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Differences in constitutive innate immunity between divergent Australian marsupials

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ABSTRACT

Understanding immunity in wildlife populations is important from both One Health and conservation perspectives. The constitutive innate immune system is the first line of defence against pathogens, and comparisons among taxa can test the impact of evolution and life history on immune function. We investigated serum bacterial killing ability (BKA) of five marsupial species that employ varying life history strategies, demonstrated to influence immunity in other vertebrates. The brushtail possum and eastern grey kangaroo had the greatest BKA, while ringtail possums and koalas had the least. These differences were independent of social structure, captivity status and phylogeny, but were associated with diet and body size. Sex and disease status had no effect on BKA in koalas, however potential for differences between wild and captive koalas warrants further investigation. The current study has provided a foundation for future investigations into how adaptive and innate immunity interact in marsupials from an eco-evolutionary perspective.

1. Introduction

Immunity plays a crucial role in determining disease outcomes, and immunological studies have proven to be useful tools for monitoring population health for conservation (Brock et al., 2012; Gervasi et al., 2014; Ohmer et al., 2021). Understanding dynamics of immunity and host disease susceptibility can allow the identification of populations that are vulnerable to certain novel pathogens (Hawley and Altizer, 2011). This is particularly important in the context of marsupials, as many of them are not only threatened by pathogens (McFarlane et al., 2013; Sukee et al., 2020; Thompson et al., 2010), but there is also the potential for them to be reservoirs to pathogens that can infect humans and livestock, thus posing a biosecurity risk (Burnard et al., 2017; Thompson et al., 2014). By knowing the strengths and weaknesses of each species' immune system, information can be gained on what type of infectious pathogens they are most susceptible to (DeWitt et al., 2016) and, therefore, which species are at highest risk. Comparing immunological strategies across species can allow an understanding of how evolutionary history, variation in immune function (Tian et al., 2015), and susceptibility to novel pathogens interact (Hawley and Altizer, 2011).

Immunity can be energetically and pathologically costly, thus it is hypothesised that animals will only invest in the immune defences they need and can afford (Martin et al., 2006a, 2006b; Schneeberger et al., 2013). This need for immunity is determined by differences in life history and how frequently animals are exposed to pathogens. Immune responses are broadly classified as either innate or adaptive, with adaptive immunity conferring a pathogen-specific response, and more sophisticated regulation of inflammation, healing, and immunological memory. Innate immunity can be either induced by a trigger, or constitutive, providing an immediate first line of defence against pathogens via mechanisms such as anti-microbial peptides and the complement system (Paludan et al., 2021). Constitutive innate immunity is seen as having a lower cost than induced innate and adaptive immunity and can regulate induced immune responses (Lochmiller and Deerenberg, 2000; Heinrich et al., 2017; Paludan et al., 2021). It is hypothesised that animals with a greater risk of repeated exposure to the same pathogens, such as social animals, may invest more into adaptive immunity (Altzier et al., 2003). Animals with less risk of repeated exposure, are expected to invest more into constitutive innate immunity (Martin et al., 2006a; 2006b).

As a diverse group of animals, marsupials employ many different life-history strategies, which makes them interesting for a comparative ecoimmunological study across the group. Diversity of life history

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strategies leads to differences in the risk of pathogen transmission. Some of the factors that have been identified to affect disease transmission and thus immunity in other vertebrate species, including placental mammals, include diet, social structure and body size. Grazing herbivores have been shown to have a high risk of pathogen transmission through the faecal oral route, whereas folivores are seen as having a cleaner diet (Nunn et al., 2003). Carnivores are exposed to pathogens in the carcasses they consume (Murray et al., 1999), with scavengers at a greater risk of exposure due to larger quantities of pathogens being found in carrion (DeVault et al., 2003). Larger animals are expected to have higher pathogen risk, as greater dietary intake increases their exposure (Poulin, 1995; Vitone et al., 2004). Social animals also have a greater risk of repeated pathogen exposure due to frequent encounters with other individuals (Altizer et al., 2003; Vitone et al., 2004; Kappeler et al., 2015).

The bacterial killing assay is commonly used to assess and compare constitutive innate immunity within and between species (Tieleman et al., 2005; Millet et al., 2007; Liebl and Martin, 2009; French and Neuman-Lee, 2012; Schneeberger et al., 2013; Heinrich et al., 2016; Jacobs and Fair, 2016). This assay quantifies the bactericidal activity of serum, plasma or blood and is popular in ecoimmunology as it measures a functional response of an animal's immune system, making it ecologically relevant (Tieleman et al., 2005). Different components of the immune response can be investigated, depending on the type of sample and microbe chosen (Saino et al., 2002; French and Neuman-Lee, 2012). For example, *Escherichia coli* is mainly killed by complement and natural antibodies (French and Neuman-Lee, 2012). As these are not species-specific responses, and no species-specific reagents are required, the assay is easily transferable across species and is suitable for cross-species comparisons (Millet et al., 2007; Schneeberger et al., 2013).

