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Proposing a new hypothesis: *Rickettsia* spp. as a mechanism maintaining parapatry between two Australian reptile tick species

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Abstract This study investigates two parasitic reptile ticks — *Bothriocroton hydrosauri* and *Amblyomma limbatum* — of the sleepy lizard (*Tiliqua rugosa*) that abut at a 1–2 km wide parapatric boundary in South Australia. Long-term research has investigated potential mechanisms to explain the maintenance of this boundary but has not uncovered why the distribution of *A. limbatum* does not extend further south. It has been previously hypothesised that pathogens may be responsible for maintaining parapatric boundaries. *Rickettsia* spp. has previously been reported in *B. hydrosauri* ticks. This study explored whether *Rickettsia* spp. occurs in co-occurring *A. limbatum*. We observed that *Rickettsia* spp. was absent from all *A. limbatum* ticks and that 83% of examined *B. hydrosauri* were found to be positive with a spotted fever group *Rickettsia* strain. This study puts forward the hypothesis that *Rickettsia* spp. could contribute to the maintenance of the Mt Mary parapatric boundary between these two tick species. Further work is required to determine whether *Rickettsia* spp. can be transmitted from *B. hydrosauri* to *A. limbatum* and — if transmission can occur — to explore whether *Rickettsia* is lethal to *A. limbatum* ticks.

Key words: *Amblyomma limbatum*, *Bothriocroton hydrosauri*, Ixodid tick, spotted fever, *Tiliqua rugosa*, transmission.

INTRODUCTION

Parapatry occurs where species ranges mostly abut but with a small overlap zone (Smith, 1995). One well-studied example of a parapatric boundary is the two-tick system studied by the late Professor C. Michael Bull (Bull & Possingham, 1995; Godfrey & Gardner, 2017). Parasitic reptile ticks *Bothriocroton hydrosauri* and *Amblyomma limbatum* of the sleepy lizard *Tiliqua rugosa* abut at a 1–2 km wide parapatric boundary near Mount Mary, South Australia (Fig. 1) — where the survival of each species is reduced on the alternative side of the boundary (Bull & Burzacott, 2001). The parapatric boundary coincides with a 250 mm rainfall isohyet and a broad ecotone of mallee to chenopod scrub, where *B. hydrosauri* is found in the more mesic south and *A. limbatum* in the more arid north (Bull & Burzacott, 2001). Long-term research has investigated potential mechanisms involved in maintaining this boundary such as desiccation stress, predation, reproductive interference, competition for resources and competition for site attachment (Godfrey & Gardner 2017). These studies show that *B. hydrosauri* is less tolerant to desiccation stress and may be at higher risk of ant predation whilst on the northern side of the boundary (Bull & Burzacott, 2001); however, no compelling reason has been found for why *A. limbatum* does not extend its range further south (Godfrey & Gardner 2017).

One potential mechanism for boundary maintenance that remains relatively unexplored is the existence of a pathogen that could be lethal to or reduce the fitness of its tick host (Smallridge & Bull 2001). Pathogens are also known to have an influence on host ranges (Bozick & Real 2015). *Rickettsia* spp. are intracellular bacterial parasites or symbionts of eukaryotes, and Ixodid ticks are both host and vector (Derne *et al.*, 2015; Weinert *et al.*, 2009). *Rickettsia* spp. have been found to alter host animal behaviour and fitness such as influencing dispersal and thermal tolerance, egg production, moulting success and offspring viability in tick hosts (Harris *et al.*, 2017). Crucially, in one study, *R. rickettsii* was found to be lethal to its tick vector *Dermacentor andersoni*

