Transmission of *Amblyomma maculatum*-Associated *Rickettsia* spp. During Cofeeding on Cattle

Jung Keun Lee,^{1,*} John V. Stokes,¹ Gail M. Moraru,^{1,†} Amanda B. Harper,¹
Catherine L. Smith,¹ Robert W. Wills,² and Andrea S. Varela-Stokes¹

Abstract

Amblyomma maculatum is the primary vector for the spotted fever group rickettsiae, *Rickettsia parkeri*, a known pathogen, and “*Candidatus Rickettsia andeanae*,” currently considered nonpathogenic. Spotted fever group rickettsiae are typically endothelial cell associated and rarely circulate in the blood. Horizontal transmission to naïve ticks through blood feeding from an infected host is likely rare. Cofeeding provides an opportunity for rickettsial transmission to naïve ticks in the absence of circulating rickettsiae. We evaluated *R. parkeri* transmission through cofeeding between *A. maculatum* adults and nymphs on beef calves. Six calves in each of two trials were infested with *A. maculatum* that had been capillary fed *R. parkeri*. Four days later, calves each received recipient *A. maculatum* that were either capillary fed “*Ca. R. andeanae*” or not capillary fed before infestation. Trials differed by whether we included a barrier to minimize adjacent feeding between recipient and donor ticks. After cofeeding, we detected *R. parkeri* in 27% of “*Ca. R. andeanae*”-free recipient ticks, whereas *R. parkeri* was not detected in any recipient ticks that were capillary fed “*Ca. R. andeanae*.” *Rickettsia parkeri* transmission efficiency to naïve ticks was greater when ticks freely cofed in proximity. No rickettsial DNA was detected in calf blood. Results confirm cofeeding as a method of horizontal transmission of *R. parkeri* in the absence of host rickettsemia and suggest no evidence of transmission by cofeeding when recipient ticks are first exposed to “*Ca. R. andeanae*” through capillary feeding. While cofeeding may provide an opportunity for maintaining the pathogen, *R. parkeri*, the mechanisms driving any potential effect of “*Ca. R. andeanae*” on *R. parkeri* transmission are unclear.

Keywords: spotted fever group rickettsia, gulf coast tick, *Rickettsia parkeri*, “*Candidatus Rickettsia andeanae*”

Introduction

THERE ARE MULTIPLE FACTORS contributing to infection rates of tick-borne pathogens and uncharacterized organisms in vector populations. Infection rates for the spotted fever group rickettsial pathogen, *Rickettsia parkeri*, vary geographically within populations of the principal tick vector, *Amblyomma maculatum* (Gulf Coast tick). In southern populations of *A. maculatum*, *R. parkeri* is common and may be detected at rates up to 56% (Sumner et al. 2007, Paddock et al. 2010, Varela-Stokes et al. 2011, Ferrari et al. 2012, Nadolny et al. 2014). This contrasts with *A. maculatum* populations from the lower Midwestern states, Kansas and

Oklahoma, where *R. parkeri* was absent from tested ticks (Paddock et al. 2015). In addition, *A. maculatum* may be infected with a spotted fever group rickettsia of unknown pathogenicity, “*Candidatus Rickettsia andeanae*.” Populations of *A. maculatum* in the southern United States typically have low infection rates of “*Ca. R. andeanae*,” with ~1–2% of sampled adult ticks typically infected (Sumner et al. 2007, Paddock et al. 2010, Fornadel et al. 2011, Varela-Stokes et al. 2011, Ferrari et al. 2012, Jiang et al. 2012, Nadolny et al. 2014). Conversely, 47% and 73% of *A. maculatum* ticks from populations in Kansas and Oklahoma, respectively, were positive for “*Ca. R. andeanae*” (Paddock et al. 2015). Coinfection of *A. maculatum* with *R. parkeri* and “*Ca. R. andeanae*” appears to be rare but

¹Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi State, Mississippi.

²Department of Pathobiology and Population Medicine, College of Veterinary Medicine, Mississippi State University, Mississippi State, Mississippi.

*Current affiliation: Department of Pathology and Population Medicine, Midwestern University, College of Veterinary Medicine, Glendale, Arizona.

†Current affiliation: Department of Biological Sciences, Linn-Benton Community College, Albany, Oregon.

has also been reported (Varela-Stokes et al. 2011, Ferrari et al. 2012, Leydet and Liang 2013, Budachetri et al. 2014, Lee et al. 2017).

Spotted fever group *Rickettsia* spp. are obligate intracellular organisms typically found in the cytoplasm and nucleus of vascular endothelial cells in vertebrate hosts (Moulder 1985, Raoult and Roux 1997). The acquisition of rickettsiae from rickettsemic vertebrate hosts may be inefficient for naïve feeding ticks since the presence of circulating rickettsiae is transient and rarely detected in animals (Horta et al. 2009, 2010, Edwards et al. 2010, Grasperge et al. 2012, Moraru et al. 2013). Thus, cofeeding is a potential method for pathogen or nonpathogen exchange between infected and naïve arthropod vectors while feeding simultaneously on a host lacking systemic infection (Jones et al. 1987). The efficiency of arthropod cofeeding for pathogen transmission may vary depending on the pathogen, tick vector, and host. For example, the efficiency of cofeeding in transmission of *Borrelia burgdorferi sensu stricto* from infected nymphs to larvae appears to be low, at 0–5% (Piesman and Happ 2001). In contrast, transmission of *R. conorii* through cofeeding may be more efficient, as between 92% and 100% of naïve *Rhipicephalus sanguineus* acquired *R. conorii* when cofeeding with infected ticks upon a seronegative dog. Interestingly, transmission of *R. conorii* dramatically decreased to 8–28.5% when the canine host was seropositive (Zemtsova et al. 2010).

Cofeeding has been evaluated as a transmission route for *R. parkeri* in studies using *A. maculatum* and *A. americanum*, where possible exclusion due to *Rickettsia amblyommatis* sp. nov. was observed (Wright et al. 2015a, Karpathy et al. 2016). Further, cofeeding was speculated as a route of transmission for *R. parkeri* among cofeeding female *A. maculatum* (Wright et al. 2015b). To our knowledge, cofeeding studies that test transmission of *R. parkeri* in the presence or absence of “*Ca. R. andeanae*,” between the primary tick vector, are not documented. Here, we present results of a study initiated to evaluate the acquisition of *R. parkeri* by naïve *A. maculatum* nymphs during cofeeding with *R. parkeri*-infected adult *A. maculatum* on nonrickettsemic hosts. In addition, we sought to assess the effect of “*Ca. R. andeanae*” infection status on *R. parkeri* acquisition during cofeeding. We hypothesized that *R. parkeri* acquisition by naïve *A. maculatum* during cofeeding with *R. parkeri*-infected *A. maculatum* would occur regardless of “*Ca. R. andeanae*” infection status or proximity in feeding, but that efficiency would be higher in “*Ca. R. andeanae*”-free *A. maculatum*. Data from this study confirm horizontal transmission of *R. parkeri* by cofeeding on beef calves, and suggest that prior exposure to “*Ca. R. andeanae*” may affect *R. parkeri* acquisition by *A. maculatum*.

