Vector of *Trypanosoma copemani* identified as *Ixodes* sp.

J. M. AUSTEN[1]*, U. M. RYAN[1], J. A. FRIEND[2], W. G. F. DITCHAM[1] *and* S. A. REID[1]

[1] *School of Veterinary and Biomedical Sciences, Division of Health Sciences, Murdoch University, South Street, Murdoch, Western Australia, 6150*
[2] *Science Division, Department of Environment and Conservation, 120 Albany Highway, Albany, Western Australia, 6330*

(*Received 17 November 2010; revised 14 January and 13 February 2011; accepted 23 February 2011; first published online 26 April 2011*)

SUMMARY

A total of 41 ticks were collected from 15 quokkas on Bald Island and 2 ticks from a Gilbert's potoroo from Two Peoples Bay. Three species of Ixodid ticks *Ixodes australiensis, Ixodes hirsti* and *Ixodes myrmecobii* were identified on the quokkas known to have a high prevalence of *Trypanosoma copemani*. Tick faeces from ticks isolated from 8 individual quokkas and a Gilbert's potoroo were examined with one identified as positive for trypanosomes. Faecal examination revealed trypanosomes similar to *in vitro* life-cycle stages of *T. copemani*. In total 12 ticks were dissected and trypanosomes found in sections of their midgut and haemolymph, 49 and 117 days after collection. Tick faeces, salivary glands and midguts from *I. australiensis* were screened using an 18S rRNA PCR with amplification seen only from the midguts. Sequencing showed 100% homology to *T. copemani* (genotype A) and 99·9% homology to the wombat (AII) isolate of *T. copemani*. Trypanosomes were only detected in *I. australiensis* as neither *I. hirsti* nor *I. myrmecobii* survived the initial 30-day storage conditions. We therefore identify a vector for *T. copemani* as *I. australiensis* and, given the detection of trypanosomes in the faeces, suggest that transmission is via the faecal-oral route.

Key words: trypanosomes, quokka, marsupial, tick, *Ixodes*, vector.

INTRODUCTION

Trypanosomes are a diverse group of protozoan parasites that can infect all classes of vertebrate. The epidemiology of diseases caused by *Trypanosoma* species are determined, in part by the ecology of the vector and the host-vector relationship that affect parasite transmission (Hoare, 1972). The majority of known vectors of mammalian trypanosomes belong to the class Insecta, which are associated with haematophagous insects of the order Hemiptera (e.g. Triatomine bugs), Diptera (e.g. flies) and Siphonaptera (e.g. flea) (Hoare, 1972). However Arachnida (ticks), bat mites and leeches are also vectors of trypanosomes (Mackerras, 1959; Lukes *et al*. 1997; Stevens *et al*. 1999; Hamilton *et al*. 2005; Thekisoe *et al*. 2007). Various leech species also play a role in the transmission of several trypanosome species such as *T. rotatorium, T. boissoni*, and *T. triglae*, which have been found in the blood of aquatic vertebrates (Lukes *et al*. 1997). The presence of trypanosome species in terrestrial blood-sucking leeches from both Asia and Australia has also been reported (Hamilton *et al*. 2005). In Australia the rate of detection of novel trypanosome species in the blood of native Australian mammals is increasing due to advances in molecular detection methods. However, little is known of these Australian trypanosomes, their life cycles and their vectors. Potential vectors such as the wombat flea (*Lycopsylla nova*) and kangaroo ticks have been implicated in the transmission of unidentified *Trypanosoma* species isolated from Australian mammals, the wombat and kangaroo (Noyes *et al*. 1999). Sandflies, leeches and the platypus tick (*Ixodes ornithorhynchi*) have been suggested as potential vectors for *T. binneyi*, which infect the platypus (Mackerras, 1959; Noyes *et al*. 1999; Jakes *et al*. 2001). Another study identified *Trypanosoma* sp. found within tick nymphs (*Ixodes holocyclus*) isolated from bandicoots infected with *T. thylacis* (Mackerras, 1959). Ticks (*Haemaphysalis hystricis*) from Japan have also been found to be naturally infected with an unidentified trypanosome species isolate KGI found to be closely related to *T. copemani* (Austen *et al*. 2009). Trypanosomes found in Australian ticks and the close phylogenetic relationship of *T. copemani* to the KGI trypanosome spread by ticks lead to the examination of ticks as potential vectors of *T. copemani*.

