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Comparative vertical transmission of Rickettsia by Dermacentor variabilis and Amblyomma maculatum

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Abstract

The geographical overlap of multiple Rickettsia and tick species coincides with the molecular detection of a variety of rickettsial agents in what may be novel tick hosts. However, little is known concerning transmissibility of rickettsial species by various tick hosts. To examine the vertical transmission potential between select tick and rickettsial species, two sympatric species of ticks, *Dermacentor variabilis* and *Amblyomma maculatum*, were exposed to five different rickettsial species, including *Rickettsia rickettsii, Rickettsia parkeri, Rickettsia montanensis, Rickettsia amblyommatis*, or flea-borne *Rickettsia felis*. Fitness-related metrics including engorgement weight, egg production index, nutrient index, and egg hatch percentage were then assessed. Subsamples of egg clutches and unfed larvae, nymphs, and adults for each cohort were assessed for transovarial and transstadial transmission of rickettsiae by qPCR. Rickettsial exposure had a minimal fitness effect in *D. variabilis* and transovarial transmission was observed for all groups except *R. rickettsii*. In contrast, rickettsial exposure negatively influenced *A. maculatum* fitness and transovarial transmission of rickettsiae was demonstrated only for *R. amblyommatis*- and *R. parkeri*-exposed ticks. Sustained maintenance of rickettsiae via transstadial transmission was diminished from F_1 larvae to F_1 adults in both tick species. The findings of this study suggest transovarial transmission specificity may not be tick species dependent, and sustained vertical transmission is not common.

Keywords

Amblyomma; Dermacentor; Rickettsia; Vertical transmission

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1. Introduction

Ticks are hematophagous arthropods known for their ability to act as reservoirs and vectors for many non-infectious and infectious microbial agents, including those within the spotted fever group (SFG) of *Rickettsia*. *Rickettsia* are gram-negative, obligate intracellular bacteria that vary in their ability to cause human disease. *Rickettsia* species are maintained within tick populations by vertical (transovarial from female to offspring or transstadially from immature stage to subsequent life cycle stage) and/or horizontal (acquired during feeding) transmission; although the role of vertical versus horizontal transmission for many rickettsial species is not well characterized. Models of vertical transmission in naturally and laboratory infected ticks demonstrate variability in stable maintenance (Burgdorfer and Brinton, 1975; Niebylski et al., 1999; Macaluso et al., 2002). Furthermore, experimental vertical transmission patterns have been shown to vary based on method of tick inoculation and life cycle stage exposed (Niebylski et al., 1999; Macaluso et al., 2002; Labruna et al., 2011; Soares et al., 2012). Rickettsial pathogenicity is posited as a key factor mediating transmission, as vertical maintenance favors non-pathogenic SFG *Rickettsia*, while horizontal transmission events are required for pathogenic SFG *Rickettsia* maintenance (Werren, 1997; Macaluso and Paddock, 2014). In the United States, the geographical range of multiple tick species continues to expand, coinciding with a rise in human cases of SFG rickettsiosis (Dumler, 2010; Paddock and Goddard, 2015; Dahlgren et al., 2016; Drexler et al., 2016). Thus, it is important to anticipate tick transmission potential for SFG *Rickettsia* to further understand the epidemiology of tick-borne rickettsial diseases (TBRDs).

The Gulf Coast tick, *Amblyomma maculatum*, is an aggressive, human-biting tick and the recognized primary vector for *Rickettsia parkeri*, the agent of *R. parkeri* rickettsiosis. In field-collected *A. maculatum, R. parkeri* infection rates range from 5 to 52% (Hooker et al., 1912; Edwards et al., 2011; Varela-Stokes et al., 2011; Nadolny et al., 2014; Paddock and Goddard, 2015; Gleim et al., 2016).

Contemporary field studies frequently detect alternative rickettsial species in *A. maculatum*, including *Rickettsia amblyommatis*, identified in 5% of ticks, and *R. montanensis*, a non-pathogenic endosymbiont *RickettsiaRickettsia*, detected in 14% of ticks (Lee et al., 2014; Gleim et al., 2016). However, whether *A. maculatum* is a competent host for these additional *Rickettsia* species remains unknown. Another tick species that also contributes to transmission of SFG *Rickettsia* within the United States is *Dermacentor variabilis*, the primary vector for *Rickettsia rickettsii*, the etiological agent of Rocky Mountain spotted fever. Interestingly, field surveys indicate that less than 1% of *D. variabilis* are infected with *R. rickettsii* (Stromdahl et al., 2011). As with *A. maculatum*, additional SFG *Rickettsia* are often identified in field-caught *D. variabilis*. For example, *R. parkeri* and *R. amblyommatis* have been detected in 28% and 1–50% of field-collected *D. variabilis*, respectively (Lee et al., 2014; Gleim et al., 2016). Additionally, *R. montanensis* is known to infect *D. variabilis*, however, the incidence in ticks is relatively low (1.5–33%), contrasting laboratory studies demonstrating high rates of sustained vertical transmission (Macaluso et al., 2001, 2002; Lee et al., 2014; Nadolny et al., 2014). Thus, although a variety of SFG rickettsial species have

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been molecularly identified in *D. variabilis* and *A. maculatum*, the transmission biology for these additional *Rickettsia* species has not been thoroughly investigated.

