



Invasive rabbits host immature *Ixodes* ticks at the urban-forest interface

Casey L. Taylor^{a,*}, Henry W. Lydecker^{a,b}, Nathan Lo^a, Dieter F. Hochuli^a, Peter B. Banks^a

^a School of Life and Environmental Sciences, The University of Sydney, Camperdown, Australia

^b Marie Bashir Institute for Infectious Disease and Biosecurity, The University of Sydney, Camperdown, Australia

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ABSTRACT

Introduced wildlife may be important alternative hosts for generalist ticks that cause health issues for humans and companion animals in urban areas, but to date are rarely considered as part of the tick-host community compared to native wildlife. In Australia, European rabbits, *Oryctolagus cuniculus*, are a widespread and abundant invasive species common to a range of human-modified ecosystems. To understand the potential role of rabbits in the life cycle of Australian ticks, we investigated the seasonal abundance of all tick life stages (larva, nymph, and adult) on rabbits collected from pest control programs in two urban forest remnants in Sydney, Australia. We also recorded whether larvae, nymphs, and adults were attached to the head, body, or limbs of rabbits to reveal patterns of tick attachment. Of the 2426 *Ixodes* ticks collected from 42 rabbits, larvae were by far the most abundant life stage (2360), peaking in abundance in autumn, while small numbers of nymphs (62) and adults (4) were present in winter and summer respectively. Larvae were found all over the body, whereas adults and nymphs were predominantly attached to the head, suggesting that the mature life stages use the host landscape differently, or that adults or nymphs may be groomed off the body. The most abundant tick species, as determined by morphology and DNA sequencing, was *Ixodes holocyclus*, a generalist tick responsible for significant human and companion animal health concerns in Australia. Our findings highlight the importance of understanding the role of introduced wildlife in tick dynamics particularly in novel ecosystems where non-native hosts may be more abundant than native hosts.

1. Introduction

Introduced vertebrates may be important tick hosts, particularly in urban and peri-urban areas where native vertebrate hosts may be less abundant (Bradley and Altizer, 2007) while introduced hosts thrive in close proximity to humans (Chapuis, 2005; Vourc'h et al., 2007). However, studies on tick ecology traditionally focus on native vertebrate hosts in tick dynamics, and few studies have considered how introduced animals contribute to the life cycle of native tick species. For example, introduced Siberian chipmunks (*Tamias sibiricus barberi*) are more heavily infested with generalist *Ixodes* ticks than native hosts (small rodents) in a suburban forest of France (Pisanu et al., 2010) and are now considered a major player in the transmission of Lyme borreliosis (Marsot et al., 2013).

Globally, rabbits and hares (Order Lagomorpha) are important hosts for a range of tick species, for example, *Ixodes dentatus* in North America and *Hyalomma lusitanicum* in Europe (Anderson, 1989; Estrada-Peña et al., 2012; González et al., 2016; Hamer et al., 2012), and they predominantly support the immature tick life stages (González et al., 2016; Horak and Fourie, 1991). *Ixodes dentatus* preferentially feed

on rabbits, are considered 'rabbit ticks', and rarely bite humans (Hamer et al., 2012), whereas ticks such as *H. lusitanicum* are generalists, they often bite humans and are implicated in tick-borne zoonoses (Toledo et al., 2009). To date, there are no records of rabbit-specialist ticks having been introduced into Australia and the ability of rabbits to host generalist ticks in Australia is poorly understood.

Domestic European rabbits, *Oryctolagus cuniculus*, arrived in Australia with the First Fleet in 1788, with their wild counterparts later released in Victoria, Australia in 1859, colonising the other five states on the Australian mainland in 60 years (King and Thompson, 1994). They can reach very high densities in suitable habitat, under favourable conditions, and in the absence of significant predation (Gibb, 1990). As such, rabbits are still widespread and common in southeastern Australia, including in human-modified landscapes, where ticks are becoming increasingly problematic (Rappo et al., 2013; Van Nunen, 2015). Due to the risks to humans and companion animals associated with broad-scale poison baiting (Twigg et al., 2001), control of urban rabbits is often limited to sporadic ground shooting and biological control, and there are high population densities of rabbits in and around almost all of Australia's major urban centres.

* Corresponding author at: School of Life and Environmental Sciences, Science Road Cottage (A10), The University of Sydney, NSW, 2006, Australia.

