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Relationships among faecal glucocorticoid metabolites, koala retrovirus and disease across a gradient of human associations

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Abstract

Koalas are now under threat of extinction from anthropological environmental change and the impacts of koala retrovirus (KoRV) induced immunosuppression and chlamydiosis, with these disease and environmental threats potentially interconnected through the effects of physiological stress. In this study, we investigated the associations among glucocorticoid metabolites (FGMs: cortisol, corticosterone and tetrahydrocortisol), KoRV load, chlamydiosis and two koala herpes viruses (PcGHV-1 and 2) across a landscape gradient of human modification/activity on the mid-north coast of NSW, Australia. Contrary to previous studies, FGM levels were not significantly correlated with any disease measures, which may be related to small sample sizes and baseline differences between individuals in hormone concentrations due to proposed intrinsic and extrinsic factors identified here. Also contrary to previous studies, KoRV load was not significantly correlated with *Chlamydia* score or body condition, potentially due to spatial variation in chlamydia prevalence. KoRV load was significantly correlated with adjusted tetrahydrocortisol levels and covaried with faecal corticosterone metabolites across the region. We found that koalas on private properties in rural/agricultural areas had significantly higher faecal corticosterone metabolite levels than koalas captured in peri-/urban areas, with the same trend seen for faecal cortisol metabolites. Koalas from peri-/urban areas had lower *Chlamydia* scores than those in national parks and koalas further from population centres had higher KoRV loads. Together these findings suggest that koalas may have the capacity to cohabitate with humans in the peri-/urban space, where sufficient suitable habitat is provided, despite the presence of conspicuous threats. Furthermore, the lower rates of disease observed in peri-/urban koalas suggests that wildlife hospitals can potentially make a meaningful difference to local wildlife population health.

INTRODUCTION

Koalas are now under threat of extinction, with wild populations in the Australian states of New South Wales (NSW) and Queensland (QLD) in steep decline[1]. The primary driver of this decline is anthropological environmental change, including habitat loss, habitat fragmentation, increased interaction with livestock and domestic animals, as well as climate change that has led to an increased severity in stochastic events such as bushfires, heatwaves, and droughts. In NSW and QLD, koala populations are also heavily impacted by *Chlamydia*-induced disease, that threaten population viability through high rates of infertility and mortality[2]. Koala retrovirus (KoRV) is endemic throughout these populations and KoRV plasma RNA load is strongly associated with the disease pathologies afflicting koalas[3,4]. We hypothesize that environmental stressors affecting koalas are intrinsically interlinked to KoRV load and disease, indicating the need for a holistic approach to conservation management of the species.

KoRV plasma RNA load can vary between koalas by 6-orders of magnitude[4]. As with other retroviruses[5], viral replication is suppressed by pre- and post-transcriptional silencing[6] and stimulated by transcription factors. For other retroviruses these transcription factors include a glucocorticoid response element that is heightened by physiological stress[5,7]. We have recently shown that this glucocorticoid response element is present in the KoRV proviral genome and that average KoRV plasma RNA load is correlated with average faecal cortisol and corticosterone metabolite levels, which have been used as indicators of physiological stress and are involved in homeostasis[3]. Thus, environmental factors leading to chronic physiological stress[8] likely impair the koala's ability to control KoRV replication resulting in immune depletion and the manifestation of disease. Further, chronic stress can directly lead to immune suppression and an increased susceptibility to infectious diseases[9–11]. As such, KoRV likely amplifies the direct negative effects of environmental stresses on koala health[12]. In this study, we investigated whether faecal glucocorticoid metabolites (FGMs: cortisol, corticosterone and tetrahydrocortisol) were associated with KoRV load and disease across a landscape gradient of human modification/activity.

Physiological stress in koalas has previously been assessed by faecal glucocorticoid metabolites (FGM) that provide an integrated measure of adrenal function and glucocorticoid production over days[8,13]. In one study examining FGM levels in koalas brought in for rehabilitation, elevated FGM levels were associated with clearance of *Eucalyptus* habitat, bushfire-related factors (dehydration and burns injury) and physical injury caused by vehicle collision or dog attacks[8]. This suggests that koalas living in peri-/urban landscapes that are exposed to vehicles, dogs and reduced tree cover may also experience a higher prevalence of chronic stressors than those living in undisturbed habitat. However, to date FGM levels have not been systematically tested between koalas living in areas with different levels of human association or variation in environmental factors to determine what constitutes an environmental stressor to a koala.

In our previous work on the phylogeography of KoRV across the koala's natural range, we determined that different KoRV subtypes are found in different regions[14], likely due to the short range dispersal of kin producing positive spatial genetic structure[15,16] and limiting the spread of locally generated subtypes. KoRV exists as both an endogenous virus, integrated into the koala germline, and as a wide diversity of exogenous subtypes that are primarily acquired from the mother at an early age[17–19]. The exogenous KoRV subtypes are correlated with KoRV plasma RNA load and both KoRV load and the exogenous subtypes are independently associated with disease in northern koala populations[4]. As such, koala populations in different regions may have differing KoRV loads, irrespective of the extent of environmental stress, due to differing subtype profiles. Therefore, we focused our comparisons on koalas from a single region, the mid-north coast of NSW, Australia.

Wild koala populations on the NSW mid-north coast are facing some of the highest rates of decline due to the black summer fires in 2020 as well as their proximity to human populations. We compared KoRV load, FGM levels, and *Chlamydia* infection and disease between koalas living in: (*i*) minimally disturbed forest habitat of national parks; (*ii)* agricultural/rural properties with low exposure to humans, primarily constituting grazing pasture with small stands of forest, (*iii*) those in peri-urban and urban environments which have more regular interactions with human-related activities, (*iv*) exhibit koalas at Port Macquarie and Port Stephens Koala Hospitals that were wild born but non-releasable and now habituated to captivity, and (*v*) koalas rescued from dangerous situations (on roads or in yards with dogs etc.) and brought to Port Macquarie Koala Hospital for assessment and treatment where needed. We also tested the wild caught koalas (cohorts *i – iii*) for phascolarctid gammaherpesvirus 1 and 2 (PcGHV-1 and PcGHV-2), which are recently characterised koala pathogens whose role in koala disease is beginning to be better appreciated[20–25]. In this study, we (1) investigated how FGM levels, KoRV and disease

(*Chlamydia* or herpes viruses) differed with the level of human activity (measured by category and distance to population centres); (2) determined if FGM levels and KoRV load are related on the regional level; and (3) assessed if KoRV load and disease (*Chlamydia* or herpes viruses) are related on the regional scale. In a preliminary analysis, we also investigated which fine-scale environmental factors were associated with higher FGM levels and KoRV load, as well as higher rates of chlamydial disease (see supplementary material).

RESULTS

Koalas

A total of 100 koalas (females = 56, males = 42, unknown = 2) were sampled as part of this study. Of these, 54 were wild caught on the NSW mid-north coast and included in the association analysis, 14 were exhibit koalas at Port Macquarie Koala Hospital or Port Stephens Koala Hospital and 32 were koalas rescued from danger in the Port Macquarie region. All wild caught and exhibit koalas were sampled outside the koala breeding season (March-July), while 24 of the koalas rescued from danger were sampled during the breeding season (September-March). The full range of tooth wear classes (1 to 7) were observed across the study koalas with 21 juveniles (tooth wear class 1–2) and 11 aged koalas (tooth wear 6–7) sampled. Overall, the majority of koalas were in moderate condition (average body condition score = 3.6 out of 5), although there were 7 koalas in very poor condition (BCS: 1–2). These poor condition koalas were found in or to the north of Port Macquarie, while koalas in fair and good condition were found across the study region (Figure S1).

