

Ghosts of Christmas past?: absence of trypanosomes in feral cats and black rats from Christmas Island and Western Australia

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SUMMARY

Trypanosomes and *Leishmania* are vector-borne parasites associated with high morbidity and mortality. *Trypanosoma lewisi*, putatively introduced with black rats and fleas, has been implicated in the extinction of two native rodents on Christmas Island (CI) and native trypanosomes are hypothesized to have caused decline in Australian marsupial populations on the mainland. This study investigated the distribution and prevalence of *Trypanosoma* spp. and *Leishmania* spp. in two introduced pests (cats and black rats) for three Australian locations. Molecular screening (PCR) on spleen tissue was performed on cats from CI ($n = 35$), Dirk Hartog Island (DHI; $n = 23$) and southwest Western Australia (swWA) ($n = 58$), and black rats from CI only ($n = 46$). Despite the continued presence of the intermediate and mechanical hosts of *T. lewisi*, there was no evidence of trypanosome or *Leishmania* infection in cats or rats from CI. Trypanosomes were not identified in cats from DHI or swWA. These findings suggest *T. lewisi* is no longer present on CI and endemic *Trypanosoma* spp. do not infect cats or rats in these locations.

Key words: *Trypanosoma*, *Leishmania*, Christmas Island, black rat, feral cat, Australia.

INTRODUCTION

Trypanosomes and *Leishmania* spp. are vector-borne parasites associated with severe disease in both animal and human hosts. The global distribution of *Trypanosoma* spp. and *Leishmania* spp. is heavily reliant on the presence of both reservoir hosts and competent vectors. Worldwide, cats (*Felis catus*) are reported to become infected by at least six *Trypanosoma* spp.; *T. brucei*, *T. congolense*, *T. gambiense*, *T. cruzi*, *T. evansi* and *T. rangeli* (Bowman *et al.* 2002). Likewise, 44 *Trypanosoma* spp. have been shown to infect rodents (Hoare, 1972; Milocco *et al.* 2013; Pumhom *et al.* 2015). *Leishmania* spp. are also zoonotic protozoan parasites (closely related both morphologically and genetically to *Trypanosoma*) that are usually transmitted by biting sand flies and are widely distributed through both tropical and temperate regions of the world. Leishmaniasis is also associated with high levels of morbidity and mortality (Svobodová *et al.* 2003; Gramiccia and Gradoni, 2005; Peacock, 2010). Over 40 mammal species are known to harbour *Leishmania* spp., including cats and rats, with the black, or 'ship' rat (*Rattus rattus*), increasingly recognized as an important natural reservoir in *Leishmania* transmission

(Oliveira *et al.* 2005; Quinnell and Courtenay, 2009; Sherry *et al.* 2011). In Australia to date, eight novel *Trypanosoma* spp. have been identified in native wildlife (Paparini *et al.* 2011; Thompson *et al.* 2013, 2014), and leishmaniasis has been reported in macropods in the Northern Territory (Rose *et al.* 2004) and is thought to be transmitted by biting midges (Dougall *et al.* 2011). It is not known whether these endemic parasites can infect feline or rodent hosts.

On Christmas Island (CI), the extinction of two native rat species has been attributed to infection with *Trypanosoma lewisi*, thought to have been introduced with black rats during incursions by sea-faring traders during the late 19th century (Durham, 1908; Andrews, 1909). The observations of these early researchers appears to have been supported recently by Wyatt *et al.* (2008) who detected *T. lewisi* DNA in skin samples from two out of six (33.3%) museum specimens of the now-extinct Maclear's rats (*R. macleari*), and one out of six (16.7%) black rats collected by Durham at the time of European colonization of the island (Wyatt *et al.* 2008). More recently, speculation that trypanosomes were still present on CI was based on the observational finding of *Trypanosoma/Leishmania*-like organisms in a blood smear of a rat on the island (Hall *et al.* 2011). Additionally, *Trypanosoma* spp. infections have been implicated in the precipitous decline of the woylie (*Bettongia penicillata*) on mainland Western Australia (Smith *et al.* 2008; Averis

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et al. 2009), although the distribution and prevalence of trypanosome and *Leishmania* species in Western Australia is not well described.