E-mail address: casey.taylor@sydney.edu.au (C.L. Taylor).

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Australian ticks can transmit a suite of bacterial pathogens (Chalada et al., 2018; Graves et al., 2016; Graves and Stenos, 2017) and can cause life-threatening allergic reactions (Van Nunen, 2015). Solutions to reduce the incidence of tick bite, like landscape management in urban greenspaces or host-targeted management, require a detailed understanding of tick ecology. Host records of Australian ticks are incomplete and lack detailed evidence of the associations between individual hosts species and the different tick life stages, limiting host-targeted tick management strategies (Lydecker et al., 2015). Native long-nosed bandicoots, *Perameles nasuta*, are suspected to be a primary host of *Ixodes holocyclus*, the Australia paralysis tick, however, this claim is not well supported by scientific evidence (Lydecker et al., 2015).

Ixodes holocyclus is a generalist tick that feeds on a wide range of animals, occasionally biting humans and companion animals and causing a range of health issues including paralysis in pets and allergic reactions in people (Barker and Walker, 2014; Van Nunen, 2015). Although long-nosed bandicoots are known hosts of *I. holocyclus*, rabbits are similar in size, their habitat preferences and foraging behaviours (diggings) overlap with long-nosed bandicoots (Chambers and Dickman, 2002; King and Wheeler, 1985; Moreno et al., 1996; Scott et al., 1999), and in many areas they are far more abundant. Rabbits utilize open grassed areas and nearby forest for both foraging opportunities and refuge (Lombardi et al., 2003), making them likely to encounter generalist ticks at the urban-forest interface, where manicured grassed areas often abut remnant vegetation.

In this study, we investigated the seasonal abundance of ticks on rabbits from two urban forest remnants in Sydney, Australia. Due to the seasonal variation in the tick life stages, we sampled rabbits during the peak season of each life stage. Adult ticks are generally more abundant in spring and early summer (Doube, 1979; Eppleston et al., 2013; Ross, 1924), larvae in summer and autumn, and nymphs in autumn and winter, though smaller cohorts of all life stages can be found year round (Doube, 1979). We aimed to determine 1) the seasonal abundance of the different life stages on rabbits from urban areas and 2) the distribution of attachment of the different life stages across the six main body regions of rabbits (head, dorsal body, ventral body, axilla, genital area, and limbs) to reveal the potential role introduced rabbits play in the life cycle of Australian ticks.

2. Methods

This study was carried out in two forest remnants in Sydney, Australia: Middle Head (0.11 km²) and North Head (3.85 km²). The two headlands are spatially independent, separated by ocean and urban development, and parts of both headlands are classified as Sydney Harbour National Park. Manicured grassed areas, roads, car parks, and buildings fragment the forest on each headland, and both areas adjoin heavily urbanised landscapes. Dominant vegetation on both headlands includes a mixture of coastal sandstone health, sandstone ridge-top woodland and sandstone gully forest. The two headlands provide habitat for a wide range of vertebrates that are capable of hosting ticks including reintroduced native bush rats (*Rattus fuscipes*) (primarily North Head) and long-nosed bandicoots, common brushtail (*Trichosurus vulpecula*) and ringtail possums (*Pseudocheirus peregrinus*), brown antechinuses (*Antechinus stuartii*) (primarily North Head), echidnas, reptiles, and birds, as well as high densities of introduced black rats (*Rattus rattus*), European foxes (*Vulpes vulpes*), and European rabbits.

We opportunistically collected ticks from 42 rabbits (32 from North Head; 10 from Middle Head) that had been shot by National Parks and Wildlife Service rangers, as part of a routine pest control program, during the three peak tick seasons: summer (December) ($n = 14$), autumn (April) ($n = 14$), and winter (July) ($n = 14$). Mean minimum temperature (min), maximum temperature (max) and relative humidity (RH) for each month rabbits were sampled was obtained from the Bureau of Meteorology (Bureau of Meteorology, 2018). December: min

= 19.9, max = 27.8, RH = 61 %, April: min = 18, max = 26.1, RH = 65 %, July: min = 8.4, max = 19.9, RH = 57 %. Shoots were conducted between 6 pm and midnight in open grassed areas, usually not far from refuge for rabbits. Culled rabbits were individually placed in sealed plastic bags immediately following shooting and were stored in a freezer to ensure ticks remained attached and could be easily collected in the laboratory.