* Corresponding author: School of Veterinary and Biomedical Sciences, Division of Health Sciences, Murdoch University, South Street, Murdoch, Western Australia, 6150. Tel: +61 8 9360 6714. Fax: +61 8 9310 4144. E-mail: J.meinema@murdoch.edu.au

MATERIALS AND METHODS

Study site and sample collection

A total of 15 quokkas (*Setonix brachyurus*), were trapped at Bald Island (34° 55′S, 118° 27′E) near Albany, and a Gilbert's potoroo from Two Peoples Bay (34° 58′S, 118° 11′E), in Western Australia during the month of June 2010. The animals used in

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doi:10.1017/S0031182011000497

https://doi.org/10.1017/S0031182011000497 Published online by Cambridge University Press

this study were live-trapped using cage traps (Mascot Wire Works collapsible rat/bandicoot traps) or long-handled nets. A total of 41 ticks were collected off quokkas from Bald Island and 2 ticks collected off a Gilbert's potoroo from Two Peoples Bay and placed into 5 ml tubes (Sarstedt, Australia) for later identification using the Australian ticks key (Roberts, 1970). Fresh whole blood samples were collected from 3 of the quokkas from Bald Island. The quokkas were anaesthetized with isoflurane and approximately 5 ml of blood was collected by venepuncture of the lateral caudal vein. The blood was added to commercial blood storage tubes containing ethylenediamine tetraacetic acid (EDTA) (Sarstedt, Australia) and stored at 4 °C for later use. Thin blood smears were prepared and stained with Modified Wright's stain using an automated slide stainer (Hematek, Bayer). The blood smears were examined at 200× magnification for the presence of trypanosomes.

Detection of trypanosomes in the tick

Once identified, a selection of 10 *Ixodes australiensis* live ticks from quokkas and 2 ticks from the Gilberts potoroo were individually mounted onto house-hold pest glue boards (TOMCAT Motomco, USA) allowing immobilization of the tick and leg restraint. The glue board was then stuck to a dissecting microscope stage with double-sided tape, and the tick dissected according to published methods (Edwards *et al*. 2009). Haemolymph was collected on the end of a scalpel blade during the removal of the tick scutum and placed onto a microscope slide for examination. The midgut and salivary glands were also removed and placed into a 1·5 ml microcentrifuge tube containing 100 μl of RPMI 1640 (pH 7·2), supplemented with 10% foetal calf serum (FCS), 1000 international units (IU)/ml of penicillin and 10 000 μg /ml of streptomycin, with final concentrations of 2 g/L sodium bicarbonate, 0·3 g/L L-glutamine and 3·6 g/L HEPES. A small region of the midgut was placed onto a microscope slide containing 10 μl of RPMI 1640 culture medium, manually pulled apart and mixed using a sterile 10 μl pipette tip. A cover-slip was placed over the dissected midgut and the preparation examined microscopically at 200× magnification to detect any motile trypanosomes. Fragments of the midgut and salivary glands were also placed into Modified Sloppy Evans Medium (MSEM) with a 500 μl overlay of RPMI 1640 (supplemented as above) and incubated in the dark at room temperature.

Detection of trypanosomes in tick faeces

Faeces from a collection of ticks isolated from 8 individual quokkas from Bald Island and a Gilbert's potoroo from Two Peoples Bay were removed from the inside of 5 ml vol. polypropylene tubes with a sterile 10 μl tip placed into 10 μl of phosphate-buffered saline (pH 7·2) (PBS) on a microscope slide and manually dispersed. The slide was air dried and stained with Modified Wright's stain using an automated slide stainer (Hematek, Bayer). A cover-slip was placed over the stained faecal matter and examined at 200× magnification to detect trypanosomes.

Morphological measurements

Digital photomicrographs were taken, at 1000× magnification, of 5 tick trypanosomes from faecal smears and 14 tick trypanosomes from the dissected midgut and bloodmeal. Measurements of key morphological features were made using Image Pro Express version 5.1 (Media Cybernetics Inc., USA). The morphological features used were based on the following morphological parameters Length (length of body measured along the mid-line including free flagellum), Breadth (maximum breadth measured at the level of the nucleus (including undulating membrane), PK (distance between the posterior end and the kinetoplast), KN (distance between the kinetoplast and posterior edge of the nucleus), NA (distance between the anterior edge of the nucleus and the anterior end of the body) and FF (length of the free flagellum) previously described (Mackerras, 1959; Hoare, 1972). Significant differences between trypanosome morphology measurements from different host species and source were determined using SPSS 17.0 (Chicago, Illinois, USA) by one-way analysis of variance (ANOVA) and the Tukey's Honestly Significant Difference test for $P<0{\cdot}05$.