Materials and Methods

Selection and sources of animal model and ticks

We selected beef cattle as our animal model due to their biological and economic importance as hosts for adult *A. maculatum*, as well as their ability to support nymphal *A. maculatum* [as reviewed in Teel et al. (2010)]. Twelve beef calves of various breeds commonly found in Mississippi (Angus, Holstein, and Brangus) were purchased from the Mississippi Agricultural and Forestry Experiment Station (MAFES) at Mississippi State University (MSU). Eight

calves were castrated males (steers), and four were females (heifers).

At the time of relocation from MAFES to MSU College of Veterinary Medicine (MSU-CVM) facilities, all calves were ~3–4 months old and in good body condition (90.4 kg; SE 3.7 kg). Before relocation, calves had been raised outdoors in an enclosed pen, received ULTRA BOSS insecticide (Merck Animal Health, Summit, NJ), and were vaccinated with Vira Shield 6 (Elanco Animal Health, Greenfield, IN). Once relocated to MSU-CVM and until the start of each study trial, we maintained calves in an outdoor, covered pen, away from pastures to minimize risk of tick acquisition. Calves were ~5–7 months of age at the beginning of each trial, with the two trials occurring 1 month apart; the effects of insecticide were considered worn off by the start of the study trials. We allocated six calves, four males and two females, to each trial and initially examined them to confirm the absence of attached ticks. We collected blood samples from calves on the first day of the study (day post-tick 0; DPT 0) to test for exposure to rickettsiae and for rickettsemia. Calves were acclimated in individual rooms of a six-room (each room ~100 ft²) climate-controlled large animal facility for 1 week and remained there for the duration of the trial. Trials were performed at an animal biosafety level 2, approved by the Institutional Biosafety Committee at MSU (Protocol 010-12). The Institutional Animal Care and Use Committee (IACUC) at MSU approved animal use for this study (Protocol 13-002).

We initially obtained adult male and female laboratory-reared pathogen-free *A. maculatum* from Biodefense and Emerging Infections Research (BEI) Resources (National Institute of Allergy and Infectious Disease, National Institutes of Health). Validation of pathogen-free status was made by routine testing of representative tick samples using PCR for *Rickettsia* spp., *Rickettsia rickettsii*, *Rickettsia parkeri*, *Rickettsia amblyommii* (*Rickettsia amblyommatis* sp. nov.), *Borrelia lonestari*, *Borrelia burgdorferi*, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, and *Anaplasma phagocytophilum*, as well as serological testing of rabbits used to maintain colonies (M. Levin, Rickettsial Zoonoses Branch, DVBD, CDC, Atlanta, GA). To generate *A. maculatum* nymphs and adults for this study, we reared pathogen-free *A. maculatum* adults on specific pathogen-free female New Zealand white rabbits, producing larval offspring, which were fed on rabbits to generate sufficient nymphs for cattle trials or for additional feeding on rabbits to produce sufficient numbers of adult stages for trials (IACUC protocol 13-002). We maintained ~500 adults and 1600 nymphal *A. maculatum* from this laboratory-reared colony in vials placed in chambers kept at ~93% humidity (using saturated potassium nitrate) until we initiated the study. We kept humidity chambers in the laboratory at room temperature and with a 10:14 (light:dark) cycle.

Rickettsia preparation

We cocultured *R. parkeri* in a Vero cell line (African green monkey kidney epithelial cells) using Eagle’s minimum essential medium (MEM) at pH 7.3 (Sigma-Aldrich, St. Louis, MO), supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA). We chose a strain of *R. parkeri* (Oktibbeha) transformed with plasmid pRAM18dRGA/Rif/GFPuv (also provided by U.G. Munderloh, University of Minnesota) to express GFPuv (*R.*

parkeri GFPuv) to allow for potential visualization in both tick and animal host cells. Cultures were maintained in a 37°C humidified incubator with 5% CO₂ (Paddock et al. 2010, Burkhardt et al. 2011). For “*Ca. R. andeanae*” propagation, we used coculture with ISE6 cells (*Ixodes scapularis* embryonic cells, originally provided by U.G. Munderloh, University of Minnesota), maintained using L15B300 medium with 20% heat-inactivated FBS (F4135; Sigma-Aldrich, St. Louis, MO) and 10% tryptose phosphate broth. The “*Ca. R. andeanae*” strain chosen was originally isolated in our laboratory from naturally infected *A. maculatum* (Ferrari et al. 2013). We prepared rickettsial suspensions for capillary feeding *R. parkeri* GFPuv to donor adult ticks and for capillary feeding “*Ca. R. andeanae*” to a subset of nymphs. Recipient ticks in the “*Ca. R. andeanae*”-free group were not capillary fed to maintain adequate numbers for the study (in the event of mortality); thus, we elected not to evaluate the effect of capillary feeding on nymphal survival in this study. To prepare suspensions, we first removed host cells from infected flasks using a cell scraper, then passed the cell suspension three times through a 21 G needle to break cell clumps, and another three times through a 30 G needle to release rickettsial organisms. The cell suspension was centrifuged at 50×g for 5 min to remove large clumps of cells, and then rickettsial organisms were collected after centrifugation at 10,000×g for 10 min and resuspended in the same cell culture medium (MEM with 10% FBS) as used for propagation. On the same day as capillary feeding, we extracted DNA from subsamples of the rickettsial preparations using a DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA), and subsequently performed quantitative (q)PCR for both *R. parkeri* and “*Ca. R. andeanae*” to evaluate rickettsial quantities (copy numbers) in the inoculum. For our qPCR, we used primers QrmpB_F and QrmpB_R and probes, CaRa_probe_FAM and Rp_probe_Hex, with concentrations and thermal profile as described by Lee et al. (2017). Rickettsial concentrations in suspensions were ~1×10⁸ and 1×10³ DNA copy numbers/mL for *R. parkeri* for feeding adult ticks and “*Ca. R. andeanae*,” respectively.