We performed post-mortem examinations of rabbits after 12 h of thawing. One person (C. Taylor) performed all tick searches, which involved systematically searching each individual for 2 h to obtain an accurate estimate of tick abundance and to record the sites of tick attachment (see protocol and recommendations by Lydecker et al., 2019a,b). Ticks were removed using 1.5 mm fine-tipped forceps, stored in ethanol, and identified to species under a light microscope using relevant morphological keys (Barker and Walker, 2014; Kwak, 2017; Roberts, 1970). Host weight and sex were also recorded during post-mortem examination.

The identification of juvenile Australian ticks remains challenging due to the lack of complete and reliable keys, thus identifications presented in this study are our best attempts using available keys.

All larvae were identified to species as accurately as possible using larval keys (Laan et al., 2011; Roberts, 1969). *Ixodes holocyclus* larvae have a rounded or blunt hypostome compared to the acutely pointed hypostome of *I. hirsti* and *I. trichosuri* (Fig. 1) (Laan et al., 2011). Distinction between *I. hirsti* and *I. trichosuri* is more challenging, relying on counting the number of Sd and Pm setae (Laan et al., 2011), which can



Fig. 1. Microscope images showing the acutely pointed hypostome of *Ixodes trichosuri* (and *Ixodes hirsti*) larvae (a) compared to the rounded hypostome of *Ixodes holocyclus* larvae.

be difficult to visualise. Nymphs were identified to species using available descriptions and comparisons with adult keys (Barker and Walker, 2014; Laan et al., 2011; Roberts, 1970).

Larvae from this study are stored in the Australian Museum for future reference (accession number: AA.812) as the morphological and molecular identification of Australian tick larvae advances. Remaining ticks will be examined for the presence of tick-borne pathogens as part of a broader long-term study on vector-borne disease in Australia.

We also performed identification using DNA sequence-based information for 13 samples, one of which was a single larva (morphologically identified as *I. trichosuri*), two of which were pools of 10 larvae (morphologically identified as *I. holocyclus*), and the rest of which were nymphs (morphologically identified as *I. holocyclus*). In each case, whole animals were used for extraction. Following homogenisation using a sterile pestle, DNA was extracted using a DNeasy Kit (Qiagen, Hilden, Germany). PCR of the ITS2 region was performed using the same primers and cycling conditions as described in Kwak et al. (2017) with the exception that Econotaq Master Mix was used. PCR products were subjected to ExoSAP-IT (Thermo Fisher Scientific, Waltham, Massachusetts) and sent to Macrogen (Seoul, Korea) for Sanger sequencing. DNA sequences were deposited in GenBank (Accession numbers MT251927-MT251939).

We calculated descriptive statistics, including index of dispersion (variance to mean ratio) to measure aggregation of ticks on rabbits in each of the three seasons. The variance to mean ratio (index of dispersion) of tick abundance data in all three seasons was > 1 , so a negative binomial distribution was used. We fitted generalized linear models (GLMs) with a negative binomial distribution and log link to test for variation in tick abundance using the categorical variables season (summer, autumn, winter), site (Middle Head, North Head), and sex (female, male).

The variation in the abundance of the different life stages was not analysed due to the small number of adults and nymphs found, however, we display these data as mean counts. A Kruskal-Wallis test was used to determine whether there was a significant difference between tick abundance on the different body sites of rabbits sampled in autumn and winter only; head, dorsal body, ventral body, axilla, genital area, or limbs. Statistical analyses were carried out in Microsoft Excel and SPSS version 24 (SPSS Inc., IBM Corp, Chicago).

3. Results

Ninety percent of rabbits in this study had ticks. All rabbits appeared to be in good condition; there were no wounds or signs of emaciation. Attached ticks ranged from unengorged, slightly engorged, to fully engorged (Fig. 2). Ticks were highly aggregated among the sampled rabbit population (Fig. 3) – only three individuals had a tick burden of > 300 ticks, whereas the vast majority had < 100 ticks.