FGM levels and disease prevalence

FGM levels

There are three FGM assays that have been used to assess putative stress levels in koalas that target different glucocorticoid metabolites; cortisol, corticosterone and tetrahydrocortisol. All these assays have been validated in different ways as indicators of changes in glucocorticoids (see methods) yet given their differing targets may show different responses to koala physiological states. As such, we used all three assays in this study to gain a comprehensive picture of koala glucocorticoid profiles across a range of human associations and disease states. Faecal cortisol metabolite concentrations ranged from 33.8 to 250.3 ng/g across the sampled koalas, while faecal corticosterone metabolites varied from 89.3 to 723.2 ng/g. Faecal tetrahydrocortisol ranged from 8.6 to 463.1 ng/g. However, there were two koalas from different national parks that had outlying high values for tetrahydrocortisol (463.1 and 217.8), which were removed from further analyses as they skewed the data distribution. After removal of these outliers the maximum value for tetrahydrocortisol was 158.2. Sex differences in faecal tetrahydrocortisol concentrations have previously been reported for koalas[26,27] and males in this study also had significantly higher faecal tetrahydrocortisol concentrations than females (t-test, $p < 0.001$). The full range of all FGMs was observed across the study region (Figs. 1A, S2 and S3).

Koala Retrovirus

KoRV RNA load in plasma ranged from 10^3 to 10^8 copies per ml across all samples with the full range observed over the study area. The distribution of values observed in the Port Macquarie exhibit koalas accorded well with that observed among the wild caught koalas in Port Macquarie (Fig. 2A). The koalas rescued from danger had significantly higher KoRV loads than the wild caught koalas from the local area ($p = 0.007$). However, this may have been the result of those samples being collected during the breeding season, which we have previously found to be associated with elevated KoRV loads [3]. KoRV load was generally low (Log10 KoRV load: 3.46–5.34) among the exhibit koalas from Port Stephens.

KoRV-A was detected in all koalas tested and ranged in relative abundance from 0.05% to 100%. Notably, all exhibit koalas from Port Stephens Koala Hospital only carried KoRV-A. KoRV-D was found in 60 of the koalas and reached high relative abundances of up to 99.95% in some koalas. KoRV-D was found throughout the study region (Figure S4). KoRV-B was detected in 33 koalas across the entire study region (Figure S5) and reached high relative abundances in some koalas of up to 96.9%. KoRV-M was found in eight koalas, that included two koalas from Port Macquarie Koala Hospital and six wild koalas captured between Port Macquarie and Kempsey (Fig. 3A). KoRV-M reached a maximum relative abundance of 90.5%. KoRV-H was detected in two koalas that were located inland from Coffs Harbour (Fig. 3A) and reached a maximum relative abundance of 23.7%. No other KoRV subtypes were detected in the region.

Chlamydia pecorum infection and chlamydiosis

C. pecorum was detected in the ocular swabs of 20 of the 73 (27.4%) koalas that returned a valid testing result (koala beta actin DNA positive) and from 34 of the 80 (42.5%) urogenital swabs, with 12 koalas testing positive at both body sites. *C. pecorum* infection was found throughout the study region (Figures S6 and S7). A total of 18 of the 86 (20.9%) koalas assessed (exhibit koalas were excluded) had ocular pathology, while 13 (15.1%) showed external signs of urogenital pathology (wet bottom). These pathologies occurred throughout the study region, although there was a particularly high incidence of ocular pathology in Limeburner's Creek National Park to the north of Port Macquarie (Figures S8 and S9). There was a single case where a koala rescued from a dangerous situation (e.g. on road or in backyard with large dog) had ocular pathology but tested negative for ocular *C. pecorum* infection. There was also one case of a female wild caught koala from Tinonee that had external signs of urogenital pathology that tested negative to urogenital *C. pecorum*.

Phascolarctid gammaherpesvirus 1 and 2

A total of 27 of the 51 (52.9%) wild caught koalas to return a valid testing result (koala beta actin DNA positive) were positive for PcGHV-1, while 34 of 52 (65.4%) had PcGHV-2 detected. These results came from pooled ocular and urogenital swabs, so it was not possible to define which body site was actively

shedding. Further, due to pooling of samples, a false negative could occur if the DNA from the infected body site swab was below quantifiable level but the DNA from the other body site swab was beta actin positive, meaning it would pass quality assurance. Both herpes viruses were detected across the study region (Figures S10 and S11).

Other infectious pathogens

Bordetella bronchiseptica was detected in the nasal swab of one (2.4%) male koala captured at Limeburner's Creek National Park from among the 41 nasal swabs that were koala beta actin positive. *Mycoplasma* spp. was also detected in a single (3.4%) swab from a male captured in Ulidarra National Park (Coffs Harbour, NSW) from among the 29 pharyngeal swabs that were koala beta actin positive. *Ureaplasma* spp. and *C. pneumoniae* were not detected in any swab types.

Variation in FGM Levels and pathogens among koalas with different human associations

Faecal corticosterone metabolite levels were found to be significantly higher among koalas that were sampled from private rural properties compared with koalas captured in peri-/urban areas, koalas that were rescued from danger or the exhibit koalas at Port Macquarie and Port Stephens koala hospitals (Table 1, Fig. 1B). Koalas captured in national parks also had significantly higher faecal corticosterone metabolite levels compared with the exhibit koalas. While koalas on rural private properties also had the highest faecal cortisol metabolite levels and the exhibit koalas the lowest, these differences were not significant when the study site was included as a random covariate in the analysis. In comparison to corticosterone and cortisol metabolite levels, there was little difference in faecal tetrahydrocortisol concentrations between the different cohorts, although the exhibit koalas did have marginally lower values than the other cohorts (Table 1).

Table 1
FGM levels, disease prevalence and severity by habitat category/ koala cohort

Category	n	Sex F:M	Average Tooth wear class	Average faecal corticosterone metabolites	Average faecal cortisol metabolites	Average adjusted faecal tetrahydro-cortisol[4]	Average KoRV load	Average percentage of KoRV load KoRV-A	Average Chlamydia Score	Proportion PcGHV-1 positive	Proportion PcGHV-2 positive
National Parks	21	14:7	3.62	199.32[3]	90.80[3]	75.90	5.30	64.07	1.86	0.50[3]	0.76
Rural	17	9:8	2.97	337.82[3]	123.92[3]	72.30	5.97	28.27[3]	0.94	0.63[3]	0.40[1]
Peri-/Urban	16	12:4	4.10	190.24	79.37	71.77	4.86	55.06[3]	0.56	0.47[3]	0.75
Rescued from Danger	8	2:6	2.75	153.65	94.55	70.22	5.83	58.21	0.13	N/A	N/A
Exhibit	14	7:5[1]	3.93[2]	132.25	66.80	59.50	5.04	75.74	N/A	N/A	N/A
p value			0.157	**0.025**	0.190*	0.949[5]	0.241[+]	0.167*	**0.008**	0.637	0.206*

1: value not available for 2 koalas 4. Adjusted for sex differences by - the female mean and + the male mean to all samples from females

2: value not available for 7 koalas 5. Linear regression model of unadjusted values but including koala sex as a covariate

3. value not available for 1 koala 6. value not available for 10 koalas

p value without site included as random factor: + 0.05–0.1, * <0.05, **<0.01, ***<0.0

Koalas captured from national parks had significantly higher *Chlamydia* scores than peri-/urban koalas or koalas rescued from danger (Table 1, Fig. 4B). There was also a non-significant trend for koalas on rural properties to have higher *Chlamydia* scores than koalas rescued from danger (post hoc t-test: p = 0.074). As *Chlamydia* score was tested by proportional odds logistic regression, the study site could not be accounted for as a random effect in the model. Nonetheless, koalas captured from national parks were significantly more likely to have ocular pathology than peri-/urban koalas or koalas on private rural properties with site included as a random effect in the model (Table S8).