As part of a larger research study into the effects of introduced species on wildlife in Australia and its islands, tissue samples from feral cats and black rats living on CI, Dirk Hartog Island (DHI) and in southwest Western Australia (swWA) were examined for the presence of *Trypanosoma* and *Leishmania* DNA. These locations represent areas of importance for wildlife conservation as well as for public health.

METHODS

Study locations

Samples were collected from three geographically and climatically distinct locations; from CI, mainland swWA, and DHI. CI is an Australian Territory, located in the Indian Ocean (10°29'S, 105°38'E) approximately 360 km south of the Indonesian capital, Jakarta, with a tropical climate. The swWA is a large ecoregion, located south of a line from Geraldton (28°46'28"S, 114°36'32"E) to Esperance (33°51'40"S, 121°33'31"E) with a Mediterranean climate. DHI is an arid inshore island (25°50'S, 113°05'E) located to the west of Shark Bay off the Western Australian coast.

Sample collection

Cat cadavers were collected from CI ($n = 35$; 8 fresh and 27 frozen), DHI ($n = 23$; all frozen) and swWA ($n = 58$; all frozen). Cats from CI and DHI were sourced from the Department of Parks and Wildlife (Parks and Wildlife) management programmes and cats from swWA were obtained during community-coordinated culling programs from 12 locations. Rats ($n = 48$; 23 fresh and 25 frozen) were collected from CI concurrently with the cats. Spleen samples were collected at necropsy and preserved in 70% ethanol.

DNA extraction

DNA was extracted from cat and rat spleen tissue using the Qiagen spin columns for blood and tissue kit according to the manufacturer's instructions (Qiagen, USA). Negative controls were used in the PCRs with the inclusion of PCR grade water in place of genomic DNA.

PCR conditions – Trypanosoma

The nested PCR protocol from Botero *et al.* (2013) was employed with generic *Trypanosoma* primers of the 18S region which have been previously described (Maslov *et al.* 1996; McInnes *et al.* 2011). External primers used were SLF (5'-GCT

TGT TTC AAG GAC TTA GC-3') and S762 (5'-GAC TTT TGC TTC CTC TAA TG-3') and internal primers were S823F (5'-CGA ACA ACT GCC CTA TAC GC-3') and S662R (5'-GAC TAC AAT GGT CTC TAA TC-3'). Cultured *Trypanosoma cruzi* and *T. lewisi* were used as positive controls.

PCR conditions – Leishmania

Subsamples of spleens were tested for *Leishmania* spp. from CI samples only, feral cats ($n = 10$) and black rats ($n = 45$), with genus-specific primers adapted from Schönian *et al.* (2003). Primers were from the internal transcriber region (ITS1), OL1853 (5'-CTG GAT CAT TTT CCG ATG-3') and OL1854 (5'-TGA TAC CAC TTA TCG CAC TT-3').

A touchdown PCR was performed on all samples using 3 μL of DNA (at 5 ng μL^{-1}) in an 11.5 μL reaction. The reaction contained 1 \times buffer, 3.0 mM MgCl₂, 0.5 mM dNTPs, 0.05 M Betaine, 0.05 μL Taq/Taq Gold and 10 μM of each primer. PCR cycling conditions were optimized under the following conditions: 1 denaturation cycle at 94 °C for 5 min (Taq)/10 min (Taq Gold) followed by 94 °C for 20 s, 63–56 °C for 60 s using 0.5 °C cycle⁻¹ increments and 72 °C for 60 s. This was then followed by 20 cycles of 94 °C at 20 s, 56 °C for 60 s, 72 °C for 60 s and a final extension of 72 °C for 5 min (Blackwell Laboratory, Australia). Positive controls included DNA extracted from *Leishmania major*, *L. braziliensis*, *L. tropica*, *L. donovani* and *L. australiensis*. All PCR products were run on a 1.5% agarose gel at 120 V for 1 h for visualization.

Statistical analysis

Confidence interval values were calculated using the exact binomial methods (Graat *et al.* 1997).

RESULTS

No *Trypanosoma* spp. were detected by PCR in any of the spleen samples (Table 1). Positive controls for *T. cruzi* and *T. lewisi* amplified at the correct product size and no amplification was detected within the negative control. Similarly, no *Leishmania* DNA was detected in either cat or rat spleen samples (Table 1). All *Leishmania*-positive controls produced amplified products at their corresponding sizes, whilst all negative controls did not produce amplification.