Cofeeding and tick placement

To generate *R. parkeri*-infected donor adult *A. maculatum*, we fitted each tick over its mouthparts with a 10 µL glass capillary tube filled with live, cultured *R. parkeri* GFPuv in suspension (~1×10⁸ DNA copy numbers/mL), with the tick secured to a glass slide using carpet tape (Kocan et al. 2005). Ticks were exposed for ~2 h in a 32°C incubator. One calf in the first trial received laboratory-reared nymphs that were naturally infected with “*Ca. R. andeanae*” (provided by K. Macaluso, Louisiana State University). No other naturally infected nymphs were available for use at the time of the study, hence the capillary feeding as our method of exposing most nymphs to “*Ca. R. andeanae*.” Nymphal *A. maculatum* in this “*Ca. R. andeanae*”-exposed group were exposed to a lower concentration of rickettsiae (~1×10³ DNA copy numbers/mL) of “*Ca. R. andeanae*,” using 5 µL glass capillary tubes fitted over their mouthparts to correspond with their smaller size (Broadwater et al. 2002, Ferrari et al. 2013). We did not attempt to achieve higher concentrations for capillary feeding due to challenges cultivating the rickettsiae and because potential pathogenic effects of “*Ca. R. andeanae*” exposure in immature ticks through capillary feeding were unknown.

After capillary feeding, adult and nymphal *A. maculatum* were transferred to vials and placed in a humidity chamber as described above, where they remained for 7 and 6 days, respectively, until being placed on calves. The mortality rate from the capillary feeding procedure in adult ticks was expected to be 30–40% based on a previous study; thus, the number of ticks capillary fed was higher than needed for the study to account for anticipated mortality in both adults and nymphs. For each of the two trials, six calves were distributed into two experimental groups (*n*=3 calves, each). On day post-tick (DPT) 0, we placed adult *A. maculatum* that had been capillary fed *R. parkeri* GFPuv 7 days before (DPT 7) in one ear each for all six calves, as described below. Four days later (DPT 4), we added nymphs to the same ear of each calf. One group (*n*=3 calves) received “*Ca. R. andeanae*”-exposed *A. maculatum* nymphs (capillary fed 6 days before, on DPT 2), and the other group (*n*=3 calves) received “*Ca. R. andeanae*”-free *A. maculatum* nymphs (reared from pathogen-free adults acquired from BEI Resources, NIAID, NIH, as described above).

Calves were sedated with xylazine (0.05 mg/kg IM) for attachment of ear chambers with initial donor tick placement, then again for placement of recipient ticks and for removal of ticks; sedation was reversed with yohimbine IV after the procedure. On DPT 0, six calves in trial 1 and six calves in trial 2 were infested with 30–32 or 46–47 donor adult *A. maculatum*, respectively. In trial 1, calves received 12 (*n*=4 calves) or 13 (*n*=2 calves) male ticks, and 18 (*n*=5 calves) or 19 (*n*=1 calf) female ticks; in trial 2, calves received 25 male ticks and 21 (*n*=1 calf) or 22 (*n*=5 calves) female ticks. Calves in the second trial were 1 month older, and thus larger, at the start of the study compared with calves in the first trial, hence the additional donor ticks used. To place ticks in the ear chamber, we first shaved the pinna and base of the ear, then fitted a sock over the ear. We adhered the sock to the ear base using OSTO-BOND skin adhesive (OSTO-BOND skin bonding latex adhesive, M.O.C. QC, Canada). Donor adult *A. maculatum* were added through an opening at the opposite end of sock attachment, and were allowed to infest all available space in the ear. On DPT 4, 65–100 nymphal recipient *A. maculatum* were added to sock chambers with donor adult stages. In addition, 10–15 adult recipient *A. maculatum* that were *R. parkeri* free and “*Ca. R. andeanae*” free and had been previously marked with paint were simultaneously added as recipient adults to evaluate *R. parkeri* acquisition by cofeeding. For recipient adult *A. maculatum* added to calves in the group with “*Ca. R. andeanae*”-exposed nymphal *A. maculatum*, we used *A. maculatum* from Oklahoma State University (OSU) (Tick Rearing Facility, Department of Entomology and Plant Pathology, OSU, Stillwater, Oklahoma), which we tested and found ticks to be naturally infected with “*Ca. R. andeanae*.” For “*Ca. R. andeanae*”-free recipient adults, we used the pathogen-free adult recipient *A. maculatum* (reared from pathogen-free adults acquired from BEI Resources, NIAID, NIH, as described in Selection and sources of animal model and ticks section).

The second calf trial differed from the first trial, in that we initially enclosed donor adult *R. parkeri*-infected *A. maculatum* in a chamber within the sock. The base of the enclosed inner chamber was ~1 cm in width and adhered to the pinna by skin adhesive; thus, there was a spatial distance of ~1 cm

separating donor from recipient ticks. In contrast, donor and recipient ticks were allowed to move freely among each other in trial 1. On DPT 12 (8 days from placement of recipient *A. maculatum*), all nymphal *A. maculatum* and fully engorged donor adult *A. maculatum* were removed from the ear sock chamber. A portion of engorged nymphs were directly frozen (-20°C) for later processing by DNA extraction and PCR detection of rickettsiae; another portion of engorged nymphs was kept in a humidity chamber for molting to the adult stage. Engorged donor female ticks were maintained in a humidity chamber for oviposition, while male ticks were frozen (-20°C). On DPT 15, the remaining recipient adult *A. maculatum* were removed; we placed females in a humidity chamber for oviposition and froze (-20°C) male ticks for later processing. After donor and recipient female *A. maculatum* oviposited, we froze a portion of the egg mass (~ 100 – 500 eggs; -20°C) for later processing, and kept the remainder in a humidity chamber to hatch to larvae. A portion of hatched larval clutches (~ 100 – 300 larvae) were also frozen (-20°C) for further processing to assess transovarial transmission.

On DPT 33 of the study, calves were euthanized by an overdose of barbiturates (intravenous injection of Beuthanasia), after initial sedation with xylazine. During necropsy we collected tissues, including ear skin, retropharyngeal lymph node, axillary lymph node, spleen, lung, heart, liver, and kidney; a portion of each tissue was fixed in 10% neutral buffered formalin for histopathology, and sections from paraffin-embedded blocks stained with hematoxylin and eosin; tissue samples were also frozen (-20°C) to archive for potential testing at a later date.