To date, the majority of marsupial immunological studies have focused mainly on adaptive and induced innate immunity and the use of molecular tools (Cheng et al., 2012; Lau et al., 2014; Hing et al., 2016; Maher and Higgins, 2016; Russell et al., 2018; Maher et al., 2019; Quigley et al., 2020), however none of these have compared immune function across species in relation to life histories. Immunogeneticists have identified the presence of complement genes in marsupials (Ong et al., 2015, 2016), however, the potential importance or evolutionary pressures on these genes and the on the innate immune phenotype have not been studied. The objective of this study is to take a functional immunological approach to investigate the bacterial killing activity of serum of brushtail and ringtail possums (*Trichosurus vulpecula* and *Pseudocheirus peregrinus*), eastern grey kangaroos (*Macropus giganteus*), koalas (*Phascolarctos cinereus*), and Tasmanian devils (*Sarcophilus harrisii*) and consider the evolutionary pressures that may have influenced them by testing various factors which might explain within and between species variances, including size, social behaviour, diet and phylogeny.

2. Materials and methods

2.1. Collection and storage of samples

Serum samples from 6 brushtail possums, 6 eastern grey kangaroos, 9 koalas, 8 ringtail possums and 7 Tasmanian devils were collected from archives at Taronga Zoo Sydney. The samples had been collected for routine blood tests from 2004 to 2019, and subsequently stored at −80 °C. These comprised a combination of captive and wild animals that had been admitted to the wildlife hospital for treatment. Animals were classified as diseased if they were showing any clinical signs at the time of blood collection, or if they had been admitted to the wildlife hospital for treatment (Supplementary Table 1).

Additionally, twelve samples were collected from free-ranging koalas (5 female, 7 male) from an area south of Gunnedah on the Liverpool Plains, NSW in July 2019 and stored at −80 °C. These samples were collected under AEC permit 2019/1547 and the NSW National Parks and Wildlife Service Scientific License SL101687. Animals were classified as diseased if they had visible signs of chlamydiosis, which include conjunctivitis or staining of the rump associated with cystitis, as well as bladder thickening and presence of paraovarian cysts as determined by ultrasonography (Marschner et al., 2014; Mathews et al., 1995). Ten samples were collected from clinically healthy free-ranging eastern grey kangaroos (10 female) from Nelson Bay, NSW in July 2019 and stored at −20 °C for 6 days, before being transferred to a −80 °C freezer. These samples were collected under AEC permit 2015/917. Wild or captive origin was known for all species but sex and disease status records were only complete for koalas (Supplementary Table 1). All samples used in the study came from adult animals, to ensure that individual age was not a confounding factor.

2.2. Bacterial killing assay

Detailed methods for the BKA can be found in supplementary material. Briefly, a tryptic soy agar (TSA) plate was inoculated with *Escherichia coli* (*E. coli,* ATCC no. 8739), incubated at 37 °C overnight, and then stored at 4 °C. A second generation (G2) colony was grown by using a single colony to inoculate a new TSA plate, which was incubated overnight at 37 °C, and stored at 4 °C until use in the assay. Bacterial growth was optimised by measuring the time it took for the *E. coli* to reach log-phase growth. Inoculated tryptic soy broth (TSB) was incubated at 37 °C overnight and 100 μL of this used to inoculate TSB broths that were then incubated at 37 °C in a shaking incubator. Aliquots were taken every 30 min for 5.5 h, and optical density was measured in a plate reader (620 nm, Halo LED 96 microplate reader), in order to graph the bacterial growth (Supplementary Fig. 1). Log-phase growth in the stock solution occurred between 90 and 210 min (Supplementary Fig. 1a).

Optimal incubation time for the assay microplate was deemed to be 360 min (see Supplementary material for details), as it provided adequate spread between samples and since log phase growth occurred between 150 and 450 min (Supplementary Fig. 1b). When the *E. coli* stock solution was in log phase growth, a 50 μL aliquot was serially diluted with PBS, using 10-fold dilutions (1:10^{-4}, 1:10^{-5}, 1:10^{-6}) plated on TSA plates. The plates were incubated overnight at 37 °C, and colony enumeration was conducted the next day. Colony forming units (CFU)/mL was calculated according to the following formula:

$$\mathrm{CFU}\Big/\mathrm{mL} = \frac{\text{no. of colonies} \times \text{dilution factor}}{\text{volume (mL)}}$$

The CFU/mL when optical density is equivalent to one was 3.585×10^8 CFU/mL and was used to calculate the dilution factor for each run of the assay, in order to make the working solution of *E. coli.* A single G2 colony of *E. coli* was used to inoculate 5 mL of TSB, and incubated overnight at 37 °C. 100 μL of this *E. coli* was used to inoculate 5 mL of prewarmed TSB and incubated at 37 °C in a shaking incubator at 150 r.p.m, to grow the stock solution. The stock solution was diluted with PBS to 10^5 CFU/mL, in order to make the working solution.