There were no other significant differences in any of the disease measures between the cohorts when study site was included as a random covariate in the analysis. There were, however, several differences between the cohorts that were significant if site was not included as a random effect in the analysis (Table 1) that may warrant further investigation with a larger number of study sites. In particular, koalas on private rural properties had higher KoRV loads, and much lower proportions of those loads were KoRV-A. Also, as above, the koalas rescued from dangerous situations still had higher KoRV loads than other peri-/urban koalas, despite those koalas sampled during the breeding season being removed from this analysis (Table 1).

Variation in FGM levels and pathogens among koalas with distance to population centres

Among the wild caught koalas, KoRV load and *Chlamydia* score were both significantly higher the further the koalas were captured from minor or major population centres (*Chlamydia* score: p = 0.037; KoRV load: p = 0.014; Fig. 2B). Whereas, body condition PcGHV-1, PcGHV-2 and all FGMs were not significantly associated with the koalas' distance to population centres (body condition: p = 0.71; PcGHV-1: p = 0.645; PcGHV-2: p = 0.629; cortisol: p = 0.89; corticosterone: p = 0.89; tetrahydrocortisol: p = 0.187).

Associations among FGM levels and pathogens

The associations between the FGMs and pathogens were assessed for the wild caught koalas. The exhibit koalas were not included in the analysis as they had been cleared of identified infectious pathogens and the koalas rescued from dangerous situations were not included as they represented a biased cohort (see above). Among the wild caught koalas, there was a strong positive correlation of 74% (p < 0.001) between faecal cortisol and corticosterone metabolite concentrations, while tetrahydrocortisol was not correlated with either of the other FGMs (Fig. 5A). To account for the higher levels of tetrahydrocortisol in males than females, sex adjusted tetrahydrocortisol concentrations were also included in the analysis (the values for females were increased by subtracting the female mean and then adding the male mean). However, the adjusted tetrahydrocortisol concentrations were also not significantly correlated with the other FGMs. Faecal cortisol metabolites were not significantly correlated with any of the disease measures, koala condition or age. By contrast, koalas with a lower proportion of their KoRV load attributed to KoRV-A had significantly higher faecal corticosterone metabolites levels (p = 0.05), whereas tetrahydrocortisol concentrations were significantly higher in koalas carrying more KoRV subtypes (p = 0.02) and adjusted tetrahydrocortisol concentrations were significantly higher in koalas with higher KoRV loads (p = 0.05). Both adjusted and unadjusted tetrahydrocortisol concentrations were also higher in koalas that were positive for KoRV-B (unadjusted: p = 0.004; adjusted: p = 0.003). In contrast to each other, faecal corticosterone metabolite levels were significantly higher in younger koalas (p = 0.01), while adjusted tetrahydrocortisol concentrations were significantly lower (p = 0.01). Corticosterone metabolite levels were also significantly higher in koalas in better condition (p = 0.04; Fig. 5A).

There was a strong and significant positive correlation between KoRV load and the number of exogenous KoRV subtypes caried by an individual (R^2 = 0.67, p < 0.001). Further, the proportion of KoRV load that was KoRV-A was strongly negatively correlated with both KoRV load (R^2 = 0.68, p < 0.001) and the number of KoRV subtypes carried (R^2 = 0.75, p < 0.001; Fig. 5A). Consistent with this, koalas that were positive for KoRV-B and KoRV-D had significantly higher KoRV loads (p < 0.001), carried more subtypes (p < 0.001–0.01) and a lower proportion of that load originated from KoRV-A (p < 0.001–0.01; Fig. 5A-B). KoRV-B and KoRV-D were also significantly associated with each other (p = 0.035). Older koalas carried significantly more KoRV subtypes and were more likely to be KoRV-B positive (p < 0.05). Interestingly, there was not a significant correlation between KoRV Load and *Chlamydia* score. Although, koalas that were KoRV-M positive had significantly higher *Chlamydia* scores (p < 0.01; Fig. 5B).

Koalas with higher *Chlamydia* scores were in significantly poorer condition (p < 0.001). PcGHV-2 was more prevalent in older koalas (p < 0.001) and older animals had significantly lower body condition (p < 0.001; Fig. 5A).

DISCUSSION

In the first study to systematically investigate faecal glucocorticoid metabolite (FGM) levels and koala retrovirus in wild caught koalas across a landscape gradient of human association, we have revealed that koalas on private properties in rural/agricultural areas have significantly higher faecal corticosterone metabolite levels than koalas captured in peri-/urban areas, with the same trend observed for faecal cortisol metabolite levels; at first glance, this finding may appear counter intuitive. While the majority of rural/agricultural koalas were located using nocturnal drone surveys and the peri-/urban koalas were located by ground searches, the method of location is unlikely to be responsible for the observed differences in these FGM levels between the groups as FGMs provide an integrated measure of glucocorticoid production over days and are not generally impacted by brief acute stessors[8,13]. Further, in alignment with our findings, Narayan and colleagues'[28] analysis of koalas brought in for rehabilitation also found that koalas in urban areas had lower cortisol metabolite levels than those from rural areas, despite their use of a different cortisol enzyme immunoassay. Our assessment of disease rates across the region further showed that koalas from peri-/urban areas had lower *Chlamydia* scores than those in national parks and koalas further from population centres had higher *Chlamydia* scores and KoRV loads. Together these findings suggest that koalas may have the capacity to cohabitate with humans in the peri-/urban space, where sufficient suitable habitat is provided, despite their proximity to and interaction with people, dogs and cars. In fact, in this study on the mid-north coast of NSW, koalas in peri-/urban areas had lower levels of some glucocorticoids and generally better health. In agreement with these findings, koalas located in a green space away from human habitation had higher rates of Chlamydial disease than those in peri-urban areas of Moreton Bay, QLD[29], as did koalas at the rural Hidden Vale site in south-east QLD[30]. By contrast, *Chlamydia* rates have been found to be higher on urbanised Raymond Island compared to Mt. Eccles National Park in Victoria[31]. Further, a study of male koalas found a higher incidence of *Chlamydia* infection and disease in peri-urban areas[32]; however, 69% of the rural koalas included in that study were from central QLD, while all peri-/urban koalas were from south-east QLD. Therefore, it is not clear whether the findings from that study[32] or those from Victoria were due to regional differences or human interaction. Nonetheless, it highlights that the association between koala health and level of human contact may differ between regions based on the nature of those interactions as well as the quality, extent and connectedness of habitat across the anthropological gradient. For instance, FGM levels and disease may be higher where human modification of the landscape leads to high koala densities in isolated habitat patches (see online supplementary material) as was suggested to explain the higher incidence of *Chlamydia* and poor body condition among hospitalised koalas from more urbanised areas in south-east Queensland[33].

The lower incidence of disease in peri-/urban koalas in this study may potentially be due to sick (and injured) koalas being more often identified by members of the public and captured for veterinary attention in peri-/urban areas compared with areas outside of urban centres. In the year of this study, 2023, 156 koalas were admitted to Port Macquarie Koala Hospital and all nine of the koalas captured in Port Macquarie for this study had previously been admitted to the hospital. Admitted koalas either undergo treatment and rehabilitation or are euthanised, preventing onward transmission of infectious pathogens, such as *Chlamydia*, and alleviating suffering, thereby reducing chronic stress levels, which then in turn reduces KoRV loads[3]. While acute threats such as vehicle

collisions, dog attacks and loss of habitat in peri-/urban areas are conspicuous to the public, leading to the perception that wildlife and humans are non-compatible, in fact it appears that suburbia can be a refuge for many species[34]. For instance, the common brushtail possum (*T. vulpecula*) is in decline in rural and arid areas of Australia due to predation by foxes and the effects of drought but is common in suburban areas where there is well watered forage and artificial denning sites[35–38]. Furthermore, the lower rates of disease observed in peri-/urban koalas suggests that wildlife hospitals and carers may be making a meaningful difference to wildlife population health, and in the case of the koala, veterinary interventions outside of urban centres could play a valuable role in conservation efforts.