The objective of the current study was to assess vertical transmission of SFG *Rickettsia* by two sympatric tick species. The hypothesis is that if diverse infection of *D. variabilis* and *A. maculatum* contribute to the epidemiology of emerging rickettsioses, then these ticks can transmit *Rickettsia* indiscriminately. To test this hypothesis, female *A. maculatum* and *D. variabilis* were exposed to individual SFG rickettsial species including *R. rickettsii*, *R. parkeri, R. montanensis*, and *R. amblyommatis. Rickettsia felis*, a flea-associated transitional group *Rickettsia* was included as an outgroup control (Gillespie et al., 2008). Post-exposure, the subsequent filial generation was monitored in order to assess infection within tick cohorts. Additionally, several biological parameters of fitness were measured for each experimental cohort in order to better understand how tick populations are affected by rickettsial exposure and infection. Vertebrate host animal exposure to *Rickettsia* was also assessed in order to evaluate the potential for infected cohorts to transmit bacteria to the host during feeding.

2. Materials and methods

2.1. Rickettsial strains and culture

Vero E6 (African Green monkey kidney-derived) and ISE6 (*Ixodes scapularis* embryo-derived) cells were routinely cultured as previously described (Pornwiroon et al., 2006, 2009). Isolates of *R. parkeri* (Portsmouth), *R. rickettsii* (Sheila Smith), *R. montanensis* (M5/6), and *R. amblyommatis* (Darkwater) were propagated in Vero cells; *R. felis* (LSU) was propagated in ISE6 cells. All *Rickettsia* species were low passage isolates (passage 5 or below), with the exception of *R. rickettsii* (passage 5) and *R. montanensis* (passage 51). Rickettsiae were semi-purified and enumerated as previously described (Sunyakumthorn et al., 2008). Briefly, infected host cells were lysed with a 27 g needle, followed by low-speed centrifugation at 275 × *g* for 10 min at 4 °C to pellet unlysed host cells. The *Rickettsia*-containing supernatant was then passed through a 2 μm filter (Whatman, Florham Park, NJ) to remove host cell debris. Total concentration of rickettsiae was quantified via LIVE/DEAD BacLight Bacterial Viability kit (Molecular Probes, Carlsbad, CA) on a Petroff Hausser counting chamber (Hausser Scientific Company, Horsham, PA) and viewed with a Leica fluorescent microscope (Kurtti et al., 2005).

2.2. Ticks and capillary feeding technique

Rickettsia-free *D. variabilis* (originally provided by Dr. Daniel Sonenshine of Old Dominion University) and *Rickettsia*-free *A. maculatum* (adult females provided by Centers for Disease Control and Prevention for distribution by BEI Resources, NIAID, NIH: *Adult A. maculatum*, NR-44382; adult males originally provided by Dr. Daniel Sonenshine of Old Dominion University) were maintained in a controlled environmental chamber at 27 °C, with 92% relative humidity, and a 12:12 h (light: dark) cycle at Louisiana State University. Propagation of ticks was completed as previously described (Troughton and Levin, 2007; Grasperge et al., 2014; Banajee et al., 2015). Experimental groups were created by designating 7 groups of 20 *D. variabilis* (10 females and 10 males) and 7 groups of

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15 *A. maculatum* (10 females and 5 males). Adult and nymphal ticks were encapsulated by attaching the top portion of a 15- or 50 ml conical tube to the dorsum of vertebrate hosts with a 3:1 rosin to beeswax mixture as previously described (Grasperge et al., 2014). Larval ticks were allowed to freely attach to an immobilized mouse. Post-immobilization the mouse was placed in proper housing with a wire-mesh grate over water from which engorged ticks could be collected. Groups of male and female ticks were encapsulated on individual Sprague Dawley (SD) rats and allowed to pre-feed for 3–4 days, after which females were forcibly removed with forceps. Females that had not attached were excluded from experimentation. Individual female ticks were restrained dorsal side down to adherent tape secured to the bottom of a petri dish. Capillary tubes containing *Rickettsia* or media control were fitted over the palps of each tick and the open end of the capillary was embedded in modeling clay (Macaluso et al., 2001). Each of the 5 rickettsial treatment groups were prepared at a final concentration of 5×10^7 rickettsiae/μl in appropriate host cell medium mixed 3:1 with 0.1% Rhodamine-B in 0.85% NaCl (w/v). Two media control groups were prepared from lysed and purified uninfected Vero or ISE6 cells mixed 3:1 with Rhodamine B. Immobilized ticks were then placed in a humidified incubator overnight (≈14 h) at 37 °C to allow acquisition of bacteria. Subsequently, ticks were separated from the capillary tube, rinsed twice with 70% EtOH, and once in deionized H_2O to remove residual medium. Solution ingestion via Rhodamine-B labeling for each treatment group was assessed by fluorescence microscopy. Ticks not positively labeled were excluded from further experimentation. Ticks positive for Rhodamine-B labeling were returned to their respective animal host and allowed to feed to repletion. Replete females were surface sterilized and weighed to calculate engorgement weight. All vials containing engorged females were stored in a controlled environmental chamber as described above.