Serum was serially diluted with PBS (1:2 to 1:128). Diluted and neat serum, or PBS in the positive control, (22 μL) was pipetted into a 96-well microplate and 10 μL working bacterial solution was pipetted into each well. Based on the intra-assay variance of 29% before exclusion of outlying replicates, samples were assayed in triplicate. PBS 32 μL without bacterial solution was used as negative control. The plate was vortexed for 1 min at 150 r.p.m and then incubated for 30 min at 37 °C. TSB 125 μL was added to all wells, including the blank and control and vortexed. Optical density was measured using the microplate reader to determine background absorbance. The plate was incubated at 37 °C for 6.5 h, after which the optical density was measured again. To calculate bacterial killing ability, the background absorbance reading was subtracted from the final optical density reading, and then the following equation was used for each sample:

$$\left(1 - \frac{\text{mean absorbance of sample}}{\text{mean absorbance of positive control}}\right) \times 100$$

2.3. Statistical analysis

Statistical analyses were conducted using R (Version 1.2.1335). Technical replicates were rejected as outliers if they lay >2SD from their mean, blinded to the biological data associated with the point. The non-parametric Kruskal-Wallis test was used to test whether bacterial killing ability was predicted by species, and in koalas only, the Kruskal-Wallis test was used to test the impact of sex, disease status or captivity status on BKA. *Posthoc* pairwise comparison of the different marsupial species was conducted using a Dunn test, with significance level set at $P < 0.05$. We used the mean BKA values at each dilution for each species to create a Euclidean distance matrix-based cluster dendrogram in R. This was compared to the phylogenetic relationships among our study species (Mitchell et al., 2014) to examine whether phylogeny was a factor in bacterial killing ability. We performed a Mantel test using the ade4 package (v 1.7–18) (Dray and Dufour, 2007) in R to compare the matrix based on BKA values to the matrix based on phylogenetic distance to determine any correlations.

To test which factors had the most impact on bacterial killing ability we conducted a generalised linear model (GLM) in R. BKA at 1:32 dilution was the continuous response variable. Life history traits of diet, social structure and body condition were included as predictors, along with origin (wild or captive) (Table 1). According to life history theory it was predicted that Eastern grey kangaroos will have the least bacterial killing ability, since they are large, social, grazing herbivores (Kaufmann, 1975; Garnick et al., 2018) that have more need for adaptive immune response and memory. Koalas were predicted to have the greatest bacterial killing ability, since they are solitary folivores that consume a low energy diet (Mitchell, 1990; Moore and Foley, 2000). Sex, captivity or disease status of the individual were not included in the model and examined for effect on inter-individual differences in koalas only due to prevalence of missing data across species (Supplementary Table 1).

3. Results

The 1:32 dilution of serum had the greatest range of bacterial killing ability, with the average killing closest to 50% (Fig. 1) and was therefore considered optimal and associated values used to conduct all statistical analyses. There was no observable association between the storage period and bacterial killing ability (Supplementary Fig. 2). In koalas, captive animals appeared to have lower BKA values than free-ranging animals, though this was not significant ($P = 0.1$) (Fig. 2a). Likewise, disease status ($P = 0.88$) (Fig. 2b) and sex ($P = 0.61$) (Fig. 2c) had no significant effect on koalas' bacterial killing ability. Individuals with a mixture of captivity status, disease status and sex were therefore pooled for further analysis.

Species differed in their BKA (Kruskal-Wallis test, $\chi^2 = 16.162$, df = 4, $P = 0.003$) (Fig. 3). *Posthoc* comparison using a Dunn test showed that eastern grey kangaroos had significantly greater BKA than koalas ($P = 0.02$) and ringtail possums ($P < 0.01$), and brushtail possums also had significantly greater BKA than koalas ($P = 0.03$) and ringtail possums ($P < 0.01$) (Table 2). GLM results indicated that diet was a significant predictor of BKA, as was body size (Table 3, Fig. 4). Phylogeny was not a predictor of BKA (Fig. 5) as clustering was not representative of species divergence times (Mitchell et al., 2014) and the Mantel test indicated that there was no correlation between mean BKA values and phylogeny ($r = -0.028$, P value $= 0.468$).

