Molecular surveillance of piroplasms in ticks from small and medium-sized urban and peri-urban mammals in Australia

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ABSTRACT

Natural landscape alterations as a consequence of urbanisation are one of the main drivers in the movements of wildlife into metropolitan and peri-urban areas. Worldwide, these wildlife species are highly adaptable and may be responsible for the transmission of tick-borne pathogens including piroplasms (Babesia, Theileria and Cytauxzoon spp.) that cause piroplasmosis in animals and occasionally in humans. Little is known about piroplasms in the ticks of urban wildlife in Australia. Ticks from long-nosed bandicoots (Perameles nasuta; n = 71), eastern-barred bandicoots (Perameles rufescens; n = 41), northern-brown bandicoots (Isoodon macrourus; n = 19), southern-brown bandicoots (Isoodon obesulus; n = 4), bandicoot sp. (n = 2), flying foxes (Pteropus sp.; n = 3), black rats (Rattus rattus; n = 7), bush rats (Rattus fuscipes; n = 4), brushtail possums (Trichosurus vulpecula; n = 19), ringtail possums (Pseudocheirus peregrinus; n = 12), short-eared possums (Trichosurus caninus; n = 6), possum sp. (Trichosurus sp.; n = 8), and red foxes (Vulpes vulpes; n = 12) were analysed using piroplasm-specific 18S primers and Sanger sequencing. Seven Ixodes tasmani ticks from long-nosed bandicoots and bandicoot sp., three I. tasmani ticks and one Ixodes holocyclus tick from brushtail possums, and one Haemaphysalis longicornis tick from a red fox were positive for piroplasms. New genotypes, with sequences sharing 98% nucleotide similarities with Theileria orientalis were identified in marsupials. Theileria orientalis was identified in the I. longicornis tick from the red fox. Babesia and Theileria spp. in the ticks parasitizing bandicoots and brushtail possums clustered closely with respective Babesia and Theileria clades derived from Australian marsupials. This represents the first detection of piroplasms in ticks parasitizing brushtail possums and a red fox in Australia.

1. Introduction

Deforestation, habitat fragmentation, and increases in human populations associated with urbanisation inevitably decrease the natural flora and fauna biodiversity (Mackenstedt et al., 2015). While some wildlife species remain urbanophobes, some have emerged as urban adapters or exploiters, and over the years have become familiar inhabitants of our towns and cities. There are various factors that favour the urban-adaptation of certain wildlife species, including the availability of anthropogenic food resources (Oro et al., 2013), shelter (Parris and Hazell, 2005), and reduction in threats from natural predators (Bateman and Fleming, 2012). Constant supplies of season-independent resources enable many successful urban-adapted wildlife species to attain higher population densities compared to their rural counterparts (Bradley and Altizer, 2007). Consequently, the growth in populations of competent hosts, reservoirs and amplifiers of vector-borne pathogens, increases the prevalence of those pathogens and the frequency of human-wildlife interactions, potentially leading to higher rates of zoonotic disease transmission (Bradley and Altizer, 2007).

Although many mammal populations have declined as a consequence of landscape alterations (Baker and Harris, 2007), others have benefited, especially from the creation of urban environments. In Australia, the most common mammals in urban areas include native brushtail possums (Trichosurus vulpecula), ringtail possums (Pseudocheirus peregrinus) (Hill et al., 2007), and bandicoots (Isoodon and Perameles spp.) (Fitzgibbon et al., 2011); as well as introduced wildlife such as European red foxes (Vulpes vulpes) (Marks and Bloomfield, 1999). In addition, bats (Pteropus sp.) and rats (Rattus spp.) also reside

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in Australian metropolitan areas (Plowright et al., 2011; Tait et al., 2014; Banks and Smith, 2015). Individuals of these species make use of urban areas either exclusively or by nesting in remnant bushland and regularly visiting nearby urban habitats (Harper, 2005).

Ticks are one of the most competent arthropod vectors that transmit a vast number of pathogenic bacteria, haemoparasites and viruses that significantly affect the health of human, livestock and companion animals worldwide (Dantas-Torres et al., 2012). Ticks are responsible for a variety of emerging zoonotic diseases and wildlife are often the amplifying hosts for these pathogens (Colwell et al., 2011). The encroachment of wild animals into urban areas as a result of urban development, brings wildlife and their ticks into close proximity with humans, consequently introducing possible spill-over of tick-borne pathogens (Banks and Smith, 2015).