Rickettsial DNA detection

We extracted genomic DNA from individual whole ticks, and samples of larval clutches using a DNeasy Blood and Tissue Kit (Qiagen, Inc.). Extracted DNA samples were stored at -20°C until PCR and qPCR testing. Before testing for the presence of rickettsial DNA, we used extracts in a PCR assay to amplify the tick mitochondrial 16S rRNA gene to confirm amplifiable DNA was present (Black and Piesman 1994) for quality control. To test for acquisition of *R. parkeri* in *A. maculatum* engorged nymphs and molted adults, we initially used primers RpompAF and RpompAR in a species-specific PCR assay targeting a portion of the rickettsial outer membrane protein A (*ompA*) gene as described (Varela-Stokes et al. 2011), then tested samples in a qPCR, as described below.

DNA detection of both *R. parkeri* and “*Ca. R. andeanae*” was performed on extracts from recipient nymphs (engorged and molted, as adults) using a TaqMan[®] multiplex qPCR. A subset of extracts from recovered adults was also tested by qPCR. The qPCR consisted of one set of *Rickettsia*-wide primers, two species-specific probes targeting a unique sequence in the rickettsial outer membrane protein B (*ompB*) gene, one set of primers amplifying a portion of the *A. maculatum* macrophage migration inhibitory factor (MIF) gene, and an MIF probe as described in Lee et al. (2017). For each qPCR assay testing extracts, we included 10-fold standard dilutions (10^7 – 10^2) consisting of a mixture of plasmid templates for *R. parkeri* GFPuv Oktibbeha strain, “*Ca. R. andeanae*” and *A. maculatum* MIF. Nontemplate

(water) controls were also included in each run for quality control. Only data from multiplex qPCR reactions with reaction efficiencies between 90% and 110% for all three targets, and R squared values above or equal to 0.985, were accepted for analysis. We determined positive and negative status of extracts only.

We collected whole blood and serum samples from calves on DPT 0, 4, 7 or 8, 12, 15, 19, 22, 26, 29 and 33 in both trials. Sera from all time-point collections were stored at -20°C or -80°C until their use in serological assay to evaluate rickettsial exposure. To evaluate acute rickettsemia (blood circulation of rickettsiae) during tick feeding, we extracted DNA from whole blood on DPT 0, 4, and 7 or 8, using a DNeasy Blood and Tissue Kit (Qiagen, Inc.), and used extracts for rickettsial qPCR assays. We performed a multiplex qPCR as above with the exception that, instead of tick MIF primers and probe, we included a set of primers and probes to detect mammalian genomic DNA (12S) for quality control. This primer/probe set, Rab 12S-F, Rab 12S-R, and Rab 12S Cy5, was previously developed for rabbit samples and found to be applicable to cattle (manuscript under review). We included 10-fold standard dilutions (10^7 – 10^2) consisting of a mixture of plasmid templates for *R. parkeri* GFPuv Oktibbeha strain, “*Ca. R. andeanae*” and the mammalian plasmid in each of our qPCR assays, as well as nontemplate controls.

Indirect fluorescent antibody assay

To evaluate exposure to rickettsiae, we used an indirect fluorescent antibody assay (IFA) with *R. parkeri* (Portsmouth) antigen from cocultures with Vero cells. We initially screened calf sera at 1:32 and 1:64 serum dilutions, where reactivity at a 1:64 dilution was considered seropositive, as previously described (Edwards et al. 2010, Moraru et al. 2013). After washing the slides with phosphate buffered saline (PBS) and water, 1:400 or 1:200 dilutions of anti-bovine secondary antibody labeled with fluorescein (KPL, Inc., Gaithersburg, MD) were applied, and slides incubated for 30 min at 37°C . After washing with PBS, slides were counterstained with Eriochrome[™] Black T (Fisher Scientific, Waltham, MA), mounted using Vectashield (Vector Laboratories, Inc., Burlingame, CA), and then observed under UV light. For quality control, known positive and negative cattle sera were included on separate wells. Samples found to be seropositive were then serially diluted twofold up to 1:1024, with highest titers defined as ≥ 1024 .

Statistical methods

The effect of “*Ca. R. andeanae*” infection status on live/dead status of both engorged nymphs and molted adults in proximity and at least 1 cm separation was tested by chi-squared analysis (or Fisher’s exact test if a cell had counts <5) using PROC FREQ in SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC). The effect of “*Ca. R. andeanae*” infection status on the detection of *R. parkeri* by DNA in ticks both in proximity and separated from donor ticks in the inner chamber (~ 1 cm apart) was tested by Fisher’s exact test using PROC FREQ in SAS for Windows 9.4. Similarly, the effect of proximity on the detection of *R. parkeri* DNA in “*Ca. R. andeanae*”-free nymphs was tested by Fisher’s exact test. An alpha level of 0.05 was used to determine statistical significance.

TABLE 1. TOTAL NUMBER OF ENGORGED *AMBLYOMMA MACULATUM* NYMPHS AFTER COFEEDING WITH ADULT *AMBLYOMMA MACULATUM*, AND NUMBER OF NYMPHS THAT MOLTED TO THE ADULT STAGE FROM BOTH TRIALS

	Feeding in proximity allowed (trial 1)		Barrier to minimize close feeding (trial 2)	
	No. of engorged nymphs out of total placed (%)	No. of molted adults out of total engorged nymphs (%)	No. of engorged nymphs out of total placed (%)	No. of molted adults out of total engorged nymphs (%)
“ <i>Ca. R. andeanae</i> ”-exposed nymphs	32/250 (12.8) Frozen: 0	2/32 (6.25)	33/228 (14.5) Frozen: 11	7/22 (31.8)
“ <i>Ca. R. andeanae</i> ”-free nymphs	95/300 (31.7) Frozen: 45	26/50 (52.0)	121/300 (40.3) Frozen: 57	35/64 (54.7)

The subset of engorged nymphs frozen for detection of *Rickettsia parkeri* is also indicated; the remaining engorged nymphs were allowed to undergo ecdysis. Both recovery of engorged nymphs and molting success were lower in “*Ca. R. andeanae*”-exposed nymphs ($p < 0.0001$).