4. Discussion

We applied a bacterial killing assay to identify differences in constitutive innate immunity among five marsupial species with differing life history strategies. Diet was strongly associated with this functional measure of constitutive innate immunity, with the two eucalypt-eating specialist folivores (koalas and ringtail possums) having the lowest bacterial killing ability. Likewise, body size had an impact on constitutive innate immunity, though this was less clear; the medium and large body-sized species had a similar range of bacterial killing abilities, but the two smallest species were quite dissimilar. Unexpectedly, social structure was a poor predictor, as the two social species, eastern grey kangaroos (Kaufmann, 1975) and ringtail possums (Roberts et al., 1990) had significantly different bacterial killing abilities and two of the three solitary species, koala and brushtail possum, were also significantly different.

In our study, diet had the greatest effect on bacterial killing ability. The koala and ringtail possum, the two specialist folivores (Moore and Foley, 2000; Marsh et al., 2003; Hermsen et al., 2016), had significantly lower BKA than the herbivorous eastern grey kangaroo (Garnick et al., 2018), and the brushtail possum, a generalist folivore (Marsh et al., 2003; Cruz et al., 2012). This is consistent with the expectation that grazing herbivores have a greater risk of pathogen transfer via the faecal-oral route (Nunn et al., 2003) and this is likely true of bin scavenging, generalist feeders, such as the urban brushtail possums included in our study (McDonald-Madden et al., 2000; Eymann et al., 2006), though there was a high degree of variation across the brushtail possums (Fig. 4a) which may be influenced by fine-scale differences in urban habitat or individual feeding preferences (Herath et al., 2021). Cost benefit theory suggests that animals with low energy diet, such as the koala (Mitchell, 1990; Moore and Foley, 2000), are less likely to invest into adaptive immunity and may instead invest into energetically cheaper defences such as constitutive innate immunity (Lochmiller and Deerenberg, 2000; Martin et al., 2006b) and the results of the current study contradict this. It is possible that trade-offs remain undiscovered. For example, monoterpene PSMs inhibit *in vitro* lymphocyte function at concentrations found in koala plasma, suggesting potential for energetic and immunological trade-offs that may limit koala habitat (Marschner et al., 2019a, 2019b). Conversely, some eucalypt PSMs have antimicrobial activity (Gilles et al., 2010; Nobakht et al., 2017), though their antimicrobial effect in koala plasma has not been investigated. Given the likely impact of climate change, rising atmospheric carbon dioxide and land management practices on the concentration of nutrients and plant secondary metabolites (PSMs) in eucalypt leaves (Bustos-Segura et al., 2017), the role of these in immunity is worthy of future study.

Phylogeny did not explain bacterial killing ability in marsupials; koalas and Tasmanian devils clustered together based on mean BKA values across all dilutions, despite having the earliest divergence times (~65 MYA) of all species tested and mean BKA values were not correlated with phylogenetic distance (Mitchell et al., 2014). Likewise, brushtail possums and eastern grey kangaroos clustered together based on mean BKA (Fig. 4), despite being phylogenetically disparate (Mitchell et al., 2014). Our results are in contrast to those found in a less dietarily diverse group, the feliform carnivores, where phylogeny was the best predictor of constitutive innate immunity (Heinrich et al., 2016).

The effect of captivity may have had an influence on our study,

Table 1
A comparison of diet, social structure and body size of the five marsupial species that were investigated.

	Brushtail possum	Ringtail possum	Koala	Tasmanian devil	Eastern grey kangaroo
Diet	Generalist folivore	Specialist folivore	Specialist folivore	Carnivore	Herbivore
Social Structure	Solitary	Social	Solitary	Solitary	Social
Body Size	Small (<5 kg)	Small (<5 kg)	Intermediate (5–20 kg)	Intermediate (5–20 kg)	Large (>20 kg)