2.3. Tick sampling, DNA extraction, and qPCR

All ticks were analyzed for rickettsial infection via species-specific quantitative real-time PCR (qPCR) assays. At the beginning of oviposition, 50 eggs were subsampled from each egg clutch (cohort) for analysis of vertical transmission. Cohorts that produced eggs negative for Rickettsia were discarded from further analysis. Post-eclosion, 50 unfed larvae were subsampled from each cohort to test for transstadial transmission. Larval cohorts that had tested positive as eggs were allowed to feed on naïve Balb/c mice. Engorged larvae were collected and stored until molting was complete. A portion of the resulting unfed nymphs were then assessed for transstadial transmission. Nymphal cohorts that tested positive were then fed on Balb/c mice or SD rats and allowed to molt to the adult life stage. Adult unfed female ticks were then individually assessed as described below.

Extraction of genomic DNA (gDNA) from eggs, larvae, nymphs, and adults was carried out using the Zymo Quick g-DNA Miniprep kit (Zymo, Irvine, CA). Adults were cut in half with a sterile scalpel blade and subjected to either gDNA extraction or stored at −80 °C for further analysis. Tick samples were added to an Eppendorf Safe-Lock micro-centrifuge tube (Eppendorf, Hauppauge, NY) containing two sterile glass beads (3 mm) and 50 μl of DNase/RNase-free H_20. All samples were placed in a TissueLyser (Qiagen, Valencia, CA) for 2 cycles of 30 Hz for 3 min. After lysis, gDNA was extracted according to the manufacturer's protocol and eluted in 40 |il of DNase/RNase-free water. A Roche LightCycler 480II

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instrument (Roche, Indianapolis, IN) was utilized for qPCR using the primers listed in Table 1. Amplicons for gene-specific primers were incorporated into pCR4 TOPO and serially diluted in order to serve as internal standards for each rickettsial assay. Serial diluted standard curves were included along with experimental samples in each set of reactions. For media control-exposed ticks, a previously published assay allowing for *pan-Rickettsia* detection was employed (Jiang et al., 2004). Template consisted of tick gDNA, extraction controls, internal standard serial dilutions, or a negative (water) control. Cycling parameters are as previously described, with the modification of the pre-incubation step to 95 °C for 1 min (Thepparit et al., 2011).

2.4. Fitness metrics

The influence of rickettsial exposure on tick fitness was determined by calculating the nutrient index (NI or real conversion index) as well as the egg production index (EPI or apparent conversion index) (Bennett, 1974). The NI (Eq. (1)) is a measurement of efficiency in bloodmeal conversion to egg mass. The EPI (Eq. (2)) is measured to determine the efficiency with which ticks oviposit egg mass. One sample of 100 eggs was also taken from each egg clutch for the calculation of egg hatch percentage (Eq. (3)). Weight from eggs sampled was added back to the total mass of the egg clutch. Molting percentage was calculated for all nymph and adult ticks in selected cohorts propagated throughout experimentation.

Equations:

$$Nutrient\ index = \frac{Weight\ of\ eggs}{Initial\ weight\ of\ engorged\ tick - residual\ weight\ of\ tick} \times 100 \quad (1)$$

$$Egg\ production\ index = \frac{Weight\ of\ eggs}{Initial\ weight\ of\ engorged\ tick} \times 100 \quad (2)$$

$$\%\ Egg\ hatch = \frac{Number\ of\ viable\ life\ stage}{Total\ number\ of\ life\ stage\ prior\ to\ eclosion/molt} \times 100 \quad (3)$$

2.5. Elisa

Indirect ELISA to detect anti-rickettsial IgM was performed as previously reported with minor modifications (Banajee et al., 2015). Briefly, *R. parkeri* was sucrose purified, followed by sonication, and protein quantification via DC assay (Bio-Rad, Hercules, CA) (Ammerman et al., 2008). Maxisorp plates (Nunc, Rochester, NY) were coated overnight with 0.5 ng/μl *R. parkeri* lysate in carbonate bicarbonate coating buffer (Sigma-Aldrich, St. Louis, MO). Individual tick cohorts were fed on single vertebrate hosts only once. Sera or plasma from host animals was collected after approximately six to ten days of feeding (e.g. six days for larvae or nymphs and ten days for adults), then diluted 1:32 and 1:64 in blocking buffer (5% skim milk/0.1% Tween-20 in PBS), and added to either a well coated with *R. parkeri* antigen or a non-antigen well coated with only carbonate bicarbonate

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buffer. Anti-rat IgM or anti-mouse and IgM conjugated to horse radish peroxidase diluted in blocking buffer was used as a secondary antibody. Positive and negative control serum was included in each plate along with experimental samples. Serum was considered positive for exposure if mean OD_{414} values were greater than two times the standard deviation for all samples.