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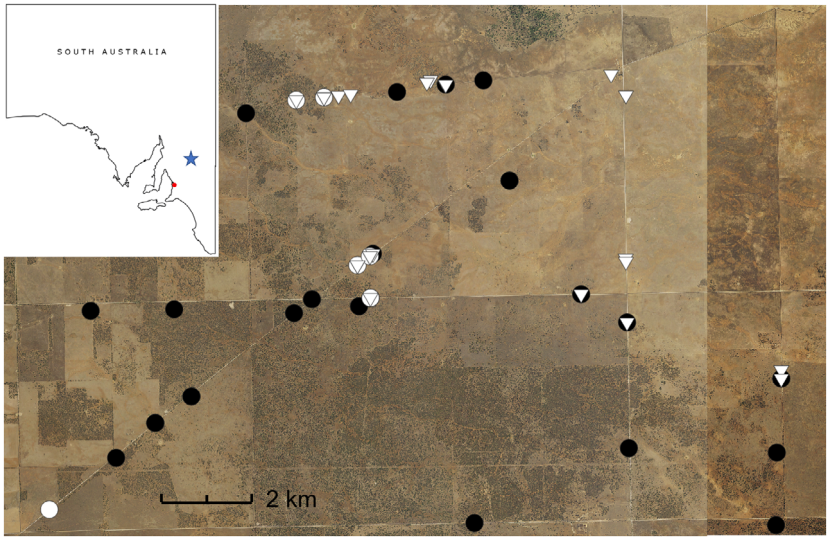

Fig. 1. Map of Mt. Mary study area showing location of collected *B. hydrosauri* (circles) and *A. limbatum* (triangles) ticks. Black and white symbols represent Rickettsia positive and negative individuals, respectively. Red dot and blue star on insert map show the position of Adelaide and the field site, respectively.

following both vertical and horizontal transmission (Niebylski *et al.*, 1999).

Spotted fever group *Rickettsia* have been detected in *B. hydrosauri* ticks in southern Australia (Stenos *et al.*, 2003; Dyer *et al.*, 2005), including a spotted fever group strain of unknown pathogenicity detected in 100% of *B. hydrosauri* ticks collected from *T. rugosa* in the Murray Mallee, South Australia (Whiley *et al.*, 2016). However, the presence of *Rickettsia* has not yet been examined in *A. limbatum*. If *Rickettsia* does not occur or has lower prevalence in *A. limbatum*, one explanation is that this *Rickettsia* spp. kills this tick species. Examining the presence of *Rickettsia* in *A. limbatum* is the first step in understanding if the hypothesis that *Rickettsia* is involved in the maintenance of the parapatric boundary has any merit.

Therefore, to further investigate the maintenance of the parapatric boundary observed near Mount Mary, South Australia, this study examined whether *Rickettsia* prevalence differs between *B. hydrosauri* and *A. limbatum* ticks collected from *T. rugosa* hosts across the parapatric boundary. From the results of our study, we propose a new hypothesis regarding the maintenance of the tick parapatric boundary.

MATERIALS AND METHODS

Study area and tick collection

Individuals of the tick species *B. hydrosauri* and *A. limbatum* were collected from sleepy lizard (*Tiliqua rugosa*) hosts near Mount Mary, South Australia (33°55′20″S, 139°17′03″E). Sampling occurred during September to December in the austral spring/summer across years 2010, 2011, 2015, 2016, 2017 and 2018. Lizards were captured by random encounter along well-established transects (Godfrey & Gardner, 2017). Ticks were removed from lizards and stored in 95% ethanol for later DNA extraction. Ticks were identified to species, life stage (larvae, nymph, adult) and sex, and GPS locations were recorded (Fig. 1). Lizards were released at capture location after being processed. All procedures were approved under Flinders University's animal ethics (currently E454/17).

DNA extraction and PCR amplification

DNA was extracted from 37 *B. hydrosauri* and 25 *A. limbatum* whole ticks using invertebrate glass fibre plate DNA extraction protocols (Ivanova *et al.*, 2006), and including an extraction blank. DNA concentrations were quantified using the Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA USA).