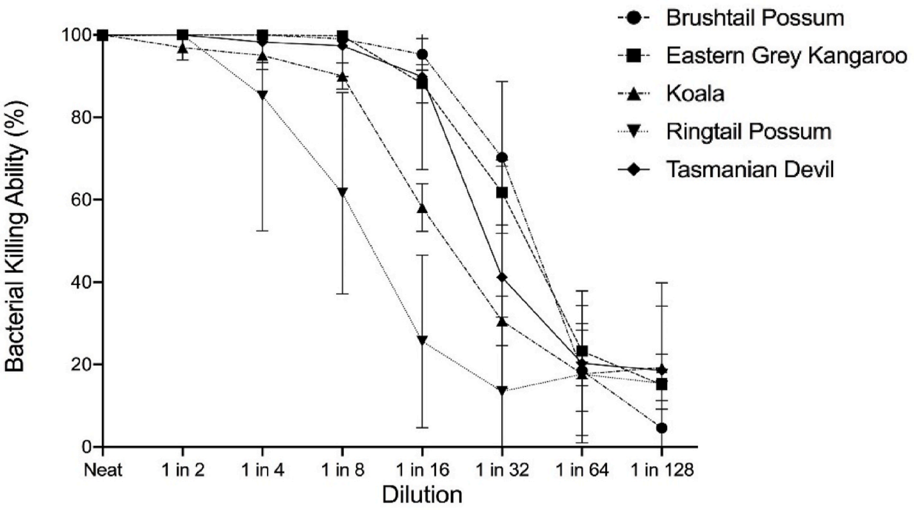

Fig. 1. Mean bacterial killing ability values (%) against *E. coli* for eight serial dilutions of serum (neat to 1 in 128) of the five marsupial species, with standard error bars displayed for each point.

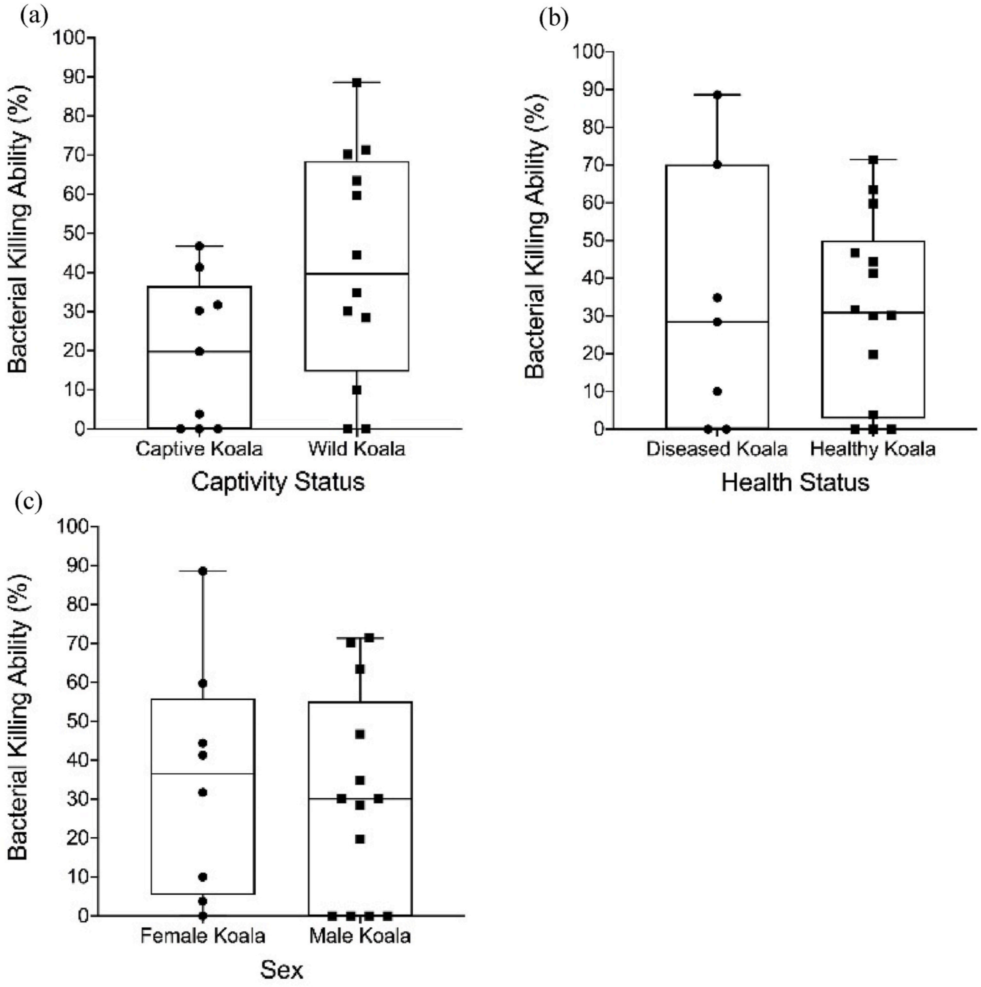

Fig. 2. Comparison of medians of bacterial killing ability (%) against *E. coli* using koala serum to investigate potential confounding factors including (a) the effect of captivity status, (b) the health status of the animal, and (c) the sex of the animal.