Interestingly, unlike the other FGMs, tetrahydocortisol concentrations were very similar across the gradient of human associations with only the exhibit koalas showing marginally lower values. Faecal tetrahydrocortisol concentrations were not correlated with faecal corticosterone or cortisol metabolite concentrations, while cortisol and corticosterone showed high concordance. This may be because the tetrahydrocorticosterone assay specifically targets faecal glucocorticoid metabolites[39], whereas the cortisol and corticosterone assays likely detect a range of metabolites in addition to their respective parent compounds. While all these FGM assays have been used as indicators of stress in koalas, corticosterone and cortisol are not only involved in the stress response but also in reproduction and homeostasis[3,40]. Further, the metabolites of these glucocorticoids that are detected in the faeces will likely vary according to the specific degradation and excretion pathways employed, which in turn will be influenced by the koala's physiological state[40]. As such, FGM concentrations are influenced by a range of intrinsic and extrinsic factors in addition to physiological stress[3,40]. Further, the effects of these factors may differ depending on the specific metabolites measured. Here we have confirmed the findings of previous studies[26,27] that tetrahydrocortisol concentrations differ between the sexes, while no sex differences were detected for faecal cortisol or corticosterone metabolites. Once these sex differences were taken into account, we also found, in contrast to a previous study[26], that tetrahydrocortisol concentrations were higher in older koalas, while corticosterone metabolites were at higher concentrations in younger koalas. Further, in our exploratory analysis of the environmental factors that influenced FGM levels (see online supplementary material) higher cortisol and corticosterone levels were associated with colder areas, while tetrahydrocortisol levels were higher in koalas in areas with lower foliar moisture content. Variation in these intrinsic and extrinsic factors across the landscape likely explain why a difference in tetrahydrocortisol concentrations was not seen across the range of human associations. They also likely contributed to why, contrary to the findings of Santamaria and collegues[27], we did not find any significant correlations between FGM levels and any disease measures. Although, disease may have also not been a major driver of FGM levels in this study as koalas in our study were wild caught, as opposed to brought in for rehabilitation, and the extent of their disease symptoms were generally less severe than in other studies.

Despite the effects of non-stress related factors on FGM concentrations, the significant positive relationship between faecal corticosterone metabolite levels and body condition score was unexpected. This result may stem from adrenal exhaustion and senescence in koalas with very poor condition. All koalas with a body condition score of < 3 were either older (tooth wear > 6) or had clinical signs of *Chlamydiosis.* Corticosterone metabolites were found at lower concentrations in aged koalas in this study, which may have contributed to the lower levels seen in poor body condition koalas. Additionally, while it might be expected that acute stress from disease would lead to higher FGM levels as seen in other koala studies[27], the effect of chronic stress on FGM levels is variable based on the species and stressor[41]. For instance, chronic stress as the result of disease has been found to lead to adrenal exhaustion and reduced FGMs in black rhinoceros (*Diceros bicornis*)[42]. As koalas in very poor body condition had likely suffered from chronic disease, adrenal dysfunction may be an additional factor contributing to lower corticosterone metabolite levels in koalas with poor body condition. As such, there is a need for further research into the effects of chronic stress associated with disease on FGM levels in koalas.

In a recent temporal study of wild koalas in south-east Queensland, we revealed that average faecal cortisol and corticosterone levels and average KoRV loads were positively correlated[3]. In this study, KoRV load and these FGMs were not significantly correlated but KoRV load was significantly associated with adjusted tetrahydrocortisol concentrations. The lack of significance for cortisol and corticosterone metabolites may have been the result of both KoRV load and FGM levels being point measures in this study rather than average measures over time. In our previous study, KoRV load was found to be very temporally stable, while FGM levels were labile. Further, while average KoRV loads and FGM levels were correlated, cortisol and corticosterone metabolite levels did not predict within koala variation in KoRV load[3]. Thus, single timepoint measures may not reliably capture the relationship between FGMs and KoRV load. Nonetheless, across the region, KoRV load and faecal corticosterone and cortisol metabolites did appear to be co-distributed, as high levels of both were found in rural areas and were predicted by a similar set of fine-scale environmental factors (see online supplementary materiel). Additionally, the proportion of KoRV load that was KoRV-A was significantly positively correlated with both faecal corticosterone metabolites measures and KoRV load; the number of KoRV subtypes was significantly positively associated with both tetrahydrocortisol concentrations and KoRV load; and KoRV-B carriage was significantly positively associated with unadjusted and adjusted tetrahydrocortisol concentrations as well as KoRV load. While together these findings are suggestive of a positive relationship between koala stress levels and KoRV load, the significance of specific associations may be affected by both the labile nature of FGMs and the impact of intrinsic and extrinsic factors on their baseline levels as discussed above.

This study has confirmed the relationship between KoRV load and the relative abundance of the exogenous subtypes, which was previously found in koalas from south-east Queensland[3,4]. Further, it adds evidence that KoRV load increases as koalas acquire more subtypes and that the number of subtypes a koala carries increases with age. As such, older koalas may be more susceptible to diseases caused by infectious pathogens due to their high KoRV burden and presumed reduced immune system function. Studies have suggested that KoRV causes immune suppression and predisposes koalas to secondary diseases such as chlamydiosis[3,4,43–45]. However, in this study KoRV load was not significantly correlated with *Chlamydia* scores. One possible explanation for this discrepancy is that on the regional scale, rates of *Chlamydia* infection and disease are more influenced by encounter rates with infected individuals, and with the locally prevalent *C. pecorum* strains rather than individual susceptibility[30]. Additionally, even within our study area, we observed geographic differences in the incidence of KoRV subtypes that may have influenced KoRV load and disease across the region. Indeed, previous studies have found that the exogenous KoRV subtypes B, D and D/F increase the risk of secondary disease independently to KoRV load[4,44–46]. In this study, KoRV-M was most prevalent in

Limeburners Creek National Park where the koalas also had a high rate of *C. pecorum* infection (qPCR positivity) and associated pathologies, leading to a significant positive correlation between KoRV-M and *Chlamydia* scores.

The role of Phascolarctid gammaherpesviruses 1 and 2 in koala disease and how they interact with KoRV is largely unknown. PcGHV-1 has been associated with KoRV *pol* positivity in Victorian koalas[20–25,47]. Further, previous studies in Vic, NSW and QLD koalas have found a positive relationship between PcGHV-1 and urogenital *C. pecorum* infection[22,47] and PcGHV-2 has been associated with both poor body condition score and *C. pecorum* infection[21,47]. While there was a positive trend between both PcGHV-1 and 2 with the number of KoRV subtypes, *Chlamydia* scores and poor body condition in our study, these trends were not significant. This may be attributed to the relatively small sample size of this study combined with the higher incidences of both herpes viruses on the NSW mid-north coast compared with other regions. The previously reported higher incidence of PcGHV-1 in older koalas[21,22,47] was also observed in this study but non-significant. We did however confirm a previous report[23] that older koalas are more likely to carry PcGHV-2 adding evidence that PcGHV-2 is horizontally rather than vertically transmitted from mother to offspring as previously postulated[47].

One unexpected finding from this study was that the koalas rescued from danger had the lowest *Chlamydia* scores. However, this finding may be an artifact of how the koalas admitted to Port Macquarie Koala Hospital were classified into the different cohorts in this study. Koalas were only categorized as being rescued from a dangerous situation (e.g. found on a road or in a backyard with a large dog) if that was the reason recorded for their hospital admission. It is likely that if a koala presented to the hospital with symptoms of chlamydiosis then this would have been recorded as their reason for admission, even if they had actually been brought in after being rescued from a dangerous situation. As such, koalas included in the removed from danger cohort were unlikely to have chlamydiosis.