DNA extraction

Total genomic DNA was extracted using a DNeasy® blood and tissue kit (Qiagen), from both the tick faeces and dissected midgut and salivary glands of the ticks collected from quokka 2322 and 4489 and from the tick collected from the Gilbert's potoroo. DNA was also extracted from blood isolated from quokka 4489. The DNA was stored at −20 °C until required.

DNA amplification and sequencing

A fragment of the 18S ribosomal RNA (rRNA) gene was amplified from extracted DNA and sequenced as previously described (Austen *et al*. 2009). Amplified products were purified using a QIAquick® PCR Purification kit (QIAGEN) and sequenced directly. Sequencing reactions were performed using an ABI Prism DyeTerminator Cycle Sequencing Core kit (Applied Biosystems, USA) and sequence data were analysed using 4peaks v 1.7.1 (A. Griekspoor and Tom Groothuis, mekentosj.com).

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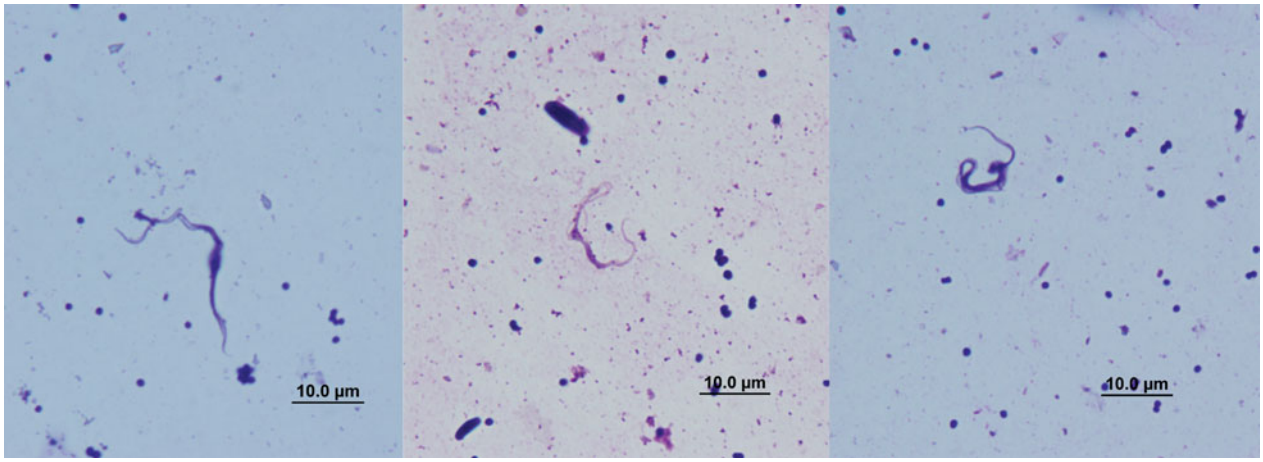


Fig. 1. Light photomicrographs of trypanosomes in Modified Wright's stain detected in the faeces of the tick.

RESULTS

Ticks

Two ticks collected from a single Gilbert's potoroo caught at Two Peoples Bay, and 25 of the 41 ticks collected from 15 quokkas captured on Bald Island, were sufficiently intact morphologically to allow identification down to the species level. *Ixodes australiensis* was identified from both Bald Island and Two peoples Bay and was the most abundant tick species collected at 72% of the total. Two other tick species with similar morphology to *Ixodes hirsti* and *Ixodes myrmecobii*, were also detected on Bald Island, at 12% and 8% of the total respectively. *Ixodes australiensis* was the only tick species dissected for examination because the specimens of *Ixodes hirsti* and *Ixodes myrmecobii* did not survive longer than 30 days in storage.

Trypanosomes present in tick faeces

Of the 8 tick faecal smears examined from ticks collected from 8 individual quokkas and 1 Gilbert's potoroo, the detection of trypanosomes was observed in 1 wet smear of dried faeces isolated from ticks collected from quokka 2322 and, to the authors' knowledge, this is the first time that trypanosomes have been identified in tick faeces. Morphologically the trypanosomes were slender in shape with a narrow undulating membrane, a large PK value (defined in Table 1) and a free flagellum (Fig. 1). The mean measurements for each morphological parameter for the trypanosomes are presented in Table 1.