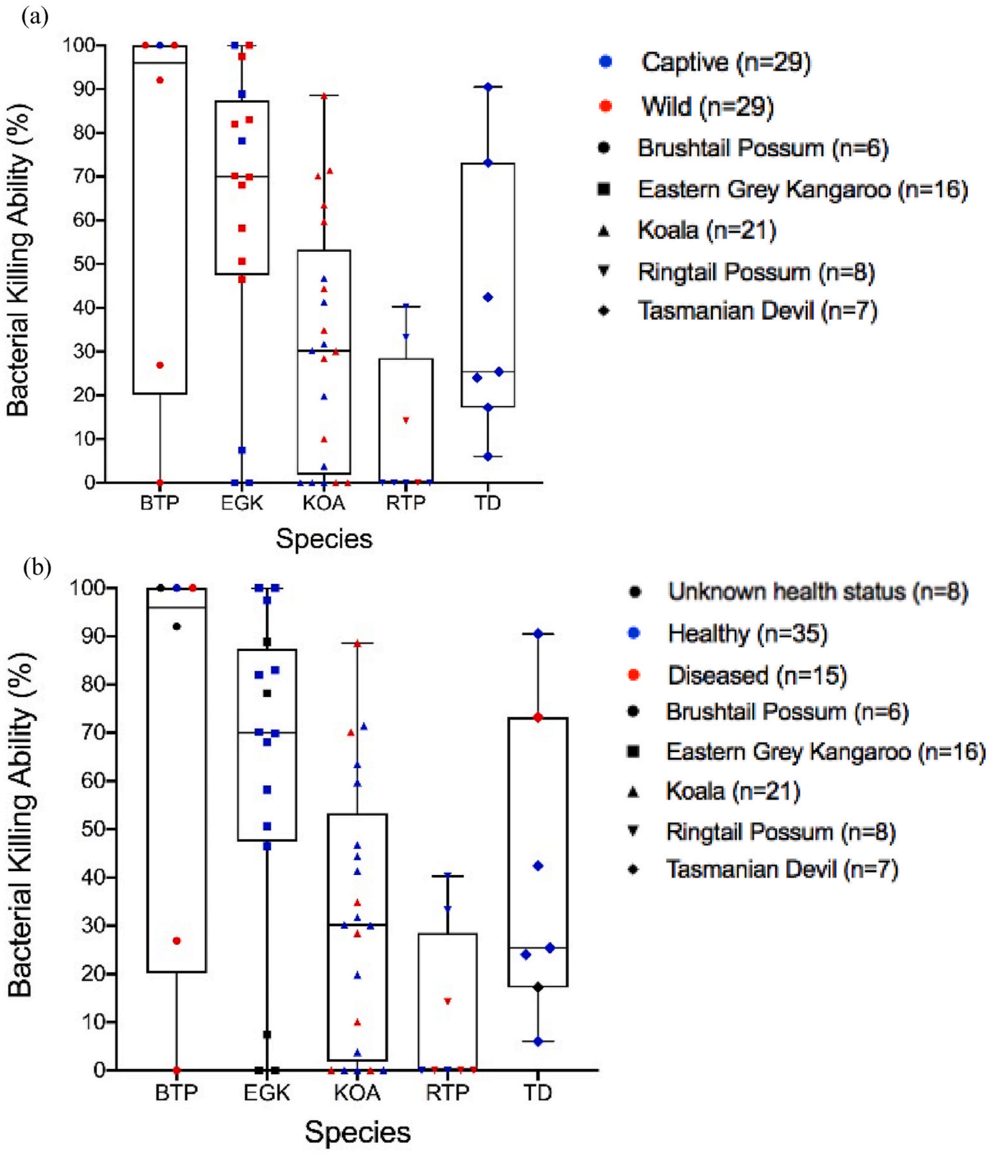

Fig. 3. Comparison of bacterial killing ability (%) against *E. coli* between marsupials with (a) the spread of captive and wild individuals shown, and (b) the spread of healthy and diseased individuals shown.

despite it not being a significant factor. For example, the BKA values for Tasmanian devils in our study may not reflect those of wild animals. As carnivores that scavenge for food, it would be expected that the Tasmanian devil would have higher bacterial killing ability, since carrion is likely to have relatively high pathogen loads (DeVault et al., 2003), however, the devil samples used in our study were all from a captive environment, which is likely to influence the types of microbes they encounter. Physiological and behavioural changes associated with captivity have previously been hypothesised to influence differences in microbiome composition between captive and wild devils (Cheng et al., 2015) and it feasible that this may influence immunological development. Although previous work has found no difference in bacterial killing ability between captive and wild hyenas (Flies et al., 2015), reduced constitutive immune response has been identified in captive birds and captive zebras (Buehler et al., 2008; Seeber et al., 2020). Our data suggested it may be worth investigating potential for an effect of captivity on koala BKA but there was no significant difference in our study.

Table 2
P-values of posthoc pairwise comparison of BKA of five marsupials, using a Dunn test.

