adults reacquire the bacteria from the water following ecdysis (Lindh et al., 2008). Many aquatic microorganisms (*e.g.*, nematodes, fungi, ciliates, viruses, and bacteria) are able to invade the hemocoel (body cavity) of larval mosquitoes by penetrating the cuticle or the intestinal epithelium

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(Granados, 1980; Kalucy and Daniel, 1972; Petersen et al., 1968; Sweeney et al., 1983; Washburn et al., 1988; Yassine et al., 2012); however, it remains unknown whether a larval-acquired infection in the hemocoel is transstadially transmitted to the hemocoel of an adult. This is a major oversight, as a hemocoelic infection during the larval stages may alter the susceptibility of an infection acquired as an adult, such as an infection with *Plasmodium* parasites (Imwong et al., 2011; Paul et al., 2002). Furthermore, although mosquito larvae mount powerful cellular and humoral immune responses against microorganisms in their hemocoel (Biron et al., 2005; Brey et al., 1988; Dimopoulos et al., 1997; Duncan et al., 2012; Kalucy and Daniel, 1972; League et al., 2017; League and Hillyer, 2016; Meredith et al., 2008; Richman et al., 1996; Shin et al., 2005), little is known about how immune stimulation during the larval stage affects development or the biology of the resultant female and male adults (Moreno-García et al., 2015).

In this study, we investigated whether an infection present in the hemocoel of the African malaria mosquito, *Anopheles gambiae*, is transstadially transmitted from larvae to adults, and whether immune stimulation in the hemocoel as a larva alters the development or biological traits of the adult, including survival and body size. Our results show that transstadial transmission of a hemocoelic infection occurs from larvae to adults, but that infection prevalence declines with each molt, and with adult age. Additionally, bacterial intensity among infected individuals increases with each molt, decreases in the days following eclosion, and then increases as adults age further. Injury, immune stimulation or infection decreases the proportion of larvae that reach adulthood, infection decreases the longevity of adult females, and treatment has complex effects on the body size of female and male adults. Taken together, these findings show that transstadial transmission of a larval-acquired hemocoel infection occurs in mosquitoes, and that a larval infection reduces overall fitness by negatively impacting life history traits.

2. Materials and methods

2.1. Mosquito rearing and maintenance

Anopheles gambiae (G3 strain) were reared and maintained in an environmental chamber as previously described (Coggins et al., 2012). Briefly, eggs were collected and placed in plastic containers with deionized water, and hatched larvae were fed daily a mixture of koi fish food and baker's yeast. During the course of this study, all treatments were initiated in early 4th instar larvae. Larvae for each treatment were separated, and after pupation and subsequent eclosion, adult mosquitoes were fed a 10% sucrose solution *ad libitum*.

2.2. Mosquito injection, inoculation of immune elicitor, and bacterial infection

Early 4th instar larvae were injected in their hemocoel at the mesothorax using a Nanoject III Auto-Nanoliter Injector (Drummond Scientific Company, Broomall, PA, USA). Larvae received 69 nl of one of the following: (1) 0.2% solids 1 μm diameter green fluorescent (505/515) carboxylate-modified microspheres (Invitrogen, Carlsbad, CA, USA) in phosphate-buffered saline (PBS); (2) PBS alone (injury control); (3) tetracycline resistant, GFP-expressing *Escherichia coli* (modified DH5α) in Luria-Bertani's rich nutrient medium (LB); or (4) LB medium alone (injury control). For bacterial infections, *E. coli* were grown overnight in a shaking incubator at 37 °C in LB broth. Infection doses were estimated prior to larval injections by measuring the optical density (OD_{600} = 5) of bacterial cultures using a BioPhotometer™ plus spectrophotometer (Eppendorf AG, Hamburg, Germany). Absolute doses were determined by spreading a 1:1000 dilution of the bacterial culture on an LB agar plate, incubating the plate overnight at 37 °C, and then counting the resultant colony forming units (CFUs).

2.3. Hemocoel transstadial transmission

Larval mosquitoes were injected in their hemocoel with either fluorescent microspheres or *E. coli*, and each subsequent life stage (late 4th instar larvae, pupae, 1-day-old adults, 5-day-old adults, and 10-day-old adults) was assessed for the presence of the challenge agent. Due to the brightness of the long-lasting fluorescent microspheres – phagocytosed microspheres remain fluorescent for > 2 weeks – the hemocoel of microsphere-injected mosquitoes from each life stage was visualized by imaging through the translucent cuticle of live individuals using bright field and fluorescence illumination on a Nikon SMZ1500 stereomicroscope (Nikon, Tokyo, Japan) connected to a Hamamatsu ORCA-Flash 2.8 digital CMOS camera (Hamamatsu Photonics, Hamamatsu, Japan) and Nikon Advanced Research NIS-Elements software. Additionally, the exuviae from treated individuals were collected following adult emergence, mounted between a microscope slide and a coverslip using Aqua Poly/Mount (Polysciences, Warrington, PA, USA), and visualized using light and fluorescence illumination on a Nikon 90i compound microscope (Nikon Corp., Tokyo, Japan) equipped with a Nikon Intensilight C-HGFI fluorescence illumination unit, a Nikon DS-Qi1Mc CCD camera, and Nikon Advanced Research NIS-Elements software. The detection of microspheres within the hemocoel of individual mosquitoes was used to determine the rate of transstadial passage of inanimate particles, and the presence in exuviae was used to indicate loss or partial loss during a molt. A total of 50 individuals were examined for each life stage and sex over the course of 10 independent trials.

In order to quantify transstadial transmission of tetracycline resistant, GFP-expressing *E. coli*, mosquitoes at each life stage that originated from infected larvae were homogenized in PBS and spread on LB agar plates containing tetracycline. Plates were incubated overnight at 37 °C, the CFUs were counted, and the number of CFUs was then used to assess infection. The state of infection was analyzed using two descriptors: prevalence (the percentage of mosquitoes infected with *E. coli*) and intensity (the mean number of CFUs in each mosquito that remained infected with *E. coli*). In order to further confirm that all colonies originated from the *E. coli* inoculums, plates were also viewed by fluorescence microscopy to confirm the expression of GFP. A total of 30 individuals were assessed at each life stage and sex over the course of 7 independent trials.

2.4. Mosquito life history traits

Using the same protocol as above, larvae were injected into their hemocoel with one of the following: fluorescent microspheres (immune elicitor), PBS (injury control for immune elicitor), *E. coli* (bacterial challenge), or LB (injury control for bacterial challenge). An additional group did not receive an injection (naïve). For the different treatments, mosquito development was measured by recording the proportion of larvae that ecdysed into pupae, and the proportion of larvae that reached adulthood (*i.e.*, eclosion). Due to the trauma associated with an injection, larvae that died within 24 h of injection were excluded from analysis. For those mosquitoes that reached adulthood, their survival was recorded for the first 24 days following eclosion. Three independent trials were conducted, with each trial starting with 120 larvae per treatment, and the data were combined for analysis.

The body size of the eclosed adults was quantified for both sexes using three complementary measurements: length of the abdomen, length of the wing, and length of the hind tibia. Adult mosquitoes were anesthetized on ice and restrained dorsal-side-up on Sylgard 184 silicone plates (Dow Corning Corp, Midland, MI, USA) by placing a 0.15 mm diameter pin through the thorax. A wing and a hind leg were removed at the base with forceps, placed on the silicone plate next to the mosquito abdomen, and all three structures were photographed individually using the Nikon SMZ1500 stereomicroscope. The lengths of all three structures were then measured using the length feature of NIS Elements. The length of the abdomen was defined as the distance