Trypanosomes present in tick

Motile trypanosomes were detected in 2 ticks of the 10 ticks collected from animals from Bald Island that were dissected and examined. Trypanosomes were observed in pieces of midgut dissected from *Ixodes australiensis* collected from quokkas 2322 and 2917. Numerous motile trypanosomes were also detected in the haemolymph (Fig. 2), as well as in the midgut and bloodmeal from 1 tick collected from a Gilbert's potoroo. Morphologically, the trypanosome tick isolates resembled the slender pleomorphic trypomastigote forms that are present in *in vitro* cultures isolated from the blood of the Gilbert's potoroo as previously described (Austen *et al*. 2009). However, the free flagellum of the trypanosomes within the midgut and bloodmeal of the tick isolated from quokka 2917 were very short or absent (Fig. 3). The mean measurements for each morphological parameter from trypanosomes isolated from within the tick collected from quokka 2917 are presented in Table 1. Attempts to culture the trypanosome tick isolate in MSEM were unsuccessful.

Comparative analysis of morphological measurements of trypanosomes from quokka blood and from ticks

Comparison of the morphological measurements taken from the trypanosomes found in tick faeces and midgut, with measurements taken from the bloodstream forms of *T. copemani* in quokkas (Table 1), showed that trypanosomes from ticks were narrower ($P<0{\cdot}001$) and their PK values were greater ($P<0{\cdot}001$) than trypanosomes from quokkas. The length of the trypanosomes both from the tick faeces, and host bloodstream were similar, but a significant difference was noted in the length of the midgut trypanosomes which were shorter ($P<0{\cdot}001$). The length of the KN parameter was highly variable; however, differences between trypanosomes from each of the 3 sources were significant ($P<0{\cdot}001$). NA was only different between trypanosomes from the midgut and tick faeces ($P<0{\cdot}001$). Comparison of the free flagellar length revealed no significant differences ($P>0{\cdot}329$) between trypanosomes from the blood of the quokka and the faeces of the tick. The midgut trypanosomes did not possess a free flagellum.

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Table 1. Mean dimensions and standard error (S.E.) of morphological features of *Trypanosoma copemani* isolated from tick faeces from quokka 2322, tick midgut and bloodmeal from quokka 2917 and bloodstream trypanosomes from quokka blood

Parameter*	Source	No. of organisms	Observed range (μm)	Mean ± S.E. (μm)
L	tick faeces	5	27·0–44·1	35·5 ± 2·7
	tick midgut	14	21·5–39·8	28·2 ± 1·5
	quokka blood	12	31·2–44·3	37·8 ± 1·1
B	tick faeces	5	0·8–1·2	1·2 ± 0·2
	tick midgut	14	0·9–1·7	1·4 ± 0·1
	quokka blood	12	1·6–6·0	4·2 ± 0·3
PK	tick faeces	5	5·5–13·4	9·9 ± 1·3
	tick midgut	14	3·6–14·4	9·0 ± 0·6
	quokka blood	12	5·2–8·9	6·5 ± 0·3
KN	tick faeces	4	0·8–4·1	2·7 ± 0·8
	tick midgut	14	5·3–10·2	7·9 ± 0·3
	quokka blood	12	4·0–7·8	5·9 ± 0·2
NA	tick faeces	4	10·2–22·8	17·1 ± 4·0
	tick midgut	14	6·3–18·3	11·3 ± 0·9
	quokka blood	12	8·8–18·1	13·7 ± 0·7
FF	tick faeces	5	7·7–16·7	10·6 ± 1·7
	quokka blood	12	8·6–14·9	12·1 ± 0·5

* L, length of body measured along the mid-line including free flagellum (total length); B, maximum breadth measured at the level of the nucleus (including undulating membrane); PK, distance between the posterior end and the kinetoplast; KN, distance between the kinetoplast and posterior edge of the nucleus; NA, distance between the anterior edge of the nucleus and the anterior end of the body; FF, length of the free flagellum.