Based on morphology, all ticks were classified into the *Ixodes* genus. *Ixodes holocyclus* was the most abundant tick species across all life stages (889, 37 %), closely followed by *I. trichosuri* (774, 32 %), then *I. hirsti* (278, 11) and *I. tasmani* (32, 1 %) (Table 1). Of the ticks identified using morphology, 453 were unable to be classified to the species level due to a damaged hypostome, and/or damage to alloscutum or main body (dorsal and ventral) preventing counting of setae required to distinguish *I. holocyclus*, *I. trichosuri*, and *I. hirsti*.

Of the 10 nymphs that were identified using DNA sequencing methods, 9 were considered *I. holocyclus* based on a 99–100 % match with ITS2 sequences in GenBank. One sequence was found to be *I. trichosuri*. The single larva examined was identified as *I. trichosuri*. Of the two pools of 10 larvae, one of the pools returned a sequence matching *I. holocyclus*, while the other returned a sequence matching *I. trichosuri*. It is possible that these pools contained a mix of both species, with the DNA of one of the other species dominating during the PCR.

Season was a significant predictor of tick abundance (Wald $\chi^2 = 59.22$, $df = 2$, $p = < 0.001$) (Table 2); tick abundance was



Fig. 2. Microscope image of *Ixodes trichosuri* larvae illustrates the varying stages of engorgement of larval ticks found on rabbits in this study.

significantly higher in autumn compared to winter (Table 2, Fig. 4). Neither site (Wald $\chi^2 = 1.79$, $df = 1$, $p = 0.18$) nor individual body weight (Wald $\chi^2 = 0.009$, $df = 1$, $p = 0.92$) was a significant predictor of tick abundance. Males had significantly more ticks than females, although tick abundance was highly variable for both sexes (Fig. 5) (sex effect Wald $\chi^2 = 5.64$, $df = 1$, $p = 0.018$). Weight was not significantly different between males and females ($t = 0.43$, $df = 40$, $p = 0.67$).

Larvae were the most abundant life stage found on rabbits (2360 individuals, 97.3 % of total), peaking in abundance in autumn with moderate numbers of nymphs present in winter (62, 2.6 %) and few adults present in summer (4, 0.2 %) (Fig. 6). There were more nymphs on rabbits sampled in winter compared to the other two seasons, reflecting the peak season for nymphs. There was no significance difference ($\chi^2_{(5)} = 5.51$, $p = 0.357$) in tick abundance among the different body regions. However, almost all adults and nymphs were found on the head, particularly around the eyes, whereas larvae were predominantly found on the body and limbs (Fig. 7).

4. Discussion

We found that introduced rabbits supported high numbers of immature *Ixodes* ticks and 90 % of rabbits in this study had ticks. Our findings suggest that rabbits have the potential to play a role in tick dynamics, particularly where rabbits reach higher densities than co-occurring native small mammals. Moreover, as rabbits move between grassed areas and forest (Lombardi et al., 2003), they have the potential to transport ticks into areas frequented by humans and companion animals.

Sequenced ticks were identified as *I. holocyclus* and *I. trichosuri*. A range of native and introduced animals can host *I. holocyclus* (Roberts, 1960) but detailed studies on the interactions between *I. holocyclus* and hosts other than bandicoots and rats are needed. The common brushtail possum is an important host of *I. trichosuri* (Baxter et al., 2009) but the species has been found on other animals including bush rats (Spratt and Haycock, 1988). Detailed studies of alternative host associations are also lacking for *I. trichosuri*. While *I. holocyclus* is responsible for a range of health concerns in humans and companion animals in Australia, little is known about if and what health implications result from bites of *I. trichosuri* (Baxter et al., 2009).

Only a few rabbits in this study had an extremely high tick burden (> 300 larval ticks) (Fig. 3). This aggregated distribution of parasites among the host population is observed in a wide range of parasite-host contexts, where most individuals in a population experience intermediate parasite levels (Poulin, 2007). A possible mechanism driving this pattern in our study could be a host encountering thousands of