2.6. Animal use statement

All experimental animals were obtained from the LSU Division of Laboratory and Animal Medicine (DLAM) and monitored in accordance with LSU IACUC 13-034.

2.7. Statistical analysis

Data were analyzed in SAS using the GLM procedure. A Levene's test of homogeneity was performed for all data. Engorgement weight was analyzed using a one-way ANOVA and a Dunnett's post hoc test. Egg production index and nutrient index data were analyzed using a Kruskall Wallis test. A p-value of 0.05 was considered significant.

3. Results

3.1. Effect of Rickettsia infection on tick engorgement weight

To determine the influence of rickettsial exposure on tick fitness, the engorgement weights of female *D. variabilis* and *A. maculatum* post-exposure to *R. rickettsii*, *R. parkeri*, *R. amblyommatis*, *R. montanensis*, or *R. felis* were compared to *Rickettsia-free D. variabilis* and *A. maculatum* exposed to vehicle control consisting of cell growth media. All *Rickettsia*-exposed *D. variabilis* resulted in a minor reduction in engorgement weight (6.4–23.3%); however, differences were not significant (Table 2). In contrast, *A. maculatum* exposed to *R. parkeri, R. amblyommatis*, *R. montanensis*, or *R. felis* had significantly lower engorgement weights, with an approximate reduction of 37% in ticks exposed to *R. montanensis* and *R. parkeri* (Table 2). Likewise, *A. maculatum* exposed to *R. amblyommatis* and *R. felis* had a 30% decrease in engorgement weight (Table 2). Exposure to *R. rickettsii* did not have a significant impact on the engorgement weight of *A. maculatum*. Overall, these results demonstrate exposure to *Rickettsia* species in *D. variabilis* had no effect on engorgement weight, while *A. maculatum* was negatively impacted.

3.2. Bloodmeal conversion in D. variabilis and A. maculatum post-rickettsial exposure

Efficient bloodmeal conversion in the engorged female is vital for the production of viable progeny from egg mass. Compared to control ticks, all *Rickettsia*-exposed *D. variabilis* had comparable NIs. In contrast, *A. maculatum* exposed to *R. amblyommatis*, *R. montanensis*, or *R. parkeri* had a significant reduction (~24%) in NI (Table 2). Nutrient index values for both *R. rickettsii* and *R. felis* on average displayed a decrease of 16%, compared to media control *A. maculatum* (Table 2). Consistent with engorgement weight, *A. maculatum* biology was negatively impacted post-rickettsial exposure, while no impact on fitness was observed for *D. variabilis*.

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3.3. Egg production index in D. variabilis and A. maculatum post-rickettsial exposure

Although the NI is a useful metric in its ability to calculate bloodmeal conversion for the production of eggs, it does not account for the ability of the tick to oviposit eggs produced. Thus, to further quantify the effects of *Rickettsia* exposure on ticks, the EPI of engorged females was calculated. EPI of D. variabilis exposed to *R. amblyommatis, R. felis, R. parkeri*, and *R. rickettsii* resulted in non-significant reduction that averaged approximately 6%, while exposure to *R. montanensis* resulted in a significant reduction (11%) compared to the control group (Table 2). For *A. maculatum*, consistent with NI reduction, exposure to *R. amblyommatis, R. parkeri*, and *R. montanensis*, but not *R. felis* or *R. rickettsii* (4%), resulted in a significant reduction in oviposited egg mass (Table 2). Overall, the egg output of *R. montanensis*-exposed *D. variabilis* was significantly reduced. Additionally, *A. maculatum*-exposed to *R. amblyommatis, R. montanensis*, and *R. parkeri* were unable to produce the same amount of eggs as ticks exposed to media alone.

3.4. Measurement of viable F_1 progeny in D. variabilis and A. maculatum cohorts exposed to and infected with Rickettsia

To quantify the effects of rickettsial exposure and infection in both tick species, the percent hatch rate was calculated (Table 3). Overall, no cohort of either *D. variabilis* or *A. maculatum* showed a significant decrease in viable larvae. Furthermore, there was no correlation between infection and a change in viability through the F_1 adult stage. Most cohorts produced between 90 and 100% molt success, with the exception of cohorts exposed to *R. parkeri* which ranged between 69 and 100% (Table 3). Statistical significance could not be assigned to emergent nymphal and adult cohorts due to smaller numbers of positive cohorts. Thus, *Rickettsia* exposure in *D. variabilis* and *A. maculatum* has a slight impact on egg hatching, but molting rates among survivors return to levels comparable to control ticks at larval and nymphal stages.