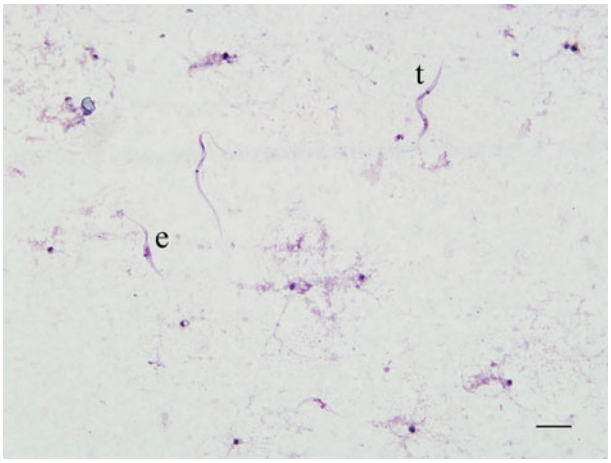


Fig. 2. Light photomicrographs of *Trypanosoma copemani* in Modified Wright's stain detected in the haemolymph of the tick collected from the Gilbert's potoroo F170. Trypomastigote form (t), epimastigote form (e). Scale bar represents 10 μm.

Characterization of Trypanosoma copemani *isolated from blood and ticks*

Two of the 3 quokkas (Q4489 and Q4539) from Bald Island were positive for trypanosomes through microscopical examination (Fig. 4) and amplification of the 18S rRNA gene. A tick from one of the same quokkas (Q4489) as well as a tick from another quokka from Bald Island (Q2322) and a tick isolate collected from a Gilbert's potoroo was also positive by PCR. The 18S rRNA sequences from the quokka blood (Q4489) and the Gilbert's potoroo tick isolate had 100% homology to each other and 100% homology to *T. copemani* (genotype A) and 99·9% homology to the *T. copemani* wombat AII isolate. The 18S rRNA sequences from the 2 Bald Island tick isolates were also confirmed as *T. copemani*. They had 100% homology to each other and just 1 bp difference compared to the trypanosome isolated from the quokka blood (Q4489) and the Gilbert's potoroo tick isolate. No amplification was detected from DNA extracted from the tick salivary glands and the faeces.

DISCUSSION

Ticks are obligate ectoparasites of vertebrates that are capable of transmitting pathogens (viruses, bacteria, rickettsiae, helminths and protozoa) of veterinary and medical importance (Roberts, 1970). In Australia, various tick species of the family Ixodidae are responsible for transmitting zoonotic diseases, such as tick typhus, and Lyme disease (Roberts, 1970). This is the first time ectoparasites from quokkas on Bald Island have been examined. *Ixodes myrmecobii* and *I. australiensis* have previously been found occurring in various regions of the South West of Western Australia and also within regions of Tasmania. To the best of our knowledge, *I. hirsti* has previously only been located in the eastern states of Australia in NSW, Victoria and Tasmania

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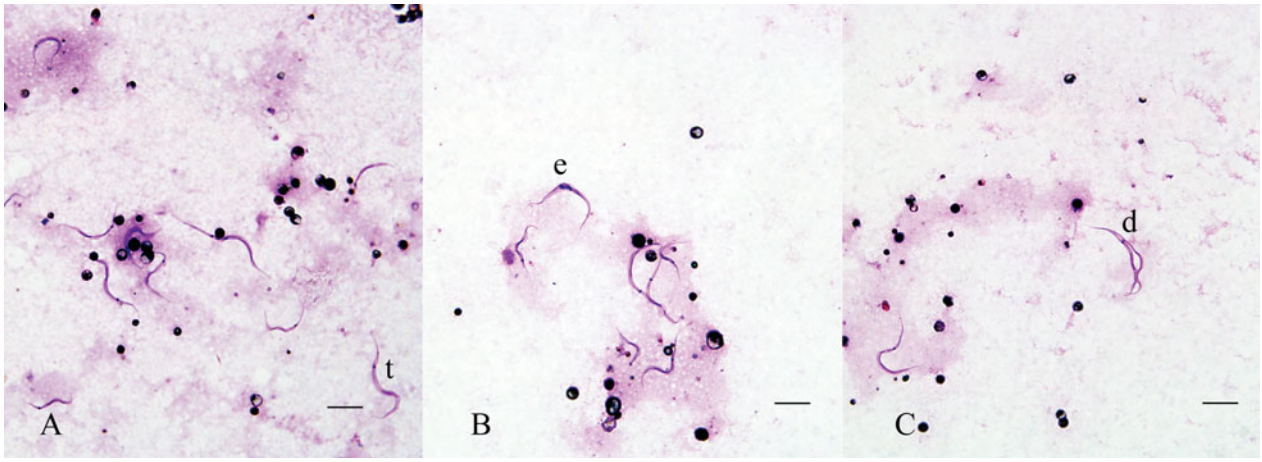


Fig. 3. Light photomicrographs of trypanosomes in Modified Wright's stain from the gut region and bloodmeal of a tick collected from quokka 2917. (A) Trypomastigote form. (B) Dividing epimastigote form (e). (C) Dividing trypomastigote form (d). Scale bar represents 10 μm.