Results

Tick recovery after cofeeding

In this study, we found that the number of nymphal *A. maculatum* that became replete after cofeeding in both trials was lower for “*Ca. R. andeanae*”-exposed ticks (12.8% in trial 1; 14.5% in trial 2) compared with “*Ca. R. andeanae*”-free ticks (31.7% in trial 1; 40.3% in trial 2) (Table 1). The success of molting (ecdysis) to the adult stage was similarly lower for the “*Ca. R. andeanae*”-exposed nymphs (6.25% in trial 1; 31.8% in trial 2) compared with “*Ca. R. andeanae*”-free nymphs (52.0% and 54.7%) in both trials. Regarding the effect of “*Ca. R. andeanae*” infection on nymphal live/dead status, for nymphs allowed to feed in proximity, significantly more “*Ca. R. andeanae*”-free nymphs engorged and survived compared with infected nymphs (chi-squared test; $p < 0.0001$). Similarly, the survival rate for adult ticks that emerged after ecdysis was significantly higher in “*Ca. R. andeanae*”-free nymphs compared with infected nymphs (Fisher’s exact test; $p < 0.0001$). We also observed a similar result in the second trial, where more “*Ca. R. andeanae*”-free nymphs engorged and survived after ecdysis (chi-squared test; $p < 0.0001$), and more uninfected nymphs had higher survival after ecdysis compared with infected nymphs (chi-squared test; $p = 0.0480$). Recovery rates for donor *A. maculatum* in trial 2 were similar between “*Ca. R. andeanae*”-exposed donor ticks and the “*Ca. R. andeanae*”-free donor ticks, at 71% and 66%, respectively.

Rickettsial DNA acquisition in recipient ticks after cofeeding

Acquisition of *R. parkeri* by recipient *A. maculatum* nymphs in samples tested after engorgement (frozen) and after ecdysis (as adults) is summarized in Table 2. To evaluate rickettsial transmission to recipient adult *A. maculatum*, we tested larval offspring from four recovered females in the second trial. All eight larval masses that were tested from the “*Ca. R. andeanae*”-exposed group ($n = 4$ masses) and “*Ca. R. andeanae*”-free group ($n = 4$ masses) were negative for both “*Ca. R. andeanae*” and *R. parkeri*. Overall, a greater percentage of ticks acquiring *R. parkeri* was observed in the “*Ca. R. andeanae*”-free group than in the “*Ca. R. andeanae*”-exposed group; however, this was not statistically significant in both trials, based on Fisher’s exact test. Rickettsial DNA was not detected in DNA extracts of engorged nymphs in the

“*Ca. R. andeanae*”-exposed group (capillary fed or naturally infected) or in DNA extracts from adults that molted from nymphs in this group, suggesting that capillary feeding was not successful or levels were below the detection limit. Regardless of whether nymphs were capillary fed “*Ca. R. andeanae*” or not, cofeeding in proximity to the donor adult *A. maculatum* resulted in significantly higher numbers of nymphs (26.8%) with detectable *R. parkeri* than when feeding proximity was restricted using the enclosure for donor ticks (4.3%) (Fisher’s exact test, $p < 0.0001$).

Indirect fluorescent antibody assay and rickettsial DNA detection from calves

All calves’ sera from both experimental groups on all collection time points were evaluated by IFA for antibodies to spotted fever group rickettsiae. For trial 1, antibody titers on DPT 0 and 4 for calves in both treatment groups were < 64 . One calf in the “*Ca. R. andeanae*”-free group seroconverted on DPT 8 with a titer of 64 and remained seropositive throughout most of the time points, with titers ≥ 1024 on DPT 15, 22, and 29, decreasing to 512 on DPT 33. Another calf in the “*Ca. R. andeanae*”-free group remained seronegative (titer < 64) at every time point. For trial 2, all six calves were seronegative for spotted fever group rickettsial antibodies.

TABLE 2. ACQUISITION OF *RICKETTSIA PARKERI* IN RECIPIENT NYMPHS, AS MEASURED BY DNA DETECTION OF *RICKETTSIA PARKERI* AFTER COFEEDING OUT OF THE TOTAL NUMBER TESTED (%)

	Feeding in proximity allowed (trial 1)	Barrier to minimize close feeding (trial 2)
“ <i>Ca. R. andeanae</i> ”-exposed nymphs	0/2 (0%) Molted: 0/2 Frozen: 0	0/18 (0%) Molted: 0/7 Frozen: 0/11
“ <i>Ca. R. andeanae</i> ”-free nymphs	19/71 (26.8%) Molted: 2/26 Frozen: 17/45	4/92 (4.3%) Molted: 1/35 Frozen: 3/57

The total number of recipient nymphs included engorged nymphs (frozen) and adults that underwent ecdysis from engorged nymphs. All larvae recovered from adult recipient *Amblyomma maculatum* were negative for both *R. parkeri* and “*Ca. R. andeanae*.” Significantly higher numbers of nymphs had detectable *R. parkeri* when feeding in proximity than when a barrier was used (Fisher’s exact test, $p < 0.0001$).

All samples were retested multiple times, by the same person, and then confirmed by at least one additional person in the laboratory.

DNA extracts from calf whole blood samples collected and tested by qPCR early in infection, on DPT 0, 4, and 8, were negative for rickettsial DNA.

Discussion

Although the ears of naturally infected cattle are not typically examined for nymphal ticks, and adult stages of *A. maculatum* are more commonly found infesting the ears, we found that nymphs could successfully feed on calves in this study. All 12 calves in these 2 trials demonstrated gross pathological changes to their ears after tick feeding, including markedly thickened, hyperemic, and edematous ear pinnae with a deformed shape, consistent with “gotch ear,” in the tick-infested ear, as compared with the uninfested ear (Edwards 2011). On histopathological examination of ears at DPT 33, inflammatory cells were noted, though vasculitis, or other lesions consistent with rickettsial infection, was not observed at this time point. Any acute changes that may have been present due to rickettsial infection likely resolved by the time of euthanasia (DPT 33). We did not detect rickettsemia in any calf at the early time points tested, specifically when donor ticks were introduced (DPT 0), recipient ticks were added (DPT 4), and all ticks were allowed the opportunity to cofeed (DPT 8). However, we did detect evidence of rickettsial exposure in the first trial, when ticks were allowed to feed in proximity. One calf in the group exposed to “*Ca. R. andeanae*”-free nymphs demonstrated higher antibody titers than the other calves. Acquisition of *R. parkeri* in “*Ca. R. andeanae*”-free nymphs was also higher in this first trial, suggesting transmission of *R. parkeri* to both calves and ticks. None of the six calves in the second trial seroconverted. Thus, transmission of *R. parkeri* to recipient ticks may have occurred under conditions where calves were not exposed to sufficient rickettsiae to mount a detectable antibody response.