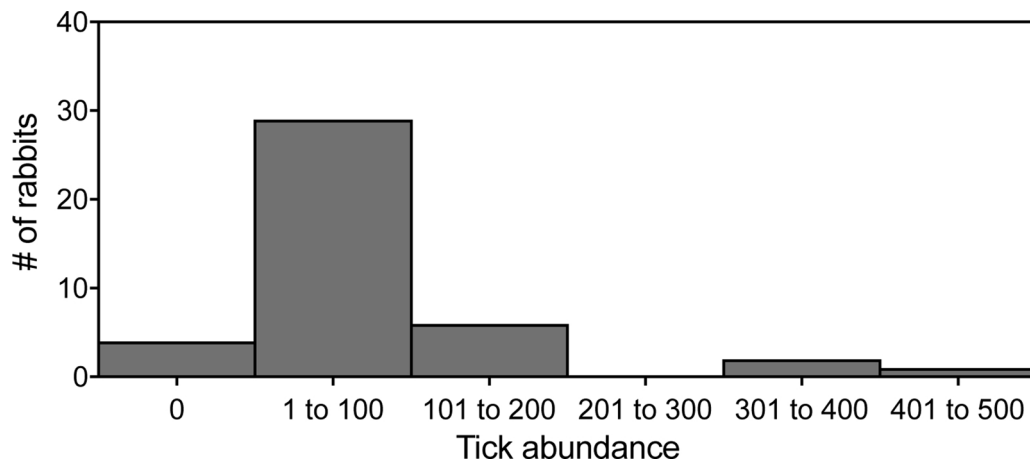


Fig. 3. Histogram of tick abundance on rabbits ($n = 42$) shows aggregation of ticks among the sampled host population. Only a few individual rabbits had high tick burdens (> 300 ticks), whereas the vast majority had far fewer ticks (< 100).

Table 1

Four *Ixodes* tick species were found on introduced rabbits with *Ixodes holocyclus* being the most abundant.

	<i>I. holocyclus</i>	<i>I. trichosuri</i>	<i>I. hirsti</i>	<i>I. tasmani</i>	Damaged/ unknown	Total
Larva	842	773	271	28	446	2360
Nymph	43	1	7	4	7	62
Adult	4	0	0	0	0	4
Total	889	774	278	32	453	2426

Table 2

Season was a significant predictor of tick abundance on rabbits using a negative binomial GLM ($p < 0.001$). Tick abundance was significantly higher on rabbits sampled in autumn compared to rabbits sampled in winter (represented by the intercept in the model output).

Predictor	Estimate	SE	Lower 95 % Wald CI	Upper 95 % Wald CI	Wald Chi- Square	df	P
(Intercept)	2.372	.2795	1.824	2.919	72.020	1	< 0.001
Summer	.171	.3939	-.601	.943	.189	1	.664
Autumn	2.642	.3873	1.883	3.401	46.548	1	< 0.001
Winter	0 ^a

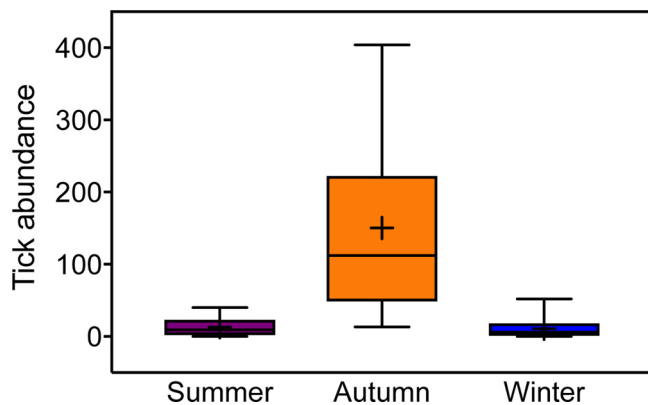


Fig. 4. Tick abundance on rabbits in summer ($n = 14$), autumn ($n = 14$), and winter ($n = 14$). Tick abundance peaked in autumn, reflecting the peak season for the larval life stage. Box-and-whisker plot shows minimum, 25th percentile, median, 7th percentile and maximum values. + denotes mean tick abundance.