This study has provided valuable insights into how disease and FGMs vary across a gradient of human associations and a preliminary explorative analysis of what fine-scale environmental factors influence FGMs and disease in koalas. It has also provided further evidence that FGM levels and KoRV load are linked across the landscape. It highlights that the different FGMs are influenced by intrinsic and extrinsic factors in different ways and that further research is needed to understand these relationships before FGMs can be used as a reliable measure of koala stress on the landscape scale. While this study provided little support for the role of KoRV in disease susceptibility, potentially due to the substantial effect of regional level factors in this dataset, it has nonetheless, detailed the prevalence of KoRV subtypes, *Chlamydia* infection and herpes viruses on the NSW mid-north coast that will be invaluable for guiding conservation efforts for this endangered species in the region.

MATERIALS AND METHODS

Reporting, ethical clearance and government permits

All methods are reported in accordance with ARRIVE guidelines[48] and were carried out in accordance with relevant guidelines and regulations. All experimental protocols were approved by the relevant institutions (Western Sydney University ethics approval: A15244; and a scientific licence from the NSW Department of Planning, Industry and Environment: SL102726).

Koala cohorts

Wild caught koalas from the mid-north coast of NSW

A total of 54 koalas were captured, screened, and sampled between April and July 2023 from 21 sites on the NSW mid-north coast (northern extent: Coffs Harbour, southern extent: Port Stephens, western extent: Nowendoc; see Figs. 1–4 and Table S9). The sites were located across the coastal plain and escarpment and bounded by the beginning of the tablelands. Of the sites, 21 were captured in National Parks, 17 were located on private rural properties and 16 were sampled from peri-urban areas. National Parks and peri-urban areas were selected for the study based on recorded previous koala sitings and the presence of habitat of high or very high koala suitability based on the NSW Department of Climate Change, Energy, the Environment and Water's model provided by Allen Mcilwee[49]. Private rural property owners were contacted through the local land service's environmental mailing lists. Interested land holders then contacted the researchers and properties which either engaged in some form of agriculture or were adjacent to agricultural land were selected for inclusion in the study.

Koalas were located for capture either through diurnal ground searching with the assistance of binoculars or by nocturnal surveys utilizing thermal camera equipped drones. Drone surveys were completed on 14 nights with up to 125 ha surveyed each night (online supplement: summary of nocturnal drone surveys). Drones were flown along a pre-programmed flight path consisting of parallel linear transects spaced 50 metres apart at a constant height of 65 metres and a maximum speed of 8m/s. When thermal signatures were identified, drones were lowered to allow for better observation to identify the detection from the drone based on appearance (e.g. size of the detection, presence of identifying features such as tails) or behaviour (e.g. location in canopy or on the ground, movement patterns such as hopping). When lowering, a distance of 20 metres from canopy height was maintained to avoid potential disturbances and collisions with arboreal fauna and the tree canopy. Drone surveys were only conducted in national parks and on private rural properties. Koalas in urban areas were spotted by ground searchers only.

Koalas were captured using the safety line and flagging technique [50]. Briefly, once a koala was found, a flag attached to a telescoping pole was gently waved in front of the koala to encourage them to back down the tree. Once the koala moved within reach, a rope loop (with safety stop to prevent overtightening) was placed over their head to prevent the koala from climbing back up the tree. Flags were then used to encourage the koala to move to the bottom of the tree where they could be negotiated into a sack.

The captured koalas were then transported from the location of capture a short distance to a field station for health assessment and sample collection. Koalas were anesthetised by the attending veterinarian using a combination of midazolam premedication (benzodiazepine) at 0.3-0.5mg/kg and Alfaxan (Jurox - Alfaxalone 10mg/ml) at 2mg/kg administered intramuscularly into either of the gluteal or quadraceps muscles. Complete anaesthesia was then achieved via mask with 3–5% isoflurane at an oxygen flow rate of 2–3 L/min. At completion of the procedure the isoflurane was turned to 0% and the koala maintained on oxygen at a flow rate of 2-3L / min until the koala regained consciousness. When able to support their head, the koala was moved into a recovery position within a supported basket and monitored visually. After full recovery from anaesthesia, koalas were released at the point of capture.

The captured koala's general health status was assessed by standardised body condition scoring[51] and the presence of any overt disease pathologies (conjunctivitis, cystitis etc.) was determined by external veterinary examination. Koala age was estimated from tooth wear[52]. Koalas were fitted with an ear tag and microchipped for individual identification. Nineteen (35%) of the captured koalas had clinical signs of chlamydiosis on external examination and were admitted for rehabilitation to the appropriate wildlife hospital or care group. Four of these koalas were subsequently included in our temporal study investigating how antibiotic treatment of chlamydia affects KoRV loads[3].

Exhibit koalas from Port Macquarie and Port Stephens Koala Hospitals

Exhibit koalas were sampled to provide a baseline reference of healthy koalas to assist in the interpretation of our findings from the wild koalas. A single sample was collected from each exhibit koala from both Port Macquarie Koala Hospital (n = 7) and Port Stephens Koala Hospital (n = 7) between May and August 2023. Five of the Port Macquarie koalas were also subsequently included in a temporal study of KoRV load and faecal glucocorticoid metabolites[3]. Samples were collected by veterinary staff during routine health checks or for the purposes of this study. The koalas were wild born from the local area and previously brought in for rehabilitation. However, while these koalas were successfully returned to health, they were deemed non-releasable due to permanent disabilities such as missing claws needed for climbing.

Koalas rescued from dangerous situations

A total of 32 koalas were assessed and sampled by veterinarians at Port Macquarie Koala Hospital between March 2023 and January 2024 that were rescued from dangerous situations by volunteers from the hospital in peri-urban areas. These dangerous situations involved a range of circumstances including but not limited to being on or adjacent to a road, found in a backyard with a large dog or in an area with no suitable habitat such as on a shopping strip. Five of these koalas were then subsequently included in our temporal study investigating how antibiotic treatment of *Chlamydia* affects KoRV loads[3].

Sample collection

Blood samples were collected to assess KoRV plasma RNA load and KoRV subtypes. Blood was drawn by a veterinarian from the cephalic vein either under anaesthesia or during conscious restraint. The blood was then transferred to an EDTA coated tube to prevent clotting and kept on ice in the field. The samples were then centrifuged at 16 000 g for 10 minutes within 12 hours of collection and the plasma collected by pipetting. Samples were stored in liquid nitrogen in the field and then transferred to a -80°C for long term storage in the laboratory.

Faecal samples were collected to assess faecal cortisol and corticosterone metabolite levels. Faecal pellets were either collected from; the base of their capture tree if the koala defecated during capture; the base of the koala's enclosure/transport cage; directly from the koala's cloaca during examination; or from plastic mats placed underneath the koala after release. All pellets were collected within 12 hours of production, placed in zip lock bags and kept on ice in the field. Pellets were transferred to a -18°C car freezer each evening and then stored at -20°C in laboratory.

Urogenital, bi-lateral ocular, pharyngeal and nasal swabs were collected from all wild caught koalas and koalas rescued from danger to test for a range of infectious pathogens including *Chlamydia pecorum*, *C. pneumoniae*, *Mycoplasma spp.*, *Ureaplasma spp.*, *Bordetella bronchiseptica*. The exhibit koalas were not tested for these infectious diseases. *Chlamydia* positivity of the koalas rescued from danger at Port Macquarie Koala Hospital was determined from urogenital and ocular swabs screened by either an in house point-of-care LAMP diagnostic test[53] or by the Koala Health Hub at the University of Sydney on a BioRad CFX (CT cutoff for a positive result < 34, while a weak positive result was recorded for CT values between 34 and 40). All other pathogens were tested for in the koalas rescued from danger as described below. Samples were kept on ice in the field and then frozen at -18°C each night followed by storage in the laboratory at -80°C prior to DNA extraction.