DISCUSSION

This study was conceived when the opportunity arose to sample cats and rats on CI after recent observations on pathology specimens suggested

Table 1. Prevalence (%) and 95% confidence interval for *Trypanosoma* and *Leishmania* in cats and rats from three geographical regions.

	swWA	DHI	CI	
	<i>F. catus</i>	<i>F. catus</i>	<i>F. catus</i>	<i>R. rattus</i>
<i>Trypanosoma</i> spp.				
Samples (<i>n</i>)	58	23	35	48
Positive samples (<i>n</i>)	0	0	0	0
Prevalence 95% confidence interval (%)	0.0, 6.2	0.0, 14.8	0.0, 10.0	0.0, 7.4
<i>Leishmania</i> spp.				
Samples (<i>n</i>)	–	–	10	43
Positive samples (<i>n</i>)	–	–	0	0
Prevalence 95% confidence interval (%)	–	–	0.0, 30.8	0.0, 8.3

swWA, southwest Western Australia; DHI, Dirk Hartog Island; CI, Christmas Island.

that *Trypanosoma* spp. may persist in this confined habitat where historical records had putatively incriminated trypanosomiasis for the extinction of two native rodents. We were also interested to investigate whether *Trypanosoma* DNA could be detected in the blood of introduced animals in regions where trypanosomiasis appears to be endemic in native marsupials upon which they undoubtedly prey. However, we found no evidence of infection with *Trypanosoma* spp. in feral cats from three geographical locations or black rats on CI. Additionally, there was also no evidence of infection by *Leishmania* spp. in feral cats or black rats on CI. These results suggest that cats and rats are not currently acting as reservoirs for trypanosomes or *Leishmania* in these locations, and questions whether *Trypanosoma* infection persists in any form on CI.

The challenge presenting molecular research when pathogen DNA is not detected in any samples is to justify that target DNA was not missed due to inhibitors and/or suitability of the tissue for that particular work. We are confident that the samples used in this study yield viable DNA as these same samples were screened successfully for the presence of other blood-borne pathogens by molecular methods (e.g. *Bartonella* species; Dybing *et al.* (2016)), thus ruling out the presence of inhibitors.

Regarding the suitability of tissues used, the spleen is regarded as a reliable location for detecting numerous different blood-borne pathogens, including *Trypanosoma*, for reasons including; the spleen is the blood filtering organ so pathogens circulating in the blood would be expected to be present (Mebius and Kraal, 2005); sampling from the spleen was more sensitive than blood for detecting vector and blood-borne pathogens including trypanosome and ehrlichial infections (Albright and Albright, 1991; Harrus *et al.* 2004), and; the primers used in this study have been previously demonstrated/proven to detect trypanosomes in both blood and tissue samples (McInnes *et al.* 2011; Botero *et al.* 2013). The nested *Trypanosoma* PCR primers used in this

study provide a highly sensitive methodology for detecting low DNA concentrations in tissues (McClatchey, 2002). Very low levels of DNA are highly likely to amplify when using nested PCR primers, compared to conventional single step PCR, as two primer pairs are required to amplify a target sequence and there is a decrease in non-specific banding (McClatchey, 2002; Pardo and Pérez-Villareal, 2004). Although the sensitivity of these primers has not been calculated for tissue, it has been calculated for blood (Dunlop *et al.* 2014). Given that Dunlop *et al.* (2014) calculated that use of these primers and amplification conditions in blood with >74 parasites/0.3 mL had a detection sensitivity of >80%, it is extremely likely that even low levels of parasitaemia would be detected in spleen samples.

Whilst the possibility of missing the presence of either *Trypanosoma* or *Leishmania* occurring at very low levels of parasitaemia cannot be discounted, we do feel confident that the PCR protocol used would detect even low levels of parasitaemia in our samples. For the scope of this paper though, quantification of parasitaemia (intensity) was not considered as important as the presence/absence of *Trypanosoma* or *Leishmania* species.

Unfortunately serum samples were not available for rats or cats in this study; serological testing may have provided additional information regarding exposure (or otherwise) of the hosts to these parasites and should be considered for future investigations.