We found that recovery rates of nymphs after cofeeding from calf ears were higher for the “*Ca. R. andeanae*”-free group than for the “*Ca. R. andeanae*”-exposed group, and were higher in the second trial compared with trial 1. We did not detect transmission of *R. parkeri* in “*Ca. R. andeanae*”-exposed recipient *A. maculatum* nymphs that were tested after cofeeding with donor adult *A. maculatum*. Although no frozen engorged “*Ca. R. andeanae*”-exposed nymphs were available for testing in the first trial, 11 frozen engorged “*Ca. R. andeanae*”-exposed nymphs were tested in the second trial. DNA extracts from these ticks were negative for *R. parkeri*. Moreover, few “*Ca. R. andeanae*”-exposed nymphs successfully molted to adults; all nine adults were negative for *R. parkeri*. In contrast, based on the data from available recipient ticks in the “*Ca. R. andeanae*”-free group, transmission of *R. parkeri* was successful for nymphal ticks that were not initially capillary fed, especially when feeding in proximity to donor ticks was not discouraged. Transmission efficiency after cofeeding may vary depending on microorganism, host antibody to *Rickettsiae*, and experimental conditions. For example, 100% of *R. sanguineus* acquired *R. conorii* while cofeeding with donor ticks on seronegative dogs, but this efficiency dramatically decreased for seropositive hosts (Piesman and Happ 2001, Kocan and de la Fuente 2003, Matsumoto et al.

2005, Zemtsova et al. 2010). Overall, the transmission rate to recipient nymphs after cofeeding in this study was low when compared with a previous study using *R. conorii israelensis* in seronegative hosts (Zemtsova et al. 2010). The calves used in this study were ~5–7 months old at the time of their entrance in the study. Although calves were raised outdoors during the prior spring season, we did not detect evidence of previous exposure to spotted fever group rickettsiae on DPT 0, based on serological assays using *R. parkeri* antigen. Previous exposure to uninfected *A. maculatum* was also considered unlikely because adult *A. maculatum* populations typically peak in summer months in Mississippi, and not while calves were outdoors before the study; no ticks were found attached to calves on arrival.

The recovery of live nymphs, and molting success of engorged live nymphs, was also lower in nymphs exposed to “*Ca. R. andeanae*” by capillary feeding (or naturally), the “*Ca. R. andeanae*”-exposed group, compared with the “*Ca. R. andeanae*”-free group, for both trials. While this may suggest that exposure to the second rickettsial species, *R. parkeri*, could hinder nymphal molting to the adult stage in “*Ca. R. andeanae*”-exposed ticks, the process of capillary feeding as having a negative impact should not be ignored, since “*Ca. R. andeanae*”-free nymphs did not undergo this procedure. In reviewing the data for natural infection as a treatment variable, we found that if the data from naturally infected ticks were removed (one calf in trial 1), the same effect on live/dead status was observed in comparison to if that data was included. No nymphs that received “*Ca. R. andeanae*” through capillary feeding or that were naturally infected with “*Ca. R. andeanae*,” and that were recovered for PCR testing were positive for either *Rickettsia* species. For capillary-fed nymphs, we were careful to select those that were viable for placement on calves at DPT 4, but some decrease in fitness may have occurred, and those that did not survive were not tested by PCR for evidence of “*Ca. R. andeanae*.” Whether there is potential for “*Ca. R. andeanae*” transmission to occur is unclear based on these data, considering that we did not have convincing evidence that “*Ca. R. andeanae*” was present. For known “*Ca. R. andeanae*”-free recipient ticks, where recovery was higher, we found that mixing donor and recipient ticks without restricting proximal feeding resulted in more efficient transmission of *R. parkeri* to recipient ticks, as compared with transmission when we hindered proximal feeding using an inner chamber. Specifically, *R. parkeri* transmission was higher under conditions where nymphs were allowed to feed adjacent to donor adults (26.8%), compared with where this was limited (4.3%). Absence of both rickettsial species in an individual recipient tick may have been due to insufficient numbers for testing, insufficient “*Ca. R. andeanae*”-exposed ticks successfully feeding, or possible exclusion of *R. parkeri* in the presence of the sympatric rickettsia. While we did not exhaustively test all donor ticks, we did test subsets of recovered adults by qPCR to evaluate infection rates with *R. parkeri*. In trial 1, we detected *R. parkeri* in 30% (10/33) and 37% (17/46) of extracts from ticks placed on calves receiving “*Ca. R. andeanae*”-exposed and uninfected recipient ticks, respectively. In addition, “*Ca. R. andeanae*” was detected in 24% (8/33) and 9% (4/46; all coinfecting with *R. parkeri*) of calves in the two groups, respectively. Unfortunately, we were unable to distinguish donor from recipient adults in trial 1, because markings did not

remain on recipient adults, but evidence of “*Ca. R. andeanae*” in adult ticks that were not known to be infected was unexpected, especially in the presence of *R. parkeri*. In trial 2, *R. parkeri* was detected in 12.5% (2/16) and 13% (3/23) of extracts from ticks placed on calves receiving “*Ca. R. andeanae*”-exposed and uninfected recipient ticks, respectively. Further, “*Ca. R. andeanae*” was detected in 6.3% (1/16) and 8.7% (2/23) of extracts in these two groups, respectively. No ticks were coinfecting. Recipient adult ticks that were tested were all males, and could be distinguished from donor ticks in trial 2. Coinfection of *R. parkeri* and “*Ca. R. andeanae*” is rare but has been reported (Varela-Stokes et al. 2011, Ferrari et al. 2012, Leydet and Liang 2013, Budachetri et al. 2014).