KoRV plasma RNA load and subtype profiles

Total RNA was extracted from the plasma using the High Pure Viral Nucleic Acid Kit (Roche) according to the manufacturer's instructions with modifications as outlined in Blyton et. al.[14]. First strand cDNA was created using Superscript III (Invitrogen) as per Blyton et. al.[14]. KoRV plasma RNA loads were estimated by quantitative PCR of a 110 bp fragment of the KoRV *pol* gene [54] amplified in triplicate from the plasma cDNA as per Blyton et. al.[4] (CT cutoff for detection < 31.5).

An approximately 500 bp region of the KoRV *env* gene containing the previously identified hypervariable domain as described in Chappell et al. [55] was amplified by nested PCR[4] and sequenced on the Illumina Nextseq. The resulting data was then processed in CLC genomics workbench 20 and QIIME 2[56] according to Blyton et. al.[14] to determine which subtypes were carried by each koala and in what proportions.

Faecal cortisol, corticosterone and tetrahydrocortisol metabolite levels

Faecal samples were dried overnight at 65°C, weighed (0.2 ± 0.01g) and extracted with 5mls of 80% methanol by overnight agitation. The samples were centrifuged and the methanol extract decanted and frozen (-20°C) until analysis. Faecal extracts were diluted 1:10 in assay buffer and analysed in duplicate for cortisol metabolite levels using a commercially available cortisol enzyme-immunoassay (EIA; ISWE002; Arbor Assays, USA), corticosterone using an in-

house enzyme-immunoassay (CJM006; Coralie Munro, UC Davis, USA) and tetrahydrocorticosterone (50c)[57] using an in-house enzyme-immunoassay with two control samples run on each plate. Results were then evaluated using a BioTek ELx808 plate reader and Gen5 software. The inter and intra assay variation for the assays were 7.8% and 4.2% for corticosterone, 8.7% and 4.8% for cortisol and 10.4% and 8.2% for tetrahydrocorticosterone respectively.

The suitability of the cortisol EIA (Arbor Assays) for detection of koala faecal cortisol metabolites has previously been evaluated through comparison with another validated cortisol EIA (C.J. Munro, UC Davis, USA)[13,58,59] and we have confirmed that the two assays yield comparable results in our laboratory[60]. An initial evaluation of the corticosterone EIA did not appear to show its suitability for monitoring faecal glucocorticoid metabolites in koalas[13]. However, subsequent assessment of faecal progesterone levels after induced ovulation revealed a lag of 24–48 hrs between production and excretion[61] that is consistent with the timing of a small peak observed in 3 of 4 koalas in faecal corticosterone metabolites during the corticosterone EIA validation by ACTH challenge. This suggests that the assay does detect biologically relevant changes in FGMs and is suitable for use in koalas but has a shorter lag than initially thought. The tetrahydrocorticosterone (50C) assay selectively targets the primary glucocorticoid metabolite present in koala faeces (tetrahydrocortisol) after injection of exogenous cortisol[39]. However, it is unknown if this is the primary metabolite produced from endogenous cortisol production or whether its concentration in the faeces is sensitive to changes in circulating cortisol levels. All three assays were therefore utilised in this study with an assumption that they likely detect different glucocorticoid metabolites due to their distinct antibodies and may differ in their magnitude of change or patterns depending on the driver of the physiological response (and its potential influence on the metabolism and excretion of the hormones).

Multi-pathogen screening

Urogenital, bi-lateral ocular, pharyngeal and nasal swabs were screened for *Chlamydia pecorum, C. pneumoniae, Mycoplasma spp., Ureaplasma spp.* and *Bordetella bronchiseptica* using two multiplex real-time PCR assays[62] on a Rotor-Gene Q (Qiagen) with a CT cutoff of < 35 for a positive result. DNA was extracted from the swabs using the MagJET genomic DNA kit (Thermo Fisher Scientific) following manufactures instructions and then purified using the KingFisher Flex (Thermo Fisher Scientific). Multiplex 1 included detection of the koala beta actin (CT cutoff < 35) as a control to confirm the presence of koala cells on the swab and no qPCR inhibitors.

Phascolarctid gammaherpes virus 1 and 2 testing

DNA extracted from urogenital and pharyngeal swabs for screening on the multi-pathogen qPCR assays was also used to detect phascolarctid gammaherpesvirus 1 and 2 (*Manticavirus phascolarctidgamma*[63]). DNA was diluted at a 1:10 ratio to reduce the impact of PCR inhibitors. Diluted samples were then pooled for each koala prior to conducting the qPCR assay. The PcGHV-1 and PcGHV-2 qPCR assays target the *dpol* gene of each of the two known phascolarctid gammaherpesviruses. Samples were run in duplicate on a Bio-Rad CFX Opus following established protocols[20,23]. Samples are deemed positive for PcGHV-1 if they have a cycle threshold (Ct) value of ≤ 37.5 and melt temperature of 81-81.5ºC. For PcGHV-2 samples are deemed positive if they have a Ct value of ≤ 35–36 and a melt temperature of 87-87.5 ºC. Any ambiguous or unclear samples were repeated on a qPCR run including the housekeeping gene beta-actin to ensure that PCR inhibitors or low DNA concentration was not impacting the prior ambiguous results.

Chlamydia scoring

To model *Chlamydia pecorum* infection across the landscape an ordered factor combing both infection and pathology across body sites (*Chlamydia* score) was formulated as follows: 0 = Not infected with *C. pecorum*, 1 = asymptomatic *C. pecorum* infection at one body site, 2 = asymptomatic *C. pecorum* infection at both body sites, 3 = symptomatic *C. pecorum* infection at one body site, 4 = symptomatic *C. pecorum* infection at one body site and asymptomatic *C. pecorum* infection at the other body site; 5 = symptomatic *C. pecorum* infection at both body sites.

Statistical Analyses

The correlation matrices of FGMs, KoRV, *Chlamydia* scores, PcGHV-1 and PcGHV-2, body condition and tooth wear were generated using prism. R^2 values were only reported for continuous or integer variables, while only the direction and strength of the association were indicated for the binary variables. The significance of the associations were determined by linear regression in prism for two continuous variables, by Wilcoxon rank sum tests for a continuous and binary variable and logistic regression for two binary variables using the stats package in R[64].

For the analysis of how FGM levels and disease measures varied between the different koala cohorts, only koalas sampled outside the koala breeding season were included to remove any potential breeding season effects that have previously been observed for KoRV load and FGM levels[3,26]; this restricted the sample size of the koalas rescued from danger to eight koalas. Significant differences among the different koala cohorts in FGM levels and diseases were determined by fitting mixed effects models using lme4 package[65] in R[64], with the cohort fitted as the explanatory variable and sampling site fitted as a random effect. For the model of faecal tetrahydrocortisol concentrations, sex was included as a fixed covariate to account for the documented higher levels in males. Continuous variables were fitted using a linear (gaussian) model with appropriate transformation (none, square root or rank) such that the resulting residuals of the model conformed to normality (as assessed using the Shapiro-Wilkes test). Count and binary responses were fitted using generalised linear models with Poisson or Logit link function, respectively. Body condition score and *Chlamydia* score was fitted as an ordered factor in a proportional odds logistic regression model using the MASS package[66,67] in R, which did not allow for the inclusion of random effects. The significance of pairwise comparisons for *Chlamydia* score and body condition score was determined using the eemeans package[68] in R.