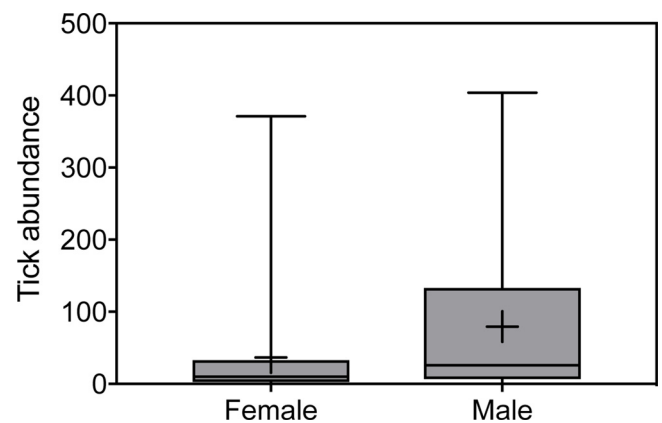


Fig. 5. Tick abundance on males ($n = 21$) was significantly higher than on females ($n = 21$) ($\chi^2 = 33.27$, $df = 1$, $p < 0.001$), though highly variable in both sexes. + indicates mean tick abundance. Box-and-whisker plot shows minimum, 25th percentile, median, 7th percentile and maximum values. + denotes mean tick abundance.

newly emerged larvae, determined by host behaviour and habitat usage. Hosts with disproportionately large tick burdens are considered most likely to be able to infect a large number of ticks, thus greatly contributing to the spread and transmission of tick-borne pathogens (Brunner and Ostfeld, 2008). In this case, where it is unknown whether rabbits harbor pathogens that can be transmitted to ticks, individual rabbits with high tick loads may amplify the local tick population, which can result in an increase in human-tick encounters. The drivers of tick aggregation among the host population are complex, and are likely caused by a range of intrinsic (sex, size, and age) and extrinsic factors (season, year, questing tick density) (Brunner and Ostfeld, 2008), some of which are outside of the scope of this study.

Tick abundance on hosts may be influenced by host factors such as their sex. Overall, we found that male rabbits had significantly higher tick abundance compared to female rabbits, though abundance was highly variable in both sexes and there was no significant difference in body weight between males and females. Males of a range of taxa can have greater parasite burdens (Bouchard et al., 2011; Lamattina et al., 2018; Vázquez et al., 2011), while other studies have shown no effect (Lutermann et al., 2012) or mixed results depending on whether host body mass was considered in analyses (Kiffner et al., 2013). Further study on host factors associated with tick infestations in rabbits, including covariates like body mass, is needed to determine whether this is a true biological trend and to reveal potential drivers.