A range of haemoparasites, i.e. piroplasms, have been identified and described in Australian native wildlife (Paparini et al., 2012), and in addition, unnamed species of piroplasm have been detected in brush-tailed bettongs ( Bettongia penicillata), burrowing bettongs ( Bettongia lesueur), and bandicoots (Paparini et al., 2012; Barbosa et al., 2017). In general, however, knowledge about the piroplasms in Australian wildlife ticks remains scarce and their zoonotic significance is far from being understood at the current time. There have been increasing reports of tick-associated illnesses in Australian humans, including a case of babesiosis reported in an Australian patient who lived in a peri-urban area and had encountered tick bites, but who had not travelled to endemic countries (Senayake et al., 2012). This emphasises the importance of understanding and examining every aspect of tick-borne disease (TBD), particularly the potential for wildlife ticks to act as reservoirs for human disease.

Urbanised wildlife contribute to the importance and health impact of TBD worldwide, both as transporters of ticks, as well as acting as reservoirs of TBD pathogens (Bradley and Altizer, 2007; Pfaffle et al., 2013; Rizzoli et al., 2014). The occurrence of wildlife in urban and peri-urban settings is becoming more evident (Harper, 2005), increasing the chances of human-wildlife interactions and the potential for human illness via tick bites. Hence, further research into TBDs in wildlife that reside in urban areas together with their ticks is essential. The objective of the present study was to conduct a survey of ticks parasitizing small and medium-sized mammalian wildlife species that are most common in urban environments in Australia, and to identify and characterise any piroplasms present.

2. Materials and methods

2.1. Ethics statements

This study was conducted under the compliance of the Australian Code for the Responsibility Conduct of Research (2007) and Australian Code for the Care and Use of Animals for Scientific Purposes, 2013. Tick collection was carried out opportunistically with the approval from the Murdoch University Animal Ethics Committee.

2.2. Sample collection and tick identification

Ticks from 18 long-nosed bandicoots (P. nasuta; n = 71), two eastern-barred bandicoots ( P. gunnii; n = 41), five northern-brown bandicoots (I. macrourus; n = 19), four southern-brown bandicoots (I. obesulus; n = 4), two bandicoot sp. (n = 2), three bat sp. ( Pteropus sp.; n = 3), three black rats ( R. rattus; n = 7), four bush rats ( R. fuscipes; n = 4), seven brushtail possums ( T. vulpecula; n = 19), six ringtail possums ( P. peregrinus; n = 12), two short-eared possums (T. caninus; n = 6), two possum sp. ( Trichosurus sp.; n = 8), and one red fox (V. vulpes; n = 12) were sampled by veterinarians, wildlife rescuers, and the public from urban and peri-urban areas across Australia. Ticks were preserved in 70% ethanol before shipment to Murdoch University and the species was subsequently identified using morphological keys

(II. t. tasmani, I. tasmani, I. holocyclus, and I. tasmani sp. B60). Of the 137 ticks collected from bandicoots, seven ticks (Table 1) were positive for both short and long piroplasm 18S fragments. Of these, five were from I. tasmani nymphs from two long-nosed bandicoots, and a single I. tasmani female removed from a bandicoot sp., at the Australia Zoo Wildlife Hospital in Queensland (QLD); and a single female I. tasmani from a bandicoot sp. from a veterinary clinic in Port Sorell, Tasmania (TAS). Using the shorter 18S gene primers, amplicons were generated in four brushtail possum ticks; comprising one I. tasmani female tick from the Royal Botanical Gardens in New South Wales (NSW), and one I. holocyclus and two I. tasmani ticks from two brush-tailed possums at the Australia Zoo Wildlife Hospital, QLD. Among the 12 tick samples from a red fox from Grosevale, NSW, only a single H. longicornis nymph was positive.