To assess if disease, FGM levels or body condition varied by the koalas' distance to population centres, regression models were constructed in R. Ordered logistic regression was used to assess body condition and *Chlamydia* score (MASS package[66,67]), while generalised linear models with Logit link functions were used to assess PcGHV-1 and PcGHV-2 (stats package) and linear models were used to assess the FGMs and KoRV load. In each analysis the distance

to all major and minor population centres was used as the explanatory variable. This measure was calculated by Allen Mcilwee (NSW Government Department of Climate Change, Energy, the Environment and Water) using an existing corporate towns mapping layer in ArcGIS. For the KoRV load analysis the number of KoRV subtypes carried by a koala was included as a covariate to account for geographic differences in KoRV subtype prevalence (lme4 package[65]).

Declarations

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Author Contribution

MB and KC designed the study and MB, TK, SJ and KC acquired funding. MB managed the project, undertook sample collection, performed the koala retrovirus laboratory analysis, completed the formal statistical analysis and wrote the manuscript. TK undertook sample collection and performed the FGM laboratory analysis. LH undertook sample collection and performed the multiplex pathogen screening. BW performed the koala herpes virus testing. AA and SF completed the veterinary assessments and assisted in sample collection. All authors reviewed and edited the manuscript.

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Data Availability

The KoRV env amplicon sequencing data is available through NCBI's SRA database under Bioproject number PRJNA1333673 (Accession numbers: SRR35579049 to SRR35579137). Tabulated data including koala location, demographic traits, health assessments, pathogen KoRV loads, screening results and FGM concentrations can be obtained from the New South Wales Government's SEED Database: https://www.seed.nsw.gov.au/

References

1. Adams-Hosking, C. et al. Use of expert knowledge to elicit population trends for the koala (Phascolarctos cinereus). *Divers. Distrib.* **22** 10.1111/ddi.12400 (2015).
2. Robbins, A., Hanger, J., Jelocnik, M., Quigley, B. L. & Timms, P. Longitudinal study of wild koalas (Phascolarctos cinereus) reveals chlamydial disease progression in two thirds of infected animals. *Sci. Rep.* **9**, 1–9 (2019).
3. Blyton, M. D. J. et al. Temporal dynamics of koala retrovirus plasma RNA load in relation to faecal glucocorticoid metabolites and Chlamydia infection. *J. Gen. Virol.* **106** 10.1099/jgv.0.002147 (2025).
4. Blyton, M. D. J., Pyne, M., Young, P. & Chappell, K. Koala retrovirus load and non-A subtypes are associated with secondary disease among wild northern koalas. *PLoS Pathog.* **18**, e1010513 (2022).
5. Pluta, A., Jaworski, J. P. & Cortes-Rubio, C. N. Balance between Retroviral Latency and Transcription: Based on HIV Model. *Pathogens* **10** 10.3390/pathogens10010016 (2020).
6. Yu, T. et al. The piRNA Response to Retroviral Invasion of the Koala Genome. *Cell* 179, 632–643 e612, (2019). 10.1016/j.cell.2019.09.002
7. Pluta, A., Jaworski, J. P. & Douville, R. N. Regulation of Expression and Latency in BLV and HTLV. *Viruses* 12, (2020). 10.3390/v12101079
8. Narayan, E. Physiological stress levels in wild koala sub-populations facing anthropogenic induced environmental trauma and disease. *Sci. Rep.* **9**, 1–9 (2019).
9. McLean, R. G. Potentiation of keystone virus infection in cotton rats by glucocorticoid-induced stress. *J. Wildl. Dis.* **18**, 141–148. 10.7589/0090-3558-18.2.141 (1982).
10. Martin, L. B. Stress and immunity in wild vertebrates: timing is everything. *Gen. Comp. Endocrinol.* **163**, 70–76. 10.1016/j.ygcen.2009.03.008 (2009).
11. Gouin, J. P., Glaser, R., Malarkey, W. B., Beversdorf, D. & Kiecolt-Glaser, J. Chronic stress, daily stressors, and circulating inflammatory markers. *Health Psychol.* **31**, 264–268. 10.1037/a0025536 (2012).
12. Blyton, M., Pyne, M., Young, P. & Chappell, K. Koala retrovirus load and non-A subtypes are associated with secondary disease among wild northern koalas. *PLoS Pathog.* **18**, e1010513 (2022).

13. Johnston, S. et al. Preliminary study of faecal cortisol and corticosterone as an index of acute cortisol secretion in the koala (Phascolarctos cinereus). *Aust. Vet. J.* **91**, 534–537 (2013).
14. Blyton, M. D. J., Moore, B. D., Young, P. & Chappell, K. Geographic patterns of koala retrovirus genetic diversity, endogenization and subtype distributions *Proceedings of the National Academy of Sciences* 119, e2122680119 (2022).
15. Blyton, M. D. J., Banks, S. C. & Peakall, R. The effect of sex-biased dispersal on opposite-sexed spatial genetic structure and inbreeding risk. *Molecular Ecology* (2015).
16. Blyton, M. D., Shaw, R. E., Peakall, R., Lindenmayer, D. B. & Banks, S. C. The role of relatedness in mate choice by an arboreal marsupial in the presence of fine-scale genetic structure. *Behav. Ecol. Sociobiol.* **70**, 313–321 (2016).
17. Tarlinton, R. E., Meers, J. & Young, P. R. Retroviral invasion of the koala genome. *Nature* **442**, 79–81. 10.1038/nature04841 (2006).
18. Joyce, B. A., Blyton, M. D. J., Johnston, S. D., Young, P. R. & Chappell, K. J. Koala retrovirus genetic diversity and transmission dynamics within captive koala populations. *Proc. Natl. Acad. Sci. U S A*. **118** 10.1073/pnas.2024021118 (2021).
19. Quigley, B. L., Ong, V. A., Hanger, J. & Timms, P. Molecular dynamics and mode of transmission of koala retrovirus as it invades and spreads through a wild Queensland koala population. *J. Virol.* **92**, e01871–e01817 (2018).
20. Wright, B. R. et al. Development of diagnostic and point of care assays for a gammaherpesvirus infecting koalas. *Plos one*. **18**, e0286407 (2023).
21. Kasimov, V. et al. Identification and prevalence of phascolarctid gammaherpesvirus Types 1 and 2 in South Australian koala populations. *Viruses* **12**, 948 (2020).
22. Wright, B. R. et al. Expanding the known distribution of phascolartid gammaherpesvirus 1 in koalas to populations across Queensland and New South Wales. *Sci. Rep.* **14**, 1223 (2024).
23. Church, C. et al. New insights into the range and transmission dynamics of a koala gammaherpesvirus, phascolarctid gammaherpesvirus 2. *Scientific Reports* **15** (2025).
24. Vaz, P. et al. Detection of a novel gammaherpesvirus in koalas (Phascolarctos cinereus). *J. Wildl. Dis.* **47**, 787–791 (2011).
25. Vaz, P. et al. Detection of a second novel gammaherpesvirus in a free-ranging koala (Phascolarctos cinereus). *J. Wildl. Dis.* **48**, 226–229 (2012).
26. Santamaria, F., Palme, R., Schlagloth, R., Klobetz-Rassam, E. & Henning, J. Seasonal variations of faecal cortisol metabolites in koalas in South East Queensland. *Animals* **11**, 1622 (2021).
27. Santamaria, F. et al. The Effect of Disease and Injury on Faecal Cortisol Metabolites, as an Indicator of Stress in Wild Hospitalised Koalas, Endangered Australian Marsupials. *Veterinary Sci.* **10**, 65 (2023).
28. Narayan, E. & Vanderneut, T. Physiological Stress in Rescued Wild Koalas Are Influenced by Habitat Demographics, Environmental Stressors, and Clinical Intervention. *Front. Endocrinol.* **10** 10.3389/fendo.2019.00018 (2019).
29. Quigley, B. L., Carver, S., Hanger, J., Vidgen, M. E. & Timms, P. The relative contribution of causal factors in the transition from infection to clinical chlamydial disease. *Sci. Rep.* **8**, 8893 (2018).
30. Robbins, A., Hanger, J., Jelocnik, M., Quigley, B. L. & Timms, P. J. S. r. Koala immunogenetics and chlamydial strain type are more directly involved in chlamydial disease progression in koalas from two south east Queensland koala populations than koala retrovirus subtypes. *Sci. Rep.* **10**, 1–13 (2020).
31. Patterson, J. L. et al. The prevalence and clinical significance of Chlamydia infection in island and mainland populations of Victorian koalas (Phascolarctos cinereus). *J. Wildl. Dis.* **51**, 309–317 (2015).
32. Hulse, L. S. et al. Epidemiology of Chlamydia-induced reproductive disease in male koalas (Phascolarctos cinereus) from southeast Queensland, Australia as assessed from penile urethral swabs and semen. *J. Wildl. Dis.* **56**, 82–92 (2020).
33. McAlpine, C. et al. Time-delayed influence of urban landscape change on the susceptibility of koalas to chlamydiosis. *Landscape Ecol.* **32**, 663–679 (2017).
34. Bateman, P. W. & Fleming, P. A. Big city life: carnivores in urban environments. *J. Zool.* **287**, 1–23 (2012).
35. Matthews, A., Lunney, D., Waples, K. & Hardy, J. *Brushtail possums:Champion of the suburbs or Our tormentors*. (2004).
36. Eymann, J., Herbert, C. A. & Cooper, D. W. Management issues of urban common brushtail possums Trichosurus vulpecula: a loved or hated neighbour. *Australian Mammalogy*. **28**, 153–171 (2006).
37. How, R. & Hillcox, S. Brushtail possum, Trichosurus vulpecula, populations in south-western Australia: demography, diet and conservation status. *Wildl. Res.* **27**, 81–89 (2000).
38. Goldingay, R. L. & Jackson, S. M. *The biology of Australian possums and gliders* (Surey Beatty and Sons Chipping Norton, NSW, 2004).
39. Santamaria, F. et al. Identification of koala (Phascolarctos cinereus) faecal cortisol metabolites using liquid chromatography-mass spectrometry and enzyme immunoassays. *Metabolites* **11**, 393 (2021).
40. Millspaugh, J. J. & Washburn, B. E. Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. *Gen. Comp. Endocrinol.* **138**, 189–199 (2004).
41. Dickens, M. J. & Romero, L. M. A consensus endocrine profile for chronically stressed wild animals does not exist. *Gen. Comp. Endocrinol.* **191**, 177–189 (2013).
42. Dorsey, C., Dennis, P., Guagnano, G., Wood, T. & Brown, J. L. Decreased baseline fecal glucocorticoid concentrations associated with skin and oral lesions in black rhinoceros (Diceros bicornis). *J. Zoo Wildl. Med.* **41**, 616–625 (2010).
43. Legione, A. R. et al. Koala retrovirus genotyping analyses reveal a low prevalence of KoRV-A in Victorian koalas and an association with clinical disease. *J. Med. Microbiol.* **66**, 236–244 (2017).