3.5. Vertical transmission of Rickettsia in D. variabilis and A. maculatum

Vertical transmission of *Rickettsia* within cohorts of *D. variabilis* and *A. maculatum* was investigated via qPCR. Initially, engorged females were allowed to oviposit and subsamples of eggs were assessed for rickettsial burden via species-specific qPCR assays (Table 4). Detection of *Rickettsia* vertically transmitted to eggs by *D. variabilis* exposed to *R. amblyommatis, R. felis, R. montanensis*, and *R. parkeri* was 25%, 17%, 14%, and 83%, respectively. Subsequent vertical transmission was detected for 20% of unfed larval and nymphal *D. variabilis* cohorts exposed to *R. parkeri*. Additionally, *R. amblyommatis* was detected in 50% of unfed *D. variabilis* nymphal cohorts. Transstadial transmission to unfed adult *D. variabilis* was not detected in either previously infected *R. amblyommatis* or *R. parkeri* cohorts. Although *D. variabilis* females were susceptible to most (4/5) Rickettsia species used in the study, contrasting results were noted for *Rickettsia*-exposed *A. maculatum*. Vertical transmission of *Rickettsia* to oviposited eggs was detected only for *A. maculatum* exposed to *R. amblyommatis* and R. parkeri at 29% and 38%, respectively. Further evidence of vertical transmission was detected in 33% unfed larval *A. maculatum* cohorts exposed to *R. parkeri*. Transstadial transmission to the unfed nymphal stage was detected in *R. amblyommatis* and *R. parkeri*-exposed *A. maculatum* cohorts at

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50% and 67%, respectively. Infection was not detected in unfed adult populations from previously infected *R. amblyommatis* and *R. parkeri* cohorts. Overall, *D. variabilis* were more susceptible to transovarial transmission by multiple *Rickettsia* species; however, maintenance of infection was restricted to only *R. amblyommatis* and *R. parkeri* at unfed larval and nymphal stages. Vertical transmission in *A. maculatum* was only successful for *R. parkeri* and *R. amblyommatis*, where subsequent detection of infection was variable in both *Rickettsia*-infected cohorts. For both *D. variabilis* and *A. maculatum*, transmission was diminished at the adult stage, as no infected unfed adult ticks were detected via qPCR.

3.6. Measurement of IgM response by mammalian hosts parasitized by exposed and infected tick cohorts

In order to further characterize the transmission patterns of *Rickettsia* exposed ticks antibody responses in the host animals used to perpetuate the positive cohorts of the F_1 generation was assayed (Table 5). Transmission to the mammalian host is an important marker for vector competence (Reisen, 2009). All animals on which initially exposed adult, females were fed had negative IgM titers for *Rickettsia*. Levels of detectable IgM (1:64) were present in serum collected from individual vertebrate hosts infested with larval *D. variabilis* exposed to R. *amblyommatis* or *D. variabilis* exposed to *R. parkeri* after approximately 6 days of tick feeding.

4. Discussion

Acquisition of rickettsial infection by ticks occurs through horizontal routes via feeding on infected hosts, or by vertical transmission in which rickettsiae are maintained through transstadial and transovarial transmission. Historically, the incidence of *Rickettsia*-infected ticks suggests that competent vector/pathogen relationships predominate, despite geographically overlapping populations of ticks with multiple rickettsial species (Macaluso and Paddock, 2014). However, the susceptibility of any given tick species to a novel rickettsial infection is not well-defined. Some rickettsial species are efficiently vertically maintained over multiple filial generations, which is often associated with selective pressure resulting in maintenance of less virulent species (Fine, 1975; Yamamura, 1993; Mather and Ginsberg, 1994; Werren, 1997). Little to no decrease in fitness is observed for these tick populations resulting in sustained levels of infected ticks. Alternatively, pathogenic rickettsial species have been shown to negatively influence the fitness of their arthropod host, a selection method that favors horizontal routes of maintenance (Burgdorfer and Brinton, 1975; Niebylski et al., 1999). The transmission utilized in each tick/Rickettsia association is important to understanding the ecology of TBRDs.

Tick fitness can be assessed by a variety of metrics including, engorgement weight, nutrient conversion, egg production, and offspring viability. In the current study, *A. maculatum* and *D. variabilis* were used to examine the biological interaction of *Rickettsia* with the vector. *Rickettsial* exposure in *D. variabilis* did not significantly impact fitness; with the exception of egg production in ticks exposed to *R. montanensis*. In contrast, *A. maculatum* fitness was significantly impacted in *Rickettsia*-exposed ticks for most species of *Rickettsia* tested. A deleterious effect of rickettsial infection has been demonstrated for various tick species,

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including *Dermacentor* species, in which sustained rickettsial infection resulted in decreased viability (Burgdorfer and Brinton, 1975; Niebylski et al., 1999). In contrast to the current study, *R. montanensis* did not elicit a fitness effect when adults were exposed and returned to host (Macaluso et al., 2001). Infection of *A. maculatum* with *R. parkeri* resulted in decreased fitness relative to bloodmeal acquisition, with variable effects on F_1 viability. These results suggest that *A. maculatu*m is less tolerant to infection. A recent study contrasts these results by reporting no adverse effects on *A. maculatum* fitness when naturally infected with *R. parkeri*. Several *Rickettsia*-dependent factors, including infection route, dose, strain variation, and isolate passage history may account for the observed differences in fitness effects. Likewise, tick-dependent variables such as colonization, which is known to affect the tick's microbiome composition and possibly susceptibility to bacterial infection, may also influence tick vector competence (Narasimhan et al., 2014; Gall et al., 2016; Zolnik et al., 2016). Additionally, the impact of established versus introduced infection requires further examination; however, the current laboratory study suggests that if adult ticks are exposed to SFG *Rickettsia*, viability may be affected, but the exposure will not interrupt the lifecycle.