	Brushtail possum	Eastern grey kangaroo	Koala	Ringtail possum	Tasmanian Devil
Brushtail possum	–	–	–	–	–
Eastern grey kangaroo	0.7	–	–	–	–
Koala	0.03[a]	0.02[a]	–	–	–
Ringtail Possum	<0.01[a]	<0.01[a]	0.11	–	–
Tasmanian Devil	0.18	0.22	0.57	0.08	–

[a] Significant at 0.05 level.

Table 3
GLM results of predictors (life history traits and wild or captive status (origin) for bacterial killing ability (assessed at 1 in 32 dilution).

	Estimate	SE	P value
Intercept	1.306	1.029	0.210
Diet	−0.843	0.287	0.005[a]
Social	−0.584	0.500	0.248
Size	0.781	0.318	0.017[b]
Origin	−0.621	0.410	0.136

[a] Significant at 0.01 level.
[b] Significant at 0.05 level.

Although body size was also a strong predictor of BKA, with BKA generally increasing with body size, this was not universal; the brushtail possum, smaller than the kangaroo, koala and Tasmanian devil, had the greatest mean BKA. It is possible that this finding stems from the fact that folivores tend to have lower bodyweight. The effect of body size was likely driven by the large-bodied kangaroos (Fig. 4b), so the inclusion of a wider range of body sizes across species may either strengthen or weaken this effect. Our finding that social structure did not play a measurable role in BKA in our study contrasts with some studies; social species are expected to have a greater risk of pathogen exposure as they encounter other individuals regularly, providing them with more opportunities for transmission (Altizer et al., 2003; Vitone et al., 2004). However, arthropod vectors transmit disease between solitary animals (Colwell et al., 2011), and solitary animals come into contact for reproduction, exposing them to sexually transmitted diseases (Ji et al., 2005). Therefore, solitary animals may not necessarily have less exposure to pathogens, especially if they are a promiscuous species (Thrall et al., 2000). The social kangaroos used in our study came from a peri-urban environment where food resources are limited and a fertility control trial has been implemented to control population density (Herbert et al., 2021), which may impact BKA in this population. Our results are also limited in interpretation as we used only one bacterial agent (*E. coli*) in the assay. Marsupial studies have found differences in anti-bacterial response to various bacterial species (Green-Barber et al., 2017; Ong et al., 2017), so our results may have differed if using a different bacterial agent.

The findings in this study provide impetus for further investigation incorporating multiple immune parameters to provide a more complete perspective of immunophenotype, and larger, well-defined groups of animals to reduce potential effects of heterogeneity of hosts. Variation between individuals within species was high in this study (Fig. 3). This may be caused by the heterogeneity inherent in wildlife populations, or by the chosen methodology. Although the bacterial killing assay is a popular ecoimmunological method, it is limited by high variation, impacting the confidence that can be drawn from results (Liebl and Martin, 2009) and intra-assay variation in the current study (29%) was