A previous *R. parkeri* cofeeding study also showed exclusion of *R. parkeri* by preacquired *R. amblyommi* (*R. amblyommatis* sp. nov.) (Wright et al. 2015a). However, Wright et al. performed cofeeding transmission between *A. maculatum* and *A. americanum*. Therefore, the dynamics of exclusion may be different in a system using two tick species. Although recovery of recipient adult *A. maculatum* was low, subsets of larvae tested from the entire egg mass of trial 2 recipient adults in both “*Ca. R. andeanae*”-exposed (laboratory reared from OSU and naturally infected) and “*Ca. R. andeanae*”-free (pathogen free from BEI Resources) groups were negative for both *R. parkeri* and “*Ca. R. andeanae*.” This shows lack of transovarial transmission for both rickettsiae in those subsets of larval cohorts tested, though not necessarily the entire larval group. Further study may help clarify the extent to which transovarial transmission may or may not occur under these cofeeding conditions. *Rickettsia parkeri* is known to be transmitted transovarially (Wright et al. 2015b, Harris et al. 2017) and our sample size was small, so it is likely that the four female ticks did not acquire rickettsiae. We also recovered low numbers of nymphs under conditions in this study and believe most died in the ears during the feeding period. This was possibly due to trauma from scratching, as we did not restrict calf movement, or desiccation of the nymphs shortly after infestation. Using cattle as hosts for nymphal *A. maculatum* may also have not been ideal because immature *A. maculatum* more typically feed on birds, rodents, and small mammals (Semtner and Hair 1973, Teel et al. 2010). Still, recovery of “*Ca. R. andeanae*”-free nymphs was higher, so the difference may have also related to the process of capillary feeding or from “*Ca. R. andeanae*” having a negative effect on tick fitness.

In summary, here we demonstrate acquisition of *R. parkeri* by naïve *A. maculatum* nymphs through cofeeding with *R. parkeri*-infected adult *A. maculatum*. We did not test ear skin during tick feeding as an indicator of rickettsiae within the endothelial cells lining blood vessels or within capillaries at the feeding site. However, seroconversion demonstrated that calves were exposed to rickettsiae in the first trial, though not necessarily in the second trial. Transovarial transmission of either *R. parkeri* or “*Ca. R. andeanae*” was not detected in the larval masses from the recipient group. One of the potential limitations in this study was the use of *R. parkeri* (Oktibbeha) transformed with a plasmid to express GFPuv. While this strain was chosen to allow for additional downstream techniques (fluorescence *in situ* hybridization and immunohistochemistry) developed in the laboratory to differentiate the two rickettsiae microscopically, transmissibility of this strain, which was transformed to express GFPuv,

may have been affected. However, we believe that a tick-derived (Oktibbeha) strain, transformed with the plasmid, was appropriate because infectivity to ticks may have been more likely. Studies utilizing *R. parkeri* (Oktibbeha) GFPuv did not find that it was deleteriously changed from the wild-type strain (Burkhardt et al. 2011). Other *Rickettsia* spp. that lack plasmids have been similarly transformed with no apparent change in virulence, most recently demonstrated with *Rickettsia typhi* GFPuv, which was similar to the wild type in viability, bacterial replication kinetics, infectivity to vertebrates (mice), and pathogenicity (Hauptmann et al. 2017).

Future studies using low passage unaltered strains may better determine the importance of cofeeding for maintenance and transmission of *A. maculatum*-associated rickettsiae. We anticipate that taken together, these data add to our knowledge of rickettsial maintenance and will contribute to a better understanding of the natural history of spotted fever rickettsiosis in the United States.

Acknowledgments

The authors appreciate the assistance from all staff of the Laboratory Animal Resources and Care (LARAC), especially Drs. Nancy Brashier and Bridget Willeford, and Mr. Mike Bassett, Ms. Amber Angelo, and Ms. Jamie Walker for critical care, animal handling, and technical support for this project. They thank Haley Parker Nabors, Katie Graham, and Jacob Hughes for technical assistance during the study. The following reagent was provided by Centers for Disease Control and Prevention for distribution by BEI Resources, NIAID, NIH: Adult *Amblyomma maculatum*, NR-44382. Funding was provided from NIH 1R15A1099928-01A1. The A.V.S. laboratory was also supported in part by NIH COBRE P20GM103646 during this study.

Author Disclosure Statement

No conflicting financial interests exist.

References

- Black WC, Piesman J. Phylogeny of hard-and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. *Proc Natl Acad Sci* 1994; 91:10034–10038.
- Broadwater AH, Sonenshine DE, Hynes WL, Ceraul S, et al. Glass capillary tube feeding: A method for infecting nymphal *Ixodes scapularis* (Acari: Ixodidae) with the Lyme disease spirochete *Borrelia burgdorferi*. *J Med Entomol* 2002; 39: 285–292.
- Budachetri K, Browning RE, Adamson SW, Dowd SE, et al. An insight into the microbiome of the *Amblyomma maculatum* (Acari: Ixodidae). *J Med Entomol* 2014; 51:119–129.
- Burkhardt NY, Baldrige GD, Williamson PC, Billingsley PM, et al. Development of shuttle vectors for transformation of diverse *Rickettsia* species. *PLoS One* 2011; 6:e29511.
- Edwards KT, Goddard J, Jones TF, Paddock CD, et al. Cattle and the natural history of *Rickettsia parkeri* in Mississippi. *Vector Borne Zoonotic Dis* 2010; 11:485–491.
- Edwards KT. Gotch ear: A poorly described, local, pathologic condition of livestock associated primarily with the Gulf Coast tick, *Amblyomma maculatum*. *Vet Parasitol* 2011; 183: 1–7.
- Ferrari FAG, Goddard J, Moraru GM, Smith WE, et al. Isolation of “*Candidatus Rickettsia andeanae*” (Rickettsiales: Rickettsiaceae)