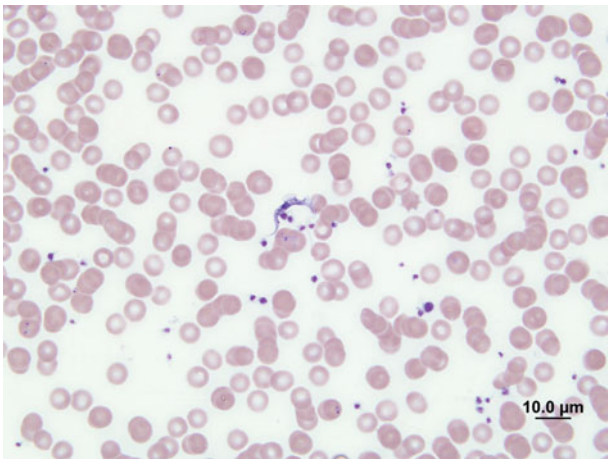


Fig. 4. Light photomicrographs of *Trypanosoma copemani* in Modified Wright's stain detected in the blood of quokka 4489 captured from Bald Island.

(Roberts, 1970). This is therefore the first report of *I. hirsti* in Western Australia.

The trypanosomes found in the midgut, haemolymph and faeces of the ticks had similar morphology (long and slender) to trypanosomes detected in tick nymphs (*Ixodes holocyclus*) removed from bandicoots (Mackerras, 1959). The long slender trypanosomes found in the faeces are likely to be the metacyclic form of *T. copemani,* as suggested by Austen *et al.* (2009). In that study, slender trypanosomes in *in vitro* cultures of blood from infected potoroos were identified and named as *T. copemani* (Austen *et al.* 2009). This parasite is now known to infect several species of marsupials including quokkas, Gilbert's potoroos, koalas and common wombats (Noyes *et al.* 1999; Austen *et al.* 2009; McInnes *et al.* 2009). Cultured trypanosomes from the blood of the potoroos were maintained at around 25 °C, which is suitable for the growth of the vector stages of trypanosomes (Vickerman, 1985). Morphological comparison of the trypanosomes within the vector and those from infected hosts shows that ticks harbour pleomorphic trypanosomes with some tick trypanosome life cycle stages found to represent the multiplication forms. The difference in PK and KN values could be due to kinetoplast migration which occurs when trypanosomes are in their multiplication and division stage (Hoare, 1972).

In the present study, motile trypanosomes were observed in the midgut of ticks dissected 49 and 117 days after removal of the ticks from the host. Motile trypanosomes were also detected in the haemolymph 117 days after removal. When xenodiagnosis is used for the detection of low levels of *T. cruzi* infection in humans or animals, first-instar *Triatoma* nymphs are allowed to feed on the suspect individual and after 30 days the bugs are examined for the presence of trypanosomes (Machado *et al.* 2001). It is unlikely that the trypanosomes observed in this study originated from the bloodmeal of the ticks examined because of the extended period between the last meal and detection of trypanosomes (49 days and 117 days). This is longer than the 30 days used in the xenodiagnostic test. It is interesting that live *T. cruzi* can not be detected in the small intestines of reduviid bugs at the fifth instar 60 days after the bug's last bloodmeal (Kollien and Schaub, 1998). The detection of motile trypanosomes at day 117 within the tick midgut, suggests that *T. copemani* may multiply in the tick. Indeed, the observation of dividing trypanosomes in stained smears from the gut and gut contents of the tick and the detection of slender trypanosomes in the tick haemolymph suggest that the ticks were systemically infected with *T. copemani*. This further supports the conclusion that *I. australiensis* is naturally infected and is the vector of *T. copemani*.