Susceptibility of *D. variabilis* and *A. maculatum* to *Rickettsia* is, in part, associated with their ability to transovarially transmit infection to their progeny. In this study, transovarial infection was detected in *D. variabilis* exposed to *R. amblyommatis*, *R. montanensis*, *R. felis*, and *R. parkeri*. Additionally, cohorts of A. *maculatum* exposed to *R. amblyommatis* and *R. parkeri* were capable of transovarially transmitting infection to their offspring. Vertical transmission of *R. parkeri* by *A. maculatum* is supported by a recent study wherein transovarial transmission was documented in three filial generations of naturally infected *R. parkeri*-A. *maculatum* (Wright et al., 2015). Additionally, although *R. felis* is primarily associated with cat fleas, it has been detected sporadically in ticks, which supports the data presented here (Ishikura et al., 2003; Jiang et al., 2012; Abarca et al., 2013; Soares et al., 2015; Roth et al., 2016). No transovarial transmission of *R. rickettsii* was identified for either tick species in the current study, consistent with previous results in which naïve female ticks were refractory to transovarial transmission, despite massive infection in the ovaries of engorged females post-rickettsial exposure (Niebylski et al., 1999). Alternatively, adult *D. andersoni* were able to produce infected *R. rickettsii*-infected offspring and the cohort maintained the infection for multiple generations demonstrating 100% filial infection rates, independent of the inoculation route (Burgdorfer and Brinton, 1975). Interestingly, larval and nymphal *D. andersoni* acquiring infection via rickettsemic hosts were able to maintain the rickettsiae through vertical transmission, suggesting that introduction of *Rickettsia* to immature life cycle stages might facilitate establishment in the vector host (Niebylski et al., 1999). Differences in vector susceptibility were observed between tick species in the current study, as *D. variabilis* was a more permissive host for transovarial transmission of *Rickettsia* species. Infection in *A. maculatum*, however, was more stringent, displaying rickettsial transmission specificity. The tick-derived molecular factors underlying tick susceptibility to rickettsial infection need to be identified.

Further elucidation of tick/*Rickettsia* specificity can be determined through investigating transstadial transmission patterns in tick populations. In the current study, continued transstadial maintenance of rickettsial infection in F_1 larval and nymphal *D. variabilis* and

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A. maculatum was detected in portions of cohorts exposed to *R. amblyommatis* and *R. parkeri*. However, infection was not detectable for either *Rickettsia* species in F_1 adults. These results are in contrast to a recent study analyzing three filial generations of naturally infected *R. parkeri*-*A. maculatum*, where infection was observed to be maintained at all life stages (Wright et al., 2015). Additionally, naturally infected *Amblyomma* have been documented to vertically maintain *R. amblyommatis* at nearly 100% prevalence (Zanettii et al., 2008). Diminishment of infection observed in the current study is similar to that observed in *D. andersoni* naturally infected with *R. montanensis* (Philip, 1959; Niebylski et al., 1999). However, the underlying factors leading to this decrease in prevalence in a tick population are currently unknown. The current and previous studies demonstrate sustained vertical transmission through immature stages and highlight the potential role larval and nymphal ticks possess in transmission of SFG *Rickettsia* to vertebrate hosts.

The results of the current study also suggest that *D. variabilis* is more susceptible to infection by multiple *Rickettsia* species. These data are consistent with field surveys in which low prevalence of *D. variabilis* infected with *R. rickettsii* and *R. montanensis* is observed, while infection with *R. parkeri* is increasingly recognized (Williamson et al., 2010; Fornadel et al., 2011; Stromdahl et al., 2011; Leydet and Liang, 2013; Henning et al., 2014). As the geographical range of *A. maculatum* increases, the role of this tick in the ecology of TBRDs is less clear (Paddock and Goddard, 2015). For *A. maculatum*, a more specific range of susceptibility as a competent vector for *R. parkeri* and *R. amblyommatis* is accompanied by significant decreases in tick fitness. While the current study examined the initial infection and transmission points for two species of ticks, there are multiple factors that require further investigation. For example, the critical point at which rickettsial exposure results in infection or clearance and how long rickettsiae remain viable in the tick is not known and may vary based on species of *Rickettsia*. Successful rickettsial maintenance may be life cycle stage dependent, as has been suggested in previous studies, implying that immature stages of ticks are more susceptible to infection (Burgdorfer and Brinton, 1975; Niebylski et al., 1999). Downstream implications of immature tick infections may include higher transstadial maintenance and the ability to transmit SFG *Rickettsia* to vertebrate hosts during subsequent feeding events. Indeed, host animal exposure, as assessed by ELISA, was positive for IgM in vertebrates parasitized by F_1 *D. variabilis* larvae infected with *R. amblyommatis* and *R. parkeri*. Thus, it is possible that immature life stages (i.e. larvae and nymphs) play a greater role in rickettsial transmission than is currently recognized. Differences in vertical transmission efficiency may also be a result of the capillary feeding inoculation route utilized in the current study; an established method that differs from the route of infection used in previous studies (Burgdorfer and Brinton, 1975; Niebylski et al., 1999; Schumacher et al., 2016). For example, ticks feeding on rickettsemic animals are likely exposed to Rickettsia over the entire course of feeding; therefore, the temporal pattern and dose in which ticks acquire rickettsiae likely contributes to successful infection and subsequent transmission. Likewise, mechanisms utilized by SFG *Rickettsia* to traverse multiple barriers within the tick in order to achieve multi-organ infection, resulting in sustained vertical maintenance have yet to be thoroughly characterized. While further studies are needed to analyze the rickettsial infection dynamics within these two tick