44. Chaban, B., Ong, V. A., Hanger, J. & Timms, P. Molecular dynamics and mode of transmission of Koala Retrovirus (KoRV) as it invades and spreads through a wild Queensland koala population. *J. Virol.* **92** (5), e01871–e01817. 10.1128/JVI.01871-17 (2017).
45. Waugh, C. A. et al. Infection with koala retrovirus subgroup B (KoRV-B), but not KoRV-A, is associated with chlamydial disease in free-ranging koalas (Phascolarctos cinereus). *Sci. Rep.* **7**, 134. 10.1038/s41598-017-00137-4 (2017).
46. Xu, W. et al. An exogenous retrovirus isolated from koalas with malignant neoplasias in a US zoo. *Proceedings of the National Academy of Sciences* 110, 11547–11552 (2013).
47. Vaz, P., Legione, A., Hartley, C. & Devlin, J. Detection and differentiation of two koala gammaherpesviruses by use of high-resolution melt (HRM) analysis reveals differences in viral prevalence and clinical associations in a large study of free-ranging koalas. *J. Clin. Microbiol.* **57** 10.1128/jcm (2019). 01478 – 01418.
48. Du Sert, N. P. et al. Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0. *PLoS Biol.* **18**, e3000411 (2020).
49. State Government of NSW and NSW Department of Climate Change, E., the Environment and Water 2019. NSW Department of Climate Change, Energy, the Environment and Water, (2025).
50. Madani, G., Ashman, K., Mella, V. & Whisson, D. A review of the 'noose and flag'method to capture free-ranging koalas. *Australian mammalogy*. **42**, 341–348 (2020).
51. Carrick, F. & Wood, A. in *12th annual conference of wildlife disease association, Australian Section.* 12.
52. Martin, R. W. Age-specific fertility in three populations of the koala, *Phascolarctos cinereus* Goldfuss, in Victoria. *Wildl. Res.* **8**, 275–283 (1981).
53. Hulse, L. S., McDonald, S., Johnston, S. D. & Beagley, K. W. J. M. Rapid point-of-care diagnostics for the detection of Chlamydia pecorum in koalas (Phascolarctos cinereus) using loop-mediated isothermal amplification without nucleic acid purification. *Microbiol. Open.* **8**, e916 (2019).
54. Kayesh, M. E. H. et al. Molecular dynamics of koala retrovirus infection in captive koalas in Japan. *Arch. Virol.* **164**, 757–765 (2019).
55. Chappell, K. et al. Phylogenetic diversity of koala retrovirus within a wild koala population. *J. Virol.* **91**, e01820–e01816 (2017).
56. Bolyen, E. et al. Reproducible, interactive, scalable, and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* **37**, 852–857 (2019).
57. Quillfeldt, P. & Möstl, E. Resource allocation in Wilson's storm-petrels Oceanites oceanicus determined by measurement of glucocorticoid excretion. *Acta ethologica*. **5**, 115–122 (2003).
58. Pahuja, H. K. & Narayan, E. J. Comparing the agreement of a commercial cortisol kit with a biologically validated assay in evaluating faecal cortisol metabolite levels in koala joeys. *Comp. Biochem. Physiol. A: Mol. Integr. Physiol.* **276**, 111353 (2023).
59. Narayan, E. J., Webster, K., Nicolson, V., Mucci, A. & Hero, J. M. Non-invasive evaluation of physiological stress in an iconic Australian marsupial: the Koala (Phascolarctos cinereus). *Gen. Comp. Endocrinol.* **187**, 39–47 (2013).
60. Keeley, T. & Johnston, S. in *68th Annual Scientific Meeting of the Australian Mammal Society*. (Australian Mammal Society).
61. Keeley, T., Barnes, M., Mucci, A., Seaton, J. & Johnston, S. D. in *Proceedings of the Australian Mammal Society Conference.*
62. Hulse, L. S. et al. Development and application of two multiplex real-time PCR assays for detection and speciation of bacterial pathogens in the koala. *J. Vet. Diagn. Invest.* **30**, 523–529 (2018).
63. Zerbini, F. M. et al. Changes to virus taxonomy and the ICTV Statutes ratified by the International Committee on Taxonomy of Viruses (2023) FM Zerbini et al. *Arch. Virol.* **168**, 175 (2023).
64. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, (2012).
65. Bates, D., Mächler, M., Bolker, B. & Walker, S. Fitting Linear Mixed-Effects Models Using lme4. *J. Stat. Softw.* **67**, 1–48. 10.18637/jss.v067.i01 (2015).
66. Venables, W. N. & Ripley, B. D. *Modern applied statistics with S-PLUS* (Springer Science & Business Media, 2013).
67. Venables, W. N. & Ripley, B. D. *Modern Applied Statistics with S*. Fourth Edition edn, (2002).
68. emmeans: Estimated Marginal Means, aka Least-Squares Means v. R package version 2.0.1. (2025).

Figures