Conventional PCR was performed targeting the *Rickettsia*-specific protein-coding gene citrate synthase (*gltA*) and spotted fever-specific outer membrane protein gene OmpA (*ompA*) using published primers (Table 1). Positive and negative extraction controls were included in all runs, and ticks negative for *Rickettsia* were confirmed as true negatives by amplifying the 16s rRNA (*rrs*) gene (Table 1). Samples were amplified using primers shown in Table 1 with 0.8 μmol L^{-1} of forward and reverse primers, 1X PCR Gold Buffer, 1.5 mmol L^{-1} $MgCl_2$, 0.8 mmol L^{-1} of dNTP and 0.5 U of AmpliTaq Gold DNA polymerase for *gltA* and 0.4 μmol L^{-1} of each forward and reverse primers, 1X PCR Gold Buffer with 2 mmol L^{-1} $MgCl_2$, 0.8 mmol L^{-1} of dNTP and 0.5 U of AmpliTaq Gold DNA polymerase for both *ompA* and *rrs*. Each reaction included 20 ng of DNA in a final volume of 25 μL per sample.

Amplification was performed on a Mastercycler® Pro Thermal Cycler (Eppendorf, Hamburg, Germany).

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Table 1. Primers used to amplify *Rickettsia* (*gltA*), spotted fever group *Rickettsia* (*ompA*) and bacterial (*rrs*) genes in *Bothriocroton hydrosauri* and *Amblyomma limbatum* ticks collected from *Tiliqua rugosa* hosts in Mount Mary, South Australia

Primer pair	Target gene	Size of PCR product (bp)	Primer sequence (5′-3′)	References
RpCS.780p	*gltA*	479	GAC CAT GAG CAG AAT GCT TCT	
RpCS.1258n			ATT GCA AAA AGT ACA GTG AAC	Ishikura *et al.* (2003)
Rr190.70p	*ompA*	532	ATG GCG AAT AAT TCT CCA AAA	
Rr190.602n			AGT GCA GCA TTC GCT CCC CCT	Vilcins *et al.* (2009)
rRNA1	*rrs*	990	AGA GTT TGA TCC TGG CTC AG	
rRNA3			CCC TCA ATT CCT TTG AGT TT	
rRNA2		1020	AAG GAG GTG ATC CAG CCG CA	Izzard *et al.* (2009)
rRNA4			CAG CAG CCG CGG TAA TAC	

Thermocycler conditions for *gltA* consisted of an initial enzyme activation at 95°C for 10 min, then 35 cycles of denaturation at 95°C for 30 s, annealing at 44°C for 30 s, extension at 65°C for 2 min and a final extension of 65°C for 10 min (Ishikura *et al.,* 2003). The *ompA* and *rrs* thermocycler conditions consisted of an initial enzyme activation at 94°C for 9 min, followed by 37 cycles of denaturation at 94°C for 45 s, annealing at 51°C for 45 s, extension at 72°C for 1 min and a final extension at 72°C for 10 min (Izzard *et al.* 2009). Amplified products were visualised on a 1.5% agarose gel, stained in GelRed (Biotium, Hayward, CA USA) and examined under a UV transilluminator (Bio-Rad, Hercules, CA USA).

Sequencing and genotyping

A selection of eight positive PCR samples for the *gltA* and *ompA* genes had their nucleotides sequenced to determine to which *Rickettsia* species they belonged. Samples were cleaned using a vacuum manifold and Multiscreen® 384-well plates (Merck, Darmstadt, Germany) following manufacturer's instructions, and a ABI Prism Big Dye Terminator Cycle Sequencing chemistry reaction was performed. The Big Dye reaction mix contained 1X Big Dye Terminator, 1X sequencing buffer, 0.4 μmol L^{-1} of 10 μmol L^{-1} forward and reverse primers, 0.12–0.16 μmol L^{-1} of template and nuclease-free water in a volume of 25 μL. Samples were run on a Mastercycler® Pro Thermal Cycler for 30 s at 96°C with 25 cycles of initial heating at 50°C for 15 s, annealing at 60°C for 4 min and extension at 25°C for 1 min 30 s. Samples were cleaned using the MultiScreen vacuum manifold and sent to the Australian Genomics Research Facility (AGRF, Adelaide) for sequencing on the ABI 3730 capillary sequence analyser (Sanger Sequencing).