Sequence analysis identified three unique genotypes in the bandicoot ticks, designated as Theliera sp. B16, Theliera sp. B43, and Theliera sp. B60. Theliera sp. B16 was identified in the I. tasmani tick from TAS, and shared 97.1% and 99.9% similarity with Theliera sp. B43 and Theliera sp. B60, respectively (Supplementary Table S1). Genotype
Table 1
Ticks identified in this study and number of positive samples for piroplasm 18S. Parentheses indicate percentage positive.

| Hosts                                      | Tick species          | Region               | Instar | n   | No. of positives |
|--------------------------------------------|-----------------------|----------------------|--------|-----|-----------------|
| Long-nosed bandicoot Perameles nasuta      | *Ixodes holocyclus*   | Sydney, NSW          | Nymph  | 8   | –               |
|                                            |                       | Castlecrag, NSW      | Nymph  | 1   | –               |
|                                            |                       | Female               | 2      | 2   | –               |
|                                            |                       | Male                 | 3      | 2   | –               |
|                                            |                       | Boorie Creek, NSW    | Female | 10  | –               |
|                                            |                       | Male                 | 14     | 2   | –               |
|                                            |                       | Bodhi, NSW           | Female | 1   | –               |
|                                            |                       | Manly, NSW           | Nymph  | 4   | 5               |
|                                            |                       | Murrah, NSW          | Nymph  | 2   | –               |
|                                            |                       | Beerwah, QLD         | Nymph  | 5   | –               |
|                                            |                       | Female               | 1      | 1   | –               |
|                                            |                       | Male                 | 3      | 1   | –               |
|                                            |                       | Beerwah, QLD         | Nymph  | 1   | –               |
|                                            |                       | Female               | 5      | 1   | –               |
|                                            |                       | Male                 | 3      | 1   | –               |
|                                            | *Ixodes tasmani*      | Sydney, NSW          | Nymph  | 5   | –               |
|                                            |                       | Beerwah, QLD         | Nymph  | 11  | 5               |
|                                            |                       | Stony Chute, NSW     | Nymph  | 1   | –               |
|                                            |                       | Female               | 1      | 1   | –               |
|                                            |                       | Male                 | 3      | 1   | –               |
|                                            | *Haemaphysalis bancroft* | Castlecrag, NSW  | Nymph  | 1   | –               |
|                                            |                       | Stony Chute, NSW     | Nymph  | 1   | –               |
|                                            |                       | Female               | 1      | 1   | –               |
|                                            |                       | Murrah, NSW          | Nymph  | 1   | –               |
|                                            | *Haemaphysalis humerosa* | Stony Chute, NSW  | Nymph  | 1   | –               |
|                                            |                       | Eastern-barred bandicoot Perameles gunnil | *Ixodes tasmani* | Devonport, TAS | Nymph  | 20  | – |
|                                            |                       | Female               | 19     | –   | –               |
|                                            |                       | Ridgeway, TAS        | Nymph  | 2   | –               |
|                                            |                       | Northern-brown bandicoot Isoodon macrourus | *Ixodes holocyclus* | Beerwah, QLD | Female | 3   | – |
|                                            |                       | Palmerston, NT       | Nymph  | 1   | –               |
|                                            |                       | Female               | 2      | 2   | –               |
|                                            |                       | Male                 | 3      | 3   | –               |
|                                            |                       | Bees Creek, NT       | Nymph  | 2   | –               |
|                                            |                       | Female               | 7      | 7   | –               |
|                                            | *Haemaphysalis humerosa* | Beerwah, QLD | Female | 1   | –               |
|                                            |                       | Male                 | 3      | 3   | –               |
|                                            | Southern-brown bandicoot Isoodon obesusus | *Ixodes falcatus* | Albany, WA | Female | 2   | – |
|                                            |                       | Maida Vale, WA       | Female | 1   | –               |
|                                            |                       | Albany, WA           | Female | 1   | –               |
|                                            | *Ixodes australiensis* | Port Sorel, TAS     | Female | 1   | 1               |
|                                            |                       | Beerwah, QLD         | Female | 1   | 1               |
| Bandicoot sp.                              | *Ixodes tasmani*      | Port Sorel, TAS      | Female | 1   | 1               |
|                                            |                       | Beerwah, QLD         | Female | 1   | 1               |
| Total bandicoot ticks                      | 137                   | 7 (5.1%)             |        |     |                 |
| Bat sp.                                    | *Rhipicephalus sanguineus* | Darwin, NT        | Female | 1   | –               |
|                                            | Flying fox *Pteropus sp.* | *Ixodes holocyclus* | Beerwah, QLD | Female | 1   | – |
|                                            | Black flying fox *Pteropus alecto* | *Ixodes tasmani* | Beerwah, QLD | Female | 1   | – |
| Total bat ticks                            | 3                     | 0                    |        |     |                 |
| Black rat                                  | *Ixodes holocyclus*   | Castlecrag, NSW      | Nymph  | 1   | –               |
|                                            |                       | Sydney, NSW          | Nymph  | 1   | –               |
|                                            | *Ixodes tasmani*      | Sydney, NSW          | Nymph  | 4   | –               |
| Bush rat                                   | *Ixodes tasmani*      | Pearl Beach, NSW     | Nymph  | 4   | –               |
| Total rat ticks                            | 11                    | 0                    |        |     |                 |
| Brushtail possum Trichosurus vulpecula     | *Ixodes holocyclus*   | Beerwah, QLD         | Female | 7   | 1               |
|                                            |                       | Male                 | 1      | 1   | –               |
|                                            | *Ixodes tasmani*      | Royal Botanic Gardens, NSW | Female | 1   | 1               |
|                                            |                       | Beerwah, QLD         | Female | 7   | 2               |
|                                            | *Ixodes trichosuri*   | Beerwah, QLD         | Female | 3   | 3               |
| Ringtail possum Pseudechirus peregrinus    | *Ixodes holocyclus*   | Beerwah, QLD         | Female | 1   | –               |
|                                            |                       | Neutral Bay, NSW     | Female | 1   | –               |
|                                            |                       | Turramurra, NSW      | Female | 6   | –               |
|                                            |                       | Beerwah, QLD         | Female | 3   | 3               |