Trypanosomes have previously been detected in the haemolymph and gut of a variety of tick species (Mackerras, 1959; Shastri and Deshpande, 1981;

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El Kady, 1998; Latif *et al*. 2004; Thekisoe *et al*. 2007). However, there is insufficient information to fully determine the mode of natural trypanosome transmission via ticks. The detection of intact trypanosomes and trypanosome DNA in smears of dried tick faeces and the detection of DNA in the midgut but not from salivary glands suggest that transmission is likely to be contaminative via tick faeces. The faecal oral route of transmission that occurs with *T. lewisi* when rats eat infected fleas and/or their droppings containing metatrypanosomes (Hoare, 1972) could be a similar process of transmission that could be occurring between *T. copemani* and its marsupial host. Ticks located on quokkas are generally found around the ears and the tail while on the Gilbert's potoroo they are more widely distributed ranging from ears, tail, rim of pouch in females and around the scrotum in males as well as amongst the fur on the body which are body parts generally accessible to grooming. The stercorarian nature of *T. copemani*, as previously suggested by Austen *et al*. (2009) is consistent with a faecal route of trypanosome transmission. However, it is not possible to rule out the salivary route of transmission because only a limited number of salivary glands were analysed. Furthermore, there is evidence that similar trypanosomes such as *T. rangeli* are transmitted via the contaminative and inoculative routes (Guhl and Vallejo, 2003).

Sequence analysis of the amplified 18S rRNA gene from the DNA extracted from the gut showed that ticks on Bald Island and Two Peoples Bay are carrying a strain of *T. copemani* (genotype A) which has previously been isolated from a quokka and a Gilbert's potoroo and is similar to *T. copemani* isolates from wombats in Victoria, Australia (Noyes *et al*. 1999). The single base-pair change may have been due to an error introduced by the polymerase. The wide geographical distribution of *T. copemani* across Australia may be accounted for by the presence of *Ixodes* tick species, which have been found in most states of Australia. Ticks show varying degrees of host specificity (Roberts, 1970). *Ixodes* ticks are generally not host-specific. *Ixodes australiensis* has been recorded from numerous hosts including small marsupials, such as the bettongs, (*Bettongia lesueuri*, and *B. penicillata*), potoroos (*Potorous tridactylus* and *P. gilbertii*), the quokka (*Setonix brachyurus*), as well as kangaroos, dogs, humans and cows (Roberts, 1970; Austen *et al*. 2009). Leeches have been identified as vectors of trypanosomes in Australia (Hamilton *et al*. 2005) and several authors have suggested various ectoparasites as potential vectors for native Australian trypanosomes (fleas, biting flies, and ticks) (Noyes *et al*. 1999; Jakes *et al*. 2001).

Australian trypanosomes have been found in a wide range of marsupials from mainland Australia (Mackerras, 1959; Noyes *et al*. 1999; Jakes *et al*. 2001; Hamilton *et al*. 2005; Austen *et al*. 2009; McInnes *et al*. 2009) as well as offshore islands (Bettiol *et al*. 1998). A recent survey of the infection status of the quokkas on Rottnest Island off the coast of Western Australia revealed that these animals are not infected with *T. copemani* (J. Austen, unpublished data) whereas quokkas on Bald Island and at Two Peoples Bay are infected. Interestingly, no ticks or fleas were detected on the quokkas from Rottnest and the only ectoparasite found on quokkas caught on the island was a biting louse (*Heterodoxus quadriseriatus*). This is in contrast to the quokka populations at Two Peoples Bay and on Bald Island, which are usually infested with ticks (J.A. Friend, unpublished observation).

The pathogenicity of *T. copemani* in naïve animals is not known and therefore the identification of the vector for this parasite is important for elucidation of the complete life cycle of *T. copemani*. Knowledge of the life cycle will aid in management decision making when re-introduction of naïve animals occurs into areas where the parasite is known to be present in the same or other species of marsupials. This is particularly important when a population of animals from the same species are genetically and morphologically different, as occurs with quokkas on Rottnest in comparison to mainland and Bald island quokka populations (Sinclair, 1998, 2001).

ACKNOWLEDGEMENTS

We would like to thank Rottnest Island Authority who funded activities on Rottnest Is. and Shane Kearney the environmental coordinator from Rottnest Island. The technical assistance of Russ Hobbs, Aileen Elliot, Simon Fleetwood and Gordon Thomson is also acknowledged.

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https://doi.org/10.1017/S0031182011000497 Published online by Cambridge University Press