Forward and reverse sequences were aligned and edited using Geneious 7 (http://www.geneiuos.com, Kearse *et al.,* 2012), and homology was compared (Fournier *et al.,* 2003) with other *Rickettsia* sequences to determine the strain or species by using the Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

DNA extraction and PCR amplification

Amplification of the *rrs* gene was successful for 36 of 37 *B. hydrosauri* and 19 of 25 *A. limbatum* tick DNA samples (Table 2). All *rrs* gene-positive *A. limbatum* ticks — which included 17 alive and two dead ticks — were confirmed *Rickettsia* negative as they did not amplify either the *gltA or ompA* genes (Table 2). Upon investigation of the *gltA* gene, 30/36 (83.3%) of *B. hydrosauri* ticks were *Rickettsia* positive and 6/36 (16.7%) negative. Positive ticks were detected in all life stages and in both sexes (Table 2). A selection

Table 2. Amplification of *Rickettsia* in *Bothriocroton hydrosauri* and *Amblyomma limbatum* ticks by PCR

	Female	Male	Nymph	Larvae	Unknown	Dead*	Total
Successful amplification							
B. hydrosauri							
Rickettsia positive	5	18	1	3	2	1	30
Rickettsia negative	1	4	1	0	0	0	6
A. limbatum							
Rickettsia positive	0	0	0	0	0	0	0
Rickettsia negative	4	7	1	0	5	2	19
Unsuccessful amplification							
B. hydrosauri	–	–	–	–	–	1	1
A. limbatum	1	–	1	–	2	2	6

*All dead ticks were males. Unsuccessful amplification of control bacterial (*rrs*) genes in the ticks is also shown.

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of 15 positive *B. hydrosauri* ticks was tested further for spotted fever group *Rickettsia* by investigation of the *ompA* gene, and all returned positive results.

Sequencing and genotyping

Sequencing of the *ompA* and the *gltA* gene was successful for six and two samples, respectively. BLAST analysis of the *ompA* gene for each sample indicated 100% homology with a *Rickettsia* strain (accession numbers: KX290294–KX290299) from *B. hydrosauri ticks* found in the Murray Mallee, South Australia (Whiley *et al.,* 2016), as well as 100% homology with undescribed *Rickettsia* species 801a and 774e (EU283837 and EU28385) from *A. fimbriatum* ticks in the Northern Territory (Vilcins *et al.,* 2009). BLAST results of the *gltA* gene returned a 99.6% and 100% match with undescribed *Rickettsia* species 774e (EU283832) (Vilcins *et al.,* 2009). The closest match with a named species was 95.8% for *ompA* and 99.5–99% for *gltA* with *R. tamurae* strain At-1 (DQ103259, Fournier *et al.,* 2006) and *R. tamurae* isolate 239 (KT753273).

DISCUSSION

The maintenance of a parapatric tick boundary between two reptile ticks on the host *Tiliqua rugosa* in the mid-north of South Australia has been a long-standing research question (Godfrey & Gardner, 2017). Here, we report that *Rickettsia* spp. was present in most *Bothriocroton hydrosauri* ticks surveyed; however, all examined *Amblyomma limbatum* ticks in Mount Mary were found to be negative for *Rickettsia*. As our sample sizes were small, it is possible the *A. limbatum* ticks were infected, but at a low prevalence. Our finding does not prove, nor refute the hypothesis that *Rickettsia* spp. are involved in the maintenance of this parapatric boundary but identifies two areas for further investigation into potential role of *Rickettsia* spp. in maintenance of the tick boundary.