(continued on next page)
Table 1 (continued)

| Hosts          | Tick species          | Region            | Instar | n   | No. of positives |
|----------------|-----------------------|-------------------|--------|-----|------------------|
|                |                       |                   |        |     |                  |
| Short-eared possum | *Ixodes holocyclus*     | Beerwah, QLD     | Female | 4   |                 |
| *Trichosurus caninus* | *Ixodes holocyclus*     | Beerwah, QLD     | Male   | 1   |                 |
|                | *Ixodes holocyclus*     | Beerwah, QLD     | Female | 1   |                 |
| Possum sp.    | *Haemaphysalis brevleri* | Beerwah, QLD     | Nymph  | 1   |                 |
|                | *Haemaphysalis humerosa* | Beerwah, QLD     | Female | 4   |                 |
|                |                       |                   | Female | 2   |                 |
| Total possum ticks |                       |                   |        | 45  | 4 (8.9%)         |
| Red fox       | *Ixodes holocyclus*     | Grosevale, NSW   | Female | 1   |                 |
| *Vulpes vulpes* |                       |                   |        |     |                  |
|                | *Haemaphysalis longicornis* | Grosevale, NSW   | Nymph  | 3   | 1                |
|                |                       |                   | Female | 8   |                 |
| Total fox ticks |                       |                   |        | 12  | 1 (8.3%)         |
| Grand total   |                       |                   |        | 205 | 12 (5.9%)        |

Theileria sp. B60 consisted of five identical sequences and shared 97.2% similarity with *Theileria* sp. B43, both of which were sourced from QLD. NCBI BLAST analyses revealed that *Theileria* sp. B16 (1371 bp), *Theileria* sp. B43 (1368 bp), and *Theileria* sp. B60 (1371 bp), shared 98% sequence homology with *Theileria* sp. K1 (JQ682879) detected in a burrowing bettong (*Bettongia lesueurii*) (Paparini et al., 2012). Of the four sequences detected in brush-tailed possum ticks, all three sequences from QLD were identical, designated as *Babesia* sp. BP7, whereas the single sequence from NSW, designated as *Babesia* sp. BP1, shared 98% similarity with the QLD genotype (Supplementary Table S1). According to NCBI BLAST, both *Babesia* sp. BP1 (1313 bp) and *Babesia* sp. BP7 (1312 bp) showed 98% similarity with *Babesia* sp. detected in marsupials (JQ682877) (Paparini et al., 2012). The sequence (1349 bp) in the *H. longicornis* tick from a red fox was identical to *T. orientalis* (XR_696404) (Hayashida et al., 2012).