The higher tick abundance on rabbits culled in autumn (Fig. 4) is

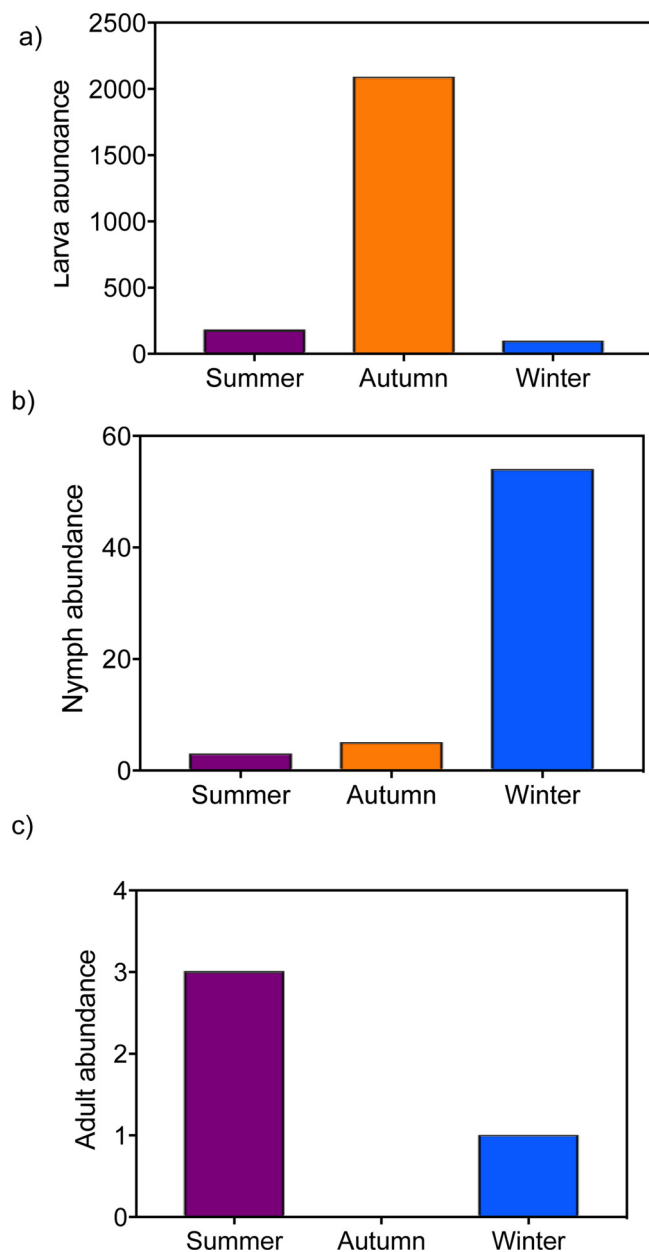


Fig. 6. Seasonal variation in the total abundance of larval (a), nymphal (b), and adult (c) ticks on rabbits. Note change in Y axis value on each graph. Larval abundance peaked in autumn, nymphal abundance peaked in winter and to a much lesser extent, and few adult ticks were found overall.

likely due to the peak in larval tick abundance following hatching during late summer and early autumn on the east coast of Australia (Doube, 1979). In autumn, *I. holocyclus* larvae are also abundant on native Northern brown bandicoots (*Isodon macrourus*) in suburban Brisbane, Australia (Doube, 1979). Compared to Doube (1979), nymphs may be more abundant on bandicoots compared to rabbits, indicating that introduced rabbits may be less capable hosts of *Ixodes* nymphs or that rabbits are able to groom them off. Note that Northern brown bandicoots are not found in our study area, thus data on the abundance of *Ixodes* on long-nosed bandicoots from Sydney, Australia would permit a more meaningful within-system comparison. Nymphs comprised 36 % of ticks collected from rabbits in winter, similar to the proportion of nymphs (45.1 %) collected from black rats during winter in the same study system (Lydecker et al., 2019c), suggesting that both introduced rabbits and rats may play relatively similar roles as hosts of generalists immature *Ixodes* ticks in a novel ecosystem.

In summer, adult ticks were present in low densities on rabbits (1.6 % of total ticks) comparable to the low numbers of adult ticks found on bandicoots (Doube, 1979) and rats (Lydecker et al., 2019c) on the east coast of Australia. This pattern could reflect the population dynamics of Australian *Ixodes* ticks, where survivability from the nymph to adult life stage may be low or adult *Ixodes* ticks may be favouring other hosts not yet studied in this detail, for example, brushtail and ringtail possums, macropods, and reptiles. While the proportion of the different tick life stages on small mammal hosts across seasons varies among species of hosts and ticks, studies on small mammals have shown that the abundance of generalist *Ixodes* nymphs is generally lower than the abundance of *Ixodes* larvae (Casher et al., 2002; González et al., 2016; Mysterud et al., 2015; Pisanu et al., 2010) and *Ixodes* adults are either absent or found at very low densities, often favouring larger vertebrates (Tälleklint and Jaenson, 1997).

We found that the site of attachment of the tick life stages varied among the different body regions: larvae were found along the entire body, while almost all of the adults and nymphs were found on the head (Fig. 7), particularly on the eyelids. These results suggest that different stages may use the host landscape differently or that rabbits can groom off adults and nymphs from other parts of the body. Ticks aggregate on particular body sites of their hosts (Reiczigel and Rózsa, 1998), possibly due to differences in vascularisation, light intensity and temperature across the skin (Heath et al., 1987), as well as interspecific competition, and host-mediated interactions such as scratching and grooming (Reiczigel and Rózsa, 1998). Rabbits groom or ‘wash’ the head by licking the forelimbs and moving them over the head, eyes, and ears (Dixon et al., 2010), which may be sufficient to interrupt the path of an unfed tick searching for a site to attach, although licking is unlikely to remove an attached tick. Rabbits can directly bite and lick fur on the other parts of the body, such as the abdomen and the hind limbs, likely to be more precise in dislodging an attached adult or nymph. This may provide an alternative explanation for the low numbers of nymphs found on rabbits in this study compared to numbers of nymphs found on bandicoots (Doube, 1979).

Our results also show that a systematic search of the entire body is required to understand infestation of rabbits by all life stages. However, if researchers are targeting nymphs or adults, sampling effort can be focused on the head.