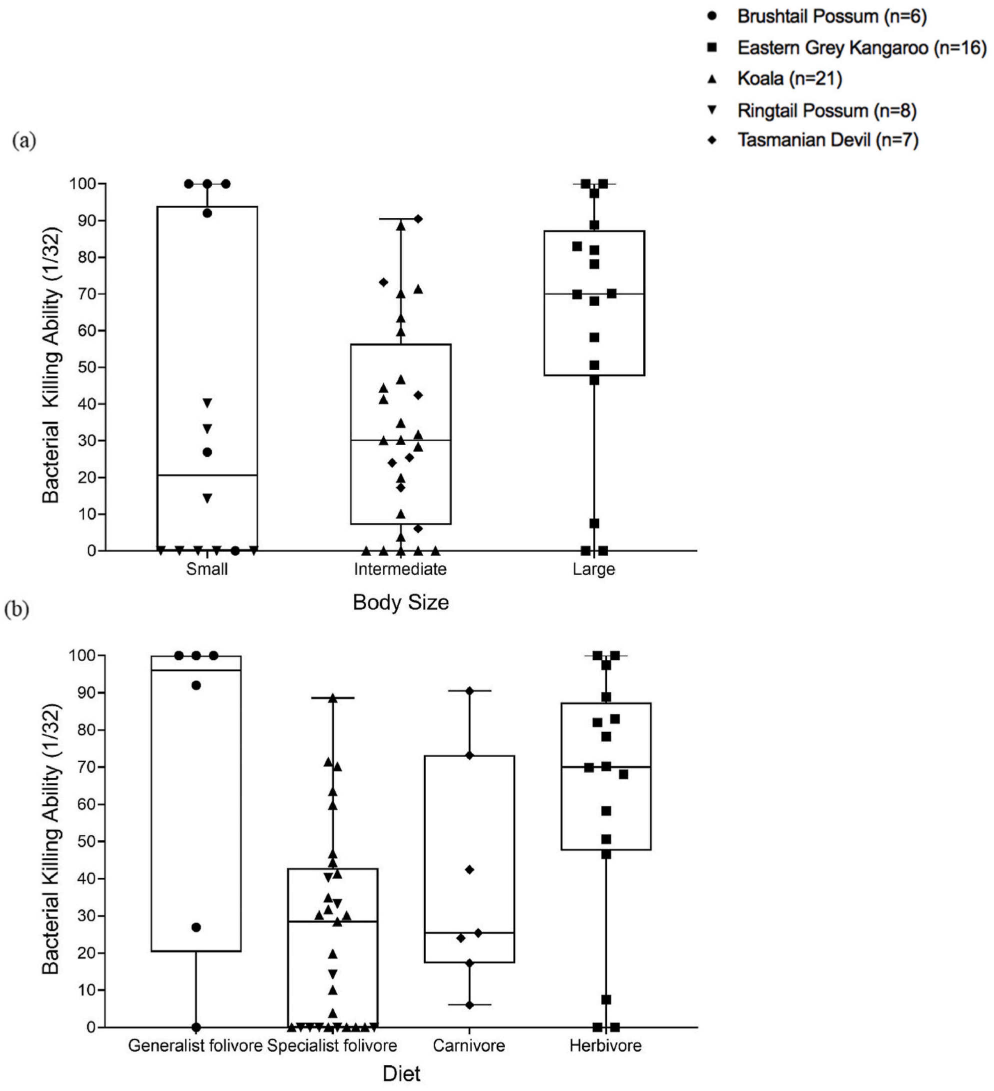

Fig. 4. Mean bacterial killing ability at 1/32 dilution across all species according to (a) diet and (b) body size.

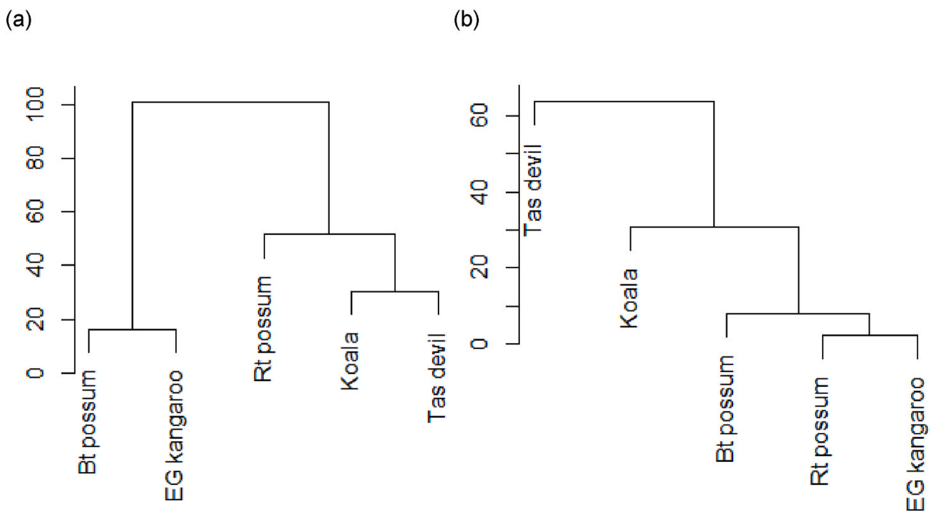

Fig. 5. Comparison of cluster dendograms based on (a) Euclidean distances calculated from mean BKA values for each species across all dilutions and (b) pictorial example based on previously published phylogeny (Mitchell et al., 2014).

consistent with this. There is evidence that sample storage can impact bacterial killing ability (O'Shaugnessy et al., 2012; Schneeberger et al., 2013; Hopkins et al., 2016; Heinrich et al., 2016; Jacobs and Fair, 2016), though our study provided no evidence to support this (see Supplementary Results).

5. Conclusion

Understanding pathogen resilience in wildlife is important for biodiversity and biosecurity, in order to predict vulnerabilities to disease in the future. Assessing constitutive innate immunity is an informative way of understanding how evolutionary and life histories may shape immunological trade-offs in different groups of animals. As a foundational study, this investigation found no evidence that captivity, sex, social structure or disease status have an impact on the bacterial killing ability of marsupials, however the effect of captivity status warrants further investigation. Rather, the study raises interesting questions about immunological trade-offs, particularly in relation to diet and body size. Further work should focus on comparing how the other branches of the immune system interact in these species and how this relates to life history strategy.

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Abbreviations

BKA	bacterial killing ability
CFU	colony forming units
GLM	generalised linear model
PBS	phosphate buffered saline,
RPM	revolutions per minute
TSA	tryptic soy agar
TSB	tryptic soy broth

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2022.104399.

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