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species, understanding the initial susceptibility of these two key tick vectors will broaden our understanding of the eco-epidemiology of TBRDs.

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Table 1

Primers and probes utilized in qPCR.

Primer (5′–3′) Probe (5′–3′)	Primer/Probe final qPCR concentration	Portion of gene amplified	Citation
ompBRm2832F-GCGGTGGTGTTCCTAATAC	0.2 μM	*R. montanensis ompB*	Petchampai et al. (2014)
ompBRm2937R-CCTAAGTTGTTATAGTCTGTAGTG	0.2 μM		
RmompB-HEX/CGGGGCAAAGATGCTAGCGCTTCACAGTTACCCCG/IABkFQ	0.3 μM		
RamompBEHF-CCGTTAACACCATTAACTATTAAAGCA	0.2 μM	*R. amblyommatis ompB*	This paper
RamompBEHR-GTGCTGCGGCTTCTACATTA	0.2 μM		
RamompB-FAM/AGAGGCGCCTTTTGAGTTGTAGGATTTGC/BHQ_1	0.3 μM		
RpompB129FJJ-CAAATGTTGCAGTTCCTCTAAATG	0.2 μM	*R. parkeri ompB*	Banajee et al. (2015)
RpompB224RJJ-AAAACAAACCGTTAAAACTACCG	0.2 μM		
RparompB-FAM/TTTG+A+G+C+A+G+CA/IABkFQ	0.3 μM		
Rf17KD135F-ATGAATAAACAAGGKACNGGHACAC	0.2 μM	*Rickettsia 17 kDa*	Jiang et al. (2004)
Rf17KD249R-AAGTAATGCRCCTACACCTACTC	0.2 μM		
R17Kbprobe-FAM/CGCGACCCGAATTGAGAACCAAGTAATGCGTCGCG/BHQ	0.3 μM		
ompBRr1370F-ATAACCCAAGACTCAAACTTTGGTA	0.2 μM	*R. rickettsii ompB*	Jiang et al. (2005)
ompBRr1494R-GCAGTGTTACCGGGATTGCT	0.2 μM		
RrompB-FAM/CGCGATCTTAAAGTTCCTAATGCTATAACCCTTACCGATCGCG/IABkFQ	0.3 μM		
AmacMIF.18F-CCAGGGCCTTCTCGATGT	0.2 μM	*A. maculatum mif*	Lee et al. (2016)
AmacMIF.99R-CCATGCGCAATTGCAAACC	0.2 μM		
AmacMIF.63-Hex-TGTTCTCCTTTGGACTCAGGCAGC/BHQ	0.3 μM		
CRTDv321F-AGGAGAAAAGCAAGGGACTG	0.2 μM	*D. variabilis* citrate synthase	Petchampai et al. (2014)
CRTDv452R-CAATGTTCTGCTCGTGCTTG	0.2 μM		
DvCRT_TYE665-TYE665/TGGAGAAGGGCTCGAACTTGGC/IAbRQsp + denotes the use of a locked nucleic acid (LNA).	0.3 μM		

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Table 2

Results of tick fitness post-rickettsial exposure in *D. variabilis* and *A. maculatum*. Ten females were initially exposed for each *Rickettsia* and tick species. *A Rhodamine* B tracer dye was used to determine positive (+) imbibement of Rickettsia or non-infected media in individual ticks. Post-engorgement the average engorgement weight, nutrient index, and egg production index values for *D. variabilis* and *A. maculatum* exposed to either rickettsial species or media control was calculated.