We found engorged ticks of all life stages on rabbits in this study, with no apparent relationship to rabbit condition (Fig. 2). European rabbits develop acquired immunity to adult female *I. ricinus* following experimental infestations, impacting tick engorgement and egg production (Bowessidjaou et al., 1977; Schorderet and Brossard, 1993). Thus, captive studies are needed to understand whether Australian *Ixodes* ticks successfully feed to repletion on introduced rabbits and moult to the next life stage (Lydecker et al., 2019a), and to reveal how rabbit grooming behaviour impacts the attachment and feeding success of the different tick life stages.

Our findings suggest invasive rabbits could be important in maintaining urban tick populations in areas where rabbits are overabundant, particularly individuals heavily infested with larvae. Additionally, the most abundant species on rabbits identified using morphology and DNA sequencing, *I. holocyclus*, is a known vector of tick-borne pathogens (Graves and Stenos, 2017), causes debilitating allergies in people (Van Nunen, 2015), and life-threatening paralysis in companion animals (Padula et al., 2020). Our results also indicate that the different life stages of tick may use the host landscape differently or that host grooming may remove adults or nymphs from the body, though experimental studies are required to confirm this phenomenon.

European rabbits persist near human dwellings and in modified and natural landscapes around the world (Frank et al., 2013), possibly allowing them to amplify tick populations in areas used by humans. As urban areas expand and the rate of tick-borne illness increases around the world, it is crucial to understand how introduced hosts that thrive in close proximity to humans fit into the life cycle of native ticks.

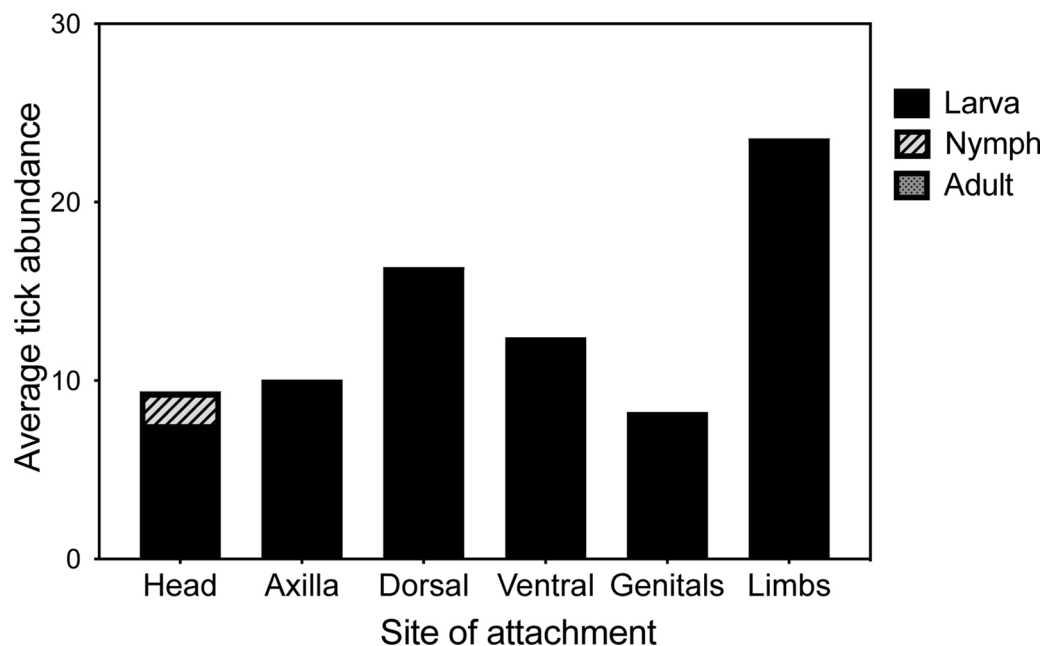


Fig. 7. Average tick abundance on the six body regions of rabbits sampled in autumn and winter ($n = 28$). Larvae were predominantly attached to the limbs and nymphs and adults, though in much smaller numbers, were mostly attached to the head.

Author contributions

C. Taylor designed the study, collected and analysed the data, and wrote and edited the manuscript. P. Banks, H. Lydecker, and D. Hochuli contributed to the study design and edited the manuscript. N. Lo facilitated DNA based identification of ticks and edited the manuscript.

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Declaration of Competing Interest

None.

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