Whilst cat and rat spleen samples were sourced from a focal area on CI, specifically from around the town site, this was representative of the initial sampling area utilized by Durham (1908). Additionally, the relatively small size of this island (135 km²) suggests that *Trypanosoma* and *Leishmania* would have expected to have been identified in our samples if they were circulating within the rat and cat populations. Assuming a prevalence of at least 10%, the probability of missing a positive animal is 0.006 (0.6%) based on the sample size of 48 rats used in this study.

The only samples tested by Wyatt *et al.* (2008) from before black rat introductions to CI were

from the bulldog rat (*R. nativitatis*; $n = 3$), with PCR failing to detect any trypanosomes. Therefore it is not possible to confirm the presence or absence of *T. lewisi* on CI prior to black rat introduction (Wyatt *et al.* 2008). However, if *T. lewisi* was endemic on CI in the native Maclear's and bulldog rats before black rat introduction, then it could be hypothesized that it was similarly lost along with the demise of the native rats.

Based on the findings of this study, the link between black rats as the source of the *Trypanosoma* spp. on CI is tenuous considering the hypothesized host (black rats), intermediate host (*Xenopsylla cheopis*; Hoare (1972)) and a mechanical vector (*Polyplax spinulosa*; Khachorian and Arakelian (1978)) are still present on the island (unpublished results), whilst our efforts to detect the presence of any *Trypanosoma* spp. was not successful. If *T. lewisi* introduced with black rats has resulted in, or at least contributed to, the extinction of two endemic rodent species, then it would not be unreasonable to expect *T. lewisi* to still be present in these hosts today.

Whilst rodent (rat and mice) trypanosomes have been studied, there is still a paucity of information in Australia. Exotic trypanosomes found in native rodents include *T. lewisi* from the bush rat (25%); a species genetically similar to *T. lewisi* in the ash-grey mouse (50%); and an unknown *Trypanosoma* spp. in the Shark Bay mouse (16.7%) (Averis *et al.* 2009). Introduced rodents infected with *T. lewisi* in Australia include the house mouse, brown rat and the black rat (Mackerras, 1959). Several novel *Trypanosoma* spp. have been identified in a range of Australian native wildlife species (Smith *et al.* 2008; Austen *et al.* 2009; Averis *et al.* 2009; Papparini *et al.* 2011). Indeed, *Trypanosoma* spp. have been implicated in the demise of marsupial populations in swWA (Smith *et al.* 2008; Averis *et al.* 2009).

In Australia, there is an absence of research into trypanosomes from cats however, natural and experimental infections of multiple species of *Trypanosoma* have been found in felines from other countries including; *T. brucei*, *T. cruzi*, *T. evansi* and *T. rangeli*. Experimental infections in cats have also been reported for *T. congolense* and *T. gambiense*. *Trypanosoma cruzi* is the most widely reported species occurring in cats with most cases originating from Latin America and prevalences ranging from 2.9 to 63.6% (Zeledón *et al.* 1975; Mott *et al.* 1978; Wisnivesky-Colli *et al.* 1985). However, many indigenous Australian mammal species are thought to be susceptible to infection by exotic *Trypanosoma* species if they were to establish in Australia (Thompson, 2013). The absence of trypanosome infection in cats sampled from swWA and DHI suggests that cats are not a major reservoir species in these areas and therefore may

not contribute to trypanosome persistence in the environment.

Domestic cats and black rats have been identified as sources of infection of the pathogenic zoonosis caused by *Leishmania infantum* (Poli *et al.* 2002; Quinnell and Courtenay, 2009; Maia and Campino, 2011). At the time of this study a significant proportion of the CI population was transitory and originated from diverse geographic locations, including many areas where *Leishmania* is endemic. However, we found no evidence of feral cats or black rats acting as reservoirs for *Leishmania* spp. on CI.

CONCLUSIONS

This study found no evidence of *Trypanosoma* spp. infection in cats and black rats from three geographically distinct locations. Of particular interest is the absence of trypanosomes from black rats on CI given that *T. lewisi* has previously been reported as occurring in rats and other rodent hosts on the island, and has been hypothesized as contributing to the extinction of two endemic rodent species. Previous studies have implicated *Trypanosoma* spp. in fauna decline; however, in the case of CI, the findings of this study do not readily support this hypothesis. This study also found no evidence of feral cats or black rats harbouring *Leishmania* spp. on CI.

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CONFLICT OF INTEREST

None.

ETHICAL STANDARDS

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and the use of laboratory animals.

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