	D. variabilis				***A. maculatum***			
Rickettsia	**Rhodamine B + females**	**Engorgement weight (mg)**	**Nutrient index (NI)**	**Egg production index (EPI)**	**Rhodamine B + females**	**Engorgement weight (mg)**	**Nutrient index (NI)**	**Egg production index (EPI)**
R. amblyommatis	8/10	498 [± 52]	28% [± 1]	55% [±3]	7/10	745* [± 60]	32%* [±4]	44%* [±3]
R. felis	6/10	509 [± 43]	27% [±3]	56% [±2]	7/10	716* [± 37]	35% [±2]	51% [±2]
R. montanensis	9/10	543 [± 14]	28% [± 1]	48%* [±3]	9/10	652* [± 92]	24%* [±6]	34%* [±5]
R. parkeri	9/10	506 [± 39]	26% [± 3]	54% [±4]	8/10	652* [± 58]	28%* [±4]	43%* [±3]
R. rickettsii	8/10	445 [± 61]	25% [± 5]	50% [±5]	5/10	740 [± 60]	37% [±4]	52% [±2]
Media Control	10/10	581 [± 24]	33% [± 1]	60% [±1]	8/10	1037 [± 85]	52% [±7]	55% [±2]

*Denotes significance of p 0.05 in comparison to medial control groups. Rhodamine B + females are expressed as number positive/total number exposed. Values in brackets represent the SEM values for each group.

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Table 3

Average percent hatch or molt of *D. variabilis* and *A. maculatum* exposed to either *Rickettsia* or media control. Subsets of each positive tick cohort were allowed to hatch or molt and the total number of viable versus non-viable ticks was counted.

	D. variabilis			***A. maculatum***		
Rickettsia	**% Hatch**	**% Molt-nymph**	**% Molt-adult**	**% Hatch**	**% Molt-nymph**	**% Molt-adult**
R. amblyommatis	76	97	100	81	94	100
R. felis	59	100	ND	92	ND	ND
R. montanensis	62	98	ND	77	ND	ND
R. parkeri	73	96	81	69	88	100
R. rickettsii	69	ND	ND	87	ND	ND
Media Control	74	86	98	90	95	100

ND (not determined) denotes a set not included in evaluation based on negative qPCR result at the preceding life stage.

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Table 4

Vertical transmission of *Rickettsia* in *D. variabilis* and *A. maculatum* through one filial generation. Female ticks were exposed to either Rickettsia or a media control and allowed to reach engorgement. Cohorts created by the progeny of individual females were followed through the egg, larval, nymphal, and adult stage to assess vertical transmission of *Rickettsia* via qPCR. Only cohorts positive as eggs were tested at the larval and nymphal life stage. Only cohorts that were positive at the nymphal stage were tested as adults. Unfed immature life stages were tested for rickettsial gDNA by pooling n = 50 eggs; n = 50 larvae; and 5 pools of n = 10 nymphs. Unfed adults were tested individually, with an n = 20-50 total ticks tested.

Rickettsia	**Life stage**	**# cohorts positive/# cohorts tested (% positive)**	
		D. variabilis	***A. maculatum***
R. amblyommatis	Egg	2/8 (25%)	2/7 (29%)
	Larva	0/2 (0%)	0/2 (0%)
	Nymph	1/2 (50%)	1/2 (50%)
	Adult	0/1 (0%)	0/1 (0%)
R. felis	Egg	1/6 (17%)	0/7 (0%)
	Larva	0/1 (0%)	N/D
	Nymph	0/1 (0%)	N/D
	Adult	N/D	N/D
R. montanensis	Egg	1/9 (14%)	0/9 (0%)
	Larva	0/1 (0%)	N/D
	Nymph	0/1 (0%)	N/D
	Adult	N/D	N/D
R. parkeri	Egg	5/6 (83%)	3/8 (38%)
	Larva	1/5 (20%)	1/3 (33%)
	Nymph	1/5 (20%)	2/3 (67%)
	Adult	0/1 (0%)	0/2 (0%)
R. rickettsii	Egg	0/8 (0%)	0/5 (0%)
	Larva	N/D	N/D
	Nymph	N/D	N/D
	Adult	N/D	N/D
Media Control	Egg	0/10 (0%)	0/8 (0%)
	Larva	0/10 (0%)	0/8 (0%)
	Nymph	0/2 (0%)	0/2 (0%)
	Adult	0/2 (0%)	0/2 (0%)

N/D = not determined based on negative qPCR result at the egg stage or after negative result at the nymphal stage.

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Table 5

Vertebrate host exposure to *Rickettsia* post tick feeding. Sera collected post tick feeding was assayed for anti-*Rickettsia* IgM via ELISA.

	D. variabilis			*A. maculatum*		
Rickettsia	**F_0 adults**	**F_1 larvae**	**F_1 nymphs**	**F_0 adults**	**F_1 larvae**	**F_1 nymphs**
R. amblyommatis	-	+	-	-	-	-
R. felis	-	-	ND	-	ND	ND
R. montanensis	-	-	ND	-	ND	ND
R. parkeri	-	+	-	-	-	-
R. rickettsii	-	ND	ND	-	ND	ND
Media Control	-	-	-	-	-	-

ND (not determined) denotes a set not included in evaluation based on negative qPCR result at the preceding life stage.