It is unknown whether *Rickettsia* spp. are transmitted between *B. hydrosauri* and *A. limbatum*. Understanding the potential transmission is a crucial factor in determining whether *Rickettsia* spp. are involved in preventing *A. limbatum* from moving south. *Rickettsia* can be transmitted via several pathways (transovarially, transtadially and horizontally), and transmission is dependent on the pathogen and the viability of its vector and host (Harris *et al.,* 2017). Studies on the transmission of *R. honei* in *B. hydrosauri* ticks from Flinders Island, Tasmania, showed that *Rickettsia* is located in the oocytes, immature eggs, Malpighian tubules, midgut epithelium and salivary glands – suggestive of transovarial and horizontal transmission (Stenos *et al.,* 2003; Whitworth *et al.,* 2003). Therefore, it is likely that transovarial and horizontal transmission of *Rickettsia* sp. occurs in the *B. hydrosauri* ticks of this current study. The presence of *Rickettsia* in *B. hydrosauri* and its absence in *A. limbatum* could indicate that transmission does not occur between these two tick species. However, this finding does not rule out transmission and subsequent death of *A. limbatum*. An alternative explanation is that Rickettsia is transmitted by the vertebrate host and that the ticks do not feed on an infected vertebrate host. However, there are other possible routes of transmission.

Co-feeding is one possible mechanism of transmission to explore. The two tick species in this system feed differently which makes transmission via the lizard host less likely. *Amblyomma limbatum* predominantly feed on the blood of their host; however, *B. hydrosauri* has been shown to feed mostly on lymph implying co-feeding could be a viable route for transmission (Smallridge & Bull, 1999). This requires investigation. Examples of co-feeding transmission of *Rickettsia* have been demonstrated in other ticks that feed on non-susceptible hosts (Lee *et al.,* 2018).

Experimental studies show that *R. ricketsii* is lethal in the ixodid tick vector *Dermacentor andersoni* following horizontal and transovarial transmission (Niebylski *et al.,* 1999). Larvae infected after feeding on rickettsemic guinea pigs survived to transmit infection as nymphs but died during the moult into adults. Furthermore, most ticks infected as nymphs died prior to feeding as an adult leading to a low prevalence of infected adult ticks (Niebylski *et al.,* 1999). If transmission does occur and this *Rickettsia* strain is lethal to *A. limbatum*, dead ticks would drop off the host, which might explain why live *A. limbatum* ticks in this study were all negative for *Rickettsia* as it was mostly adults that were examined.

This study proposes that a potentially fruitful area for further research in the understanding of boundary maintenance is the exploration of whether *Rickettsia* spp. can be transmitted to *A. limbatum*, and if so whether *Rickettsia* spp. is lethal to *A. limbatum*. If these two conditions were met, *Rickettsia* spp. could prevent *A. limbatum* from dispersing further south. Lethality of *Rickettsia* spp. could be tested by experimentally infecting ticks of all life stages in the laboratory. Additional evidence could come from investigating dead *A. limbatum* ticks at the boundary. Further to this, future studies should investigate *Rickettsia* spp. in the blood of *T. rugosa* hosts and the potential for co-feeding as a route of transmission and should consider if other vertebrate species in the area act as a source of *Rickettisa* infection for ticks. Further work should also investigate the role of tick-

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host diversity and the influence of the whole microbiota within this system and within the wider geographic distribution of the two tick species.

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AUTHOR CONTRIBUTION

Morgan Staines: Data curation (equal); Formal analysis (lead); Investigation (equal); Methodology (equal); Writing-original draft (equal). **Tessa Bradford:** Data curation (supporting); Formal analysis (supporting); Methodology (supporting); Writing-review & editing (supporting). **Stephen R. Graves:** Conceptualization (supporting); Methodology (supporting); Project administration (supporting); Writing-review & editing (supporting). **Simon Bull:** Data curation (supporting); Investigation (supporting); Writing-review & editing (supporting). **Michael G. Gardner:** Conceptualization (lead); Data curation (supporting); Funding acquisition (lead); Investigation (supporting); Project administration (lead); Writing-original draft (equal); Writing-review & editing (lead).

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