The Bayesian phylogenetic tree constructed using a multiple nucleotide alignment of 1701 bases (Fig. 1) revealed *Babesia* sp. BP1 and *Babesia* sp. BP7 were group in a clade with other *Babesia* spp. previously identified in marsupials. *Theileria* sp. B16, *Theileria* sp. B43, and *Theileria* sp. B60 from the bandicoot ticks grouped closely with the marsupial *Theileria* clade along with the *Theileria* sp. derived from *I. australensis* ticks from kangaroos and the *Theileria* sp. from burrowing bettongs with a high posterior probability value (Fig. 1). Likewise, the *Theileria* sequence derived from the *H. longicornis* tick from the red fox clustered closely with the *T. orientalis*, *T. buffeli*, and *T. sergenti* clade with a high posterior probability value of 1.0.

A shorter nucleotide alignment (856 bp) was used to construct inset trees in order to incorporate species (*Babesia macropus*, *Theileria fulginosus*, *Theileria brachyurii*, and *Theileria penicillata*) for which longer sequences were not available (Fig. 1a and b). In the case of the marsupial *Babesia* group, the inset tree generated from the shorter alignment exhibited a polytomous topology (i.e. the relationships could not be fully resolved to dichotomies). However, the *Babesia* sequences from the brushtail possum ticks remained clearly distinct from *B. macropus* and other *Babesia* spp. (Fig. 1a). In this analysis, *B. macropus* (KM206778; KM206780; KM206783) (Donahoe et al., 2015) showed 94.6%–94.8% and 92.8%–92.9% similarity with *Babesia* sp. BP1 and *Babesia* sp. BP7, respectively (Supplementary Table S2). Similarly, *Theileria* sp. B16, *Theileria* sp. B43, and *Theileria* sp. B60 shared over 95% similarities with *T. brachyurii* (95.4%, 95.1%, and 95.4%, respectively) and *T. penicillata* (97.6%, 95.8%, and 97.6%, respectively); and lowest similarities with *T. fulginosus* (90.8%, 89.7%, and 90.8%, respectively) (Supplementary Table S2). Consistent with the larger phylogenetic tree topology, the *Theileria* spp. identified in the present study branched out to form a subgroup with a strong posterior probability (0.99), and with *T. penicillata* and *Theileria* sp. from the burrowing bettong as a sister subgroup (Fig. 1b).

4. Discussion

The present study provides the first molecular survey and characterisation of the genera *Babesia* and *Theileria* in ticks from Australian brush-tailed possums and bandicoots, respectively. Additionally, to the best of our knowledge, this is also the first report of the presence of *T. orientalis* in a *H. longicornis* tick parasitizing a red fox in Australia. To date, very few studies have explored the presence of piroplasms in Australian wildlife ticks, therefore the current findings contribute further to our understanding of the capacity of wildlife ticks to harbour microorganisms in Australia.

Although the classification of Piroplasmida is yet to be fully resolved, this order is currently divided into several clades on the basis of concatenated mitochondrial and 18S sequences. Five distinct clades have been established, comprising *Babesia* sensu stricto., *Theileria* and *Cytauxzoon*, *Theileria equi*, the Western *Babesia* group, and the *Babesia microti* group (Schreg et al., 2016). Previous analyses have also identified a novel marsupial piroplasm clade (Paparini et al., 2012). Results from the present study have further confirmed that the piroplasm species derived from marsupial ticks are closely related and able to form their own respective clades, thus, increasing the sample size in future studies may further support the hypothesis that marsupial piroplasms are unique from other well described Piroplasmida groups.