- in embryonic cells of naturally infected *Amblyomma maculatum* (Ixodida: Ixodidae). *J Med Entomol* 2013; 50:1118–1125.
- Ferrari FAG, Goddard J, Paddock CD, Varela-Stokes AS. *Rickettsia parkeri* and “*Candidatus Rickettsia andeanae*” in Gulf Coast Ticks, Mississippi, USA. *Emerg Infect Dis* 2012; 18:1705–1707.
- Fornadel CM, Zhang X, Smith JD, Paddock CD, et al. High rates of *Rickettsia parkeri* infection in Gulf Coast ticks (*Amblyomma maculatum*) and identification of “*Candidatus Rickettsia andeanae*” from Fairfax County, Virginia. *Vector Borne Zoonotic Dis* 2011; 11:1535–1539.
- Graspege BJ, Wolfson W, Macaluso KR. *Rickettsia parkeri* infection in domestic dogs, Southern Louisiana, USA, 2011. *Emerg Infect Dis* 2012; 18:995–997.
- Harris EK, Verhoeve VI, Banajee KH, Macaluso JA, et al. Comparative vertical transmission of *Rickettsia* by *Dermacentor variabilis* and *Amblyomma maculatum*. *Ticks Tick Borne Dis* 2017; 8:598–604.
- Hauptmann M, Burkhardt N, Munderloh U, Kuehl S, et al. GFPuv-expressing recombinant *Rickettsia typhi*: A useful tool for the study of pathogenesis and CD8⁺ T cell immunology in *R. typhi* infection. *Infect Immun* 2017; 85: e00156–17.
- Horta MC, Moraes-Filho J, Casagrande RA, Saito TB, et al. Experimental infection of opossums *Didelphis aurita* by *Rickettsia rickettsii* and evaluation of the transmission of the infection to ticks *Amblyomma cajennense*. *Vector Borne Zoonotic Dis* 2009; 9:109–118.
- Horta MC, Sabatini GS, Moraes-Filho J, Ogrzewalska M, et al. Experimental infection of the opossum *Didelphis aurita* by *Rickettsia felis*, *Rickettsia bellii*, and *Rickettsia parkeri* and evaluation of the transmission of the infection to ticks *Amblyomma cajennense* and *Amblyomma dubitatum*. *Vector Borne Zoonotic Dis* 2010; 10:959–967.
- Jiang J, Stromdahl EY, Richards AL. Detection of *Rickettsia parkeri* and *Candidatus Rickettsia andeanae* in *Amblyomma maculatum* Gulf Coast ticks collected from humans in the United States. *Vector Borne Zoonotic Dis* 2012; 12:175–182.
- Jones LD, Davies CR, Steele GM, Nuttall RA. A novel mode of arbovirus transmission involving a nonviremic host. *Science* 1987; 237:775–777.
- Karpathy SE, Slater KS, Goldsmith CS, Nicholson WL, et al. *Rickettsia amblyommatis* sp. nov., a spotted fever group *Rickettsia* associated with multiple species of *Amblyomma* ticks in North, Central and South America. *Int J Syst Evol Microbiol* 2016; 66:5236–5243.
- Kocan KM, de la Fuente J. Co-feeding studies of ticks infected with *Anaplasma marginale*. *Vet Parasitol* 2003; 112:295–305.
- Kocan KM, Yoshioka J, Sonenshine DE, de la Fuente J, et al. Capillary tube feeding system for studying tick-pathogen interactions of *Dermacentor variabilis* (Acari: Ixodidae) and *Anaplasma marginale* (Rickettsiales: Anaplasmataceae). *J Med Entomol* 2005; 42:864–874.
- Lee JK, Moraru GM, Stokes JV, et al. *Rickettsia parkeri* and “*Candidatus Rickettsia andeanae*” in questing *Amblyomma maculatum* (Acari: Ixodidae) from Mississippi. *J Med Entomol* 2017; 54:476–480.
- Leydet BF, Liang FT. Detection of human bacterial pathogens in ticks collected from Louisiana black bears (*Ursus americanus luteolus*). *Ticks Tick Borne Dis*. 2013; 4:191–196.
- Matsumoto K, Brouqui P, Raoult D, Parola P. Experimental infection models of ticks of the *Rhipicephalus sanguineus* group with *Rickettsia conorii*. *Vector Borne Zoonotic Dis* 2005; 5:363–372.
- Moraru GM, Goddard J, Paddock CD, Varela-Stokes A. Experimental infection of cotton rats and bobwhite quail with *Rickettsia parkeri*. *Parasit Vectors* 2013; 6:1–5.
- Moulder JW. Comparative biology of intracellular parasitism. *Microbiol Rev* 1985; 49:298–337.
- Nadolny RM, Wright CL, Sonenshine DE, Hynes WL, et al. Ticks and spotted fever group rickettsiae of southeastern Virginia. *Ticks Tick-Borne Dis* 2014; 5:53–57.
- Paddock CD, Denison AM, Dryden MW, Noden BH, et al. High prevalence of “*Candidatus Rickettsia andeanae*” and apparent exclusion of *Rickettsia parkeri* in adult *Amblyomma maculatum* (Acari: Ixodidae) from Kansas and Oklahoma. *Ticks Tick-Borne Dis* 2015; 6:297–302.
- Paddock CD, Fournier PE, Sumner JW, Goddard J, et al. Isolation of *Rickettsia parkeri* and identification of a novel spotted fever group *Rickettsia* sp. from Gulf Coast ticks (*Amblyomma maculatum*) in the United States. *Appl Environ Microbiol* 2010; 76:2689–2696.
- Piesman J, Happ CM. The efficacy of co-feeding as a means of maintaining *Borrelia burgdorferi*: A North American model system. *J Vector Ecol* 2001; 26:216–220.
- Raoult D, Roux V. Rickettsioses as paradigms of new or emerging infectious diseases. *Clin Microbiol Rev* 1997; 10: 694–719.
- Semtner PJ, Hair JA. Distribution, seasonal abundance, and hosts of the Gulf Coast tick in Oklahoma. *Ann Entomol Soc Am* 1973; 66:1264–1268.
- Sumner JW, Durden LA, Goddard J, Stromdahl EY, Clark KL, Reeves WK, Paddock CD. Gulf Coast ticks (*Amblyomma maculatum*) and *Rickettsia parkeri*, United States. *Emerg Infect Dis* 2007; 13:751–753.
- Teel PD, Ketchum HR, Mock ED, Wright RE, et al. The gulf coast tick: A review of the life history, ecology, distribution, and emergence as an arthropod of medical and veterinary importance. *J Med Entomol* 2010; 47:707–722.
- Varela-Stokes AS, Paddock CD, Engber B, Toliver M. *Rickettsia parkeri* in *Amblyomma maculatum* ticks, North Carolina, USA, 2009–2010. *Emerg Infect Dis* 2011; 17:2350–2353.
- Wright CL, Gaff HD, Sonenshine DE, Hynes WL. Experimental vertical transmission of *Rickettsia parkeri* in the Gulf Coast tick, *Amblyomma maculatum*. *Ticks Tick-Borne Dis* 2015b; 6:568–573.
- Wright CL, Sonenshine DE, Gaff HD, Hynes WL. *Rickettsia parkeri* transmission to *Amblyomma americanum* by cofeeding with *Amblyomma maculatum* (Acari: Ixodidae) and potential for spillover. *J Med Entomol* 2015a; 52:1090–1095.
- Zemtsova, G, Killmaster LF, Mumcuoglu KY, Levin ML. Co-feeding as a route for transmission of *Rickettsia conorii israelensis* between *Rhipicephalus sanguineus* ticks. *Exp Appl Acarol* 2010; 52:383–392.

Address correspondence to:
 Andrea S. Varela-Stokes
 Department of Basic Sciences
 College of Veterinary Medicine
 Mississippi State University
 Mississippi State, MS 39762

E-mail: stokes@cvm.msstate.edu