In Australia, haemoproteozoon infections are frequently identified in possums (Hill et al., 2008; Paparini et al., 2011; Barbossa et al., 2017); however, to the best of our knowledge, piroplasms have not been detected in possum ticks. This study represents the first detection of a *Babesia* sp. from a tick from a brushtail possum at the Royal Botanical Gardens. This landmark in Sydney is a popular tourist attraction with an estimated 3 million visitors per annum (Paustasso and Parmentier, 2007). As a consequence, there is potential for humans to interact with these possums, and indirectly with their ticks. Unlike their rural counterparts, urban possums have been reported to host more than three ectoparasites species, and can have significant tick infestations (Webster et al., 2014; Hillman et al., 2017). In these environments, possums frequently inhabit food spaces in homes and damage gardens (Hill et al., 2007). This close association with people increases the chances of exposure by possum ticks to humans and companion pets.
animals. *Ixodes holocyclus* ticks, in particular, possess a liberal feeding behaviour (Roberts, 1970; Barker and Walker, 2014) and may be associated with the transmission of TBD. Anecdotal evidence suggests that damaged lawns and tick transmission caused by bandicoots are the biggest human-wildlife conflicts in Australian metropolitan areas (FitzGibbon and Jones, 2006; Dowle and Deane, 2009). Thus, urban bandicoots are often considered a nuisance or pest for some residents. In one study, urban residents had interactions with urban bandicoots at least once or more on a daily basis (Dowle and Deane, 2009). Again, as with the brush-tailed possums, piroplasms are well studied in bandicoot hosts, but less so in bandicoot ticks. Similar to the *I. holocyclus* tick, *I. tasmani*, the common marsupial tick, parasitises a wide range of hosts including humans (Roberts, 1970; Barker and Walker, 2014). This tick species was previously reported to harbour a *Hepatozoon* sp. from Tasmanian devil (*Sarcophilus harrisii*) (Vilcins et al., 2009). *Theileria peramelis* has also been identified in bandicoots with *I. tasmani* as its associated vector (Mackerras, 1959; Weilgama, 1986). Unfortunately, nucleotide sequences for the type species of *T. peramelis* are unavailable, therefore it is not possible to determine if the *Theileria* sp. detected in the present study is *T. peramelis*.

*Theileria orientalis* species complex, vectored by *H. longicornis* ticks, causes bovine theileriosis worldwide. Based on the sequence analysis of the major piroplasm surface protein (MPSP) gene, at least 11 distinct genotypes of the *T. orientalis* (types 1–8 and N1–N3) have been recognized globally (Sivakumar et al., 2014). The genotypes Ikeda and Chitose have been considered to be the pathogenic strains responsible for several outbreaks in the Eastern states of Australia (Izzo et al., 2010; Kamau et al., 2011). Outbreaks in Victoria were associated with the introduction of cattle from NSW (where the disease is endemic in some regions) to a beef cattle farm near Seymour, Victoria (Cufos et al., 2012). Theileriosis is now considered as an emerging TBD of high importance for livestock and human health.
significance in the beef and dairy industries (Rogers and Callow, 1966; Izzo et al., 2010; Islam et al., 2011). Haemaphysalis longicornis is a non-native tick in Australia and is suspected to have been introduced from Japan via livestock importation, along with the Thelidia species it harbours (Seddon, 1952; Hoostraaal et al., 1968). In the 1870s, red foxes were also imported into Australia for recreational hunting (Rolls, 1977; Mulley and Starr, 1984; Marks and Bloomfield, 1999). In Europe, B. microti-like and Thelidia annae are commonly reported in red foxes and their ticks (Cardoso et al., 2013; Najm et al., 2014; Farkas et al., 2015; Bartley et al., 2016). Therefore, further research is essential to better determine the range of piromplasms in foxes and their ticks in Australia.

The data from the present study emphasises the significance of tick studies in Australia and the importance of determining whether these newly characterised Babesia and Thelidia spp. can cause disease. Piromplasmosis-positive ticks in the present study originated from the eastern states of Australia. There is evidence from previous studies that kangaaroos infected with $B. macropus$ in QLD and NSW may exhibit clinical babesiosis in some situations (Dawood et al., 2013; Donahoe et al., 2015). In the present study, the health of the animals from which the ticks were removed was not recorded. In future studies, additional details on host condition at the time of sample collection and examination of blood smears could be incorporated for a more comprehensive investigation. In addition, the biology and the relationship between the tick and the protozoa, as well as the competence of the tick as a possible vector should to be investigated. Further epidemiological studies are required on piromplasmosis in wildlife living at the interface of urban settlements to gain further knowledge about potential emerging TBD and to provide new opportunities for targeted wildlife management.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijppaw.2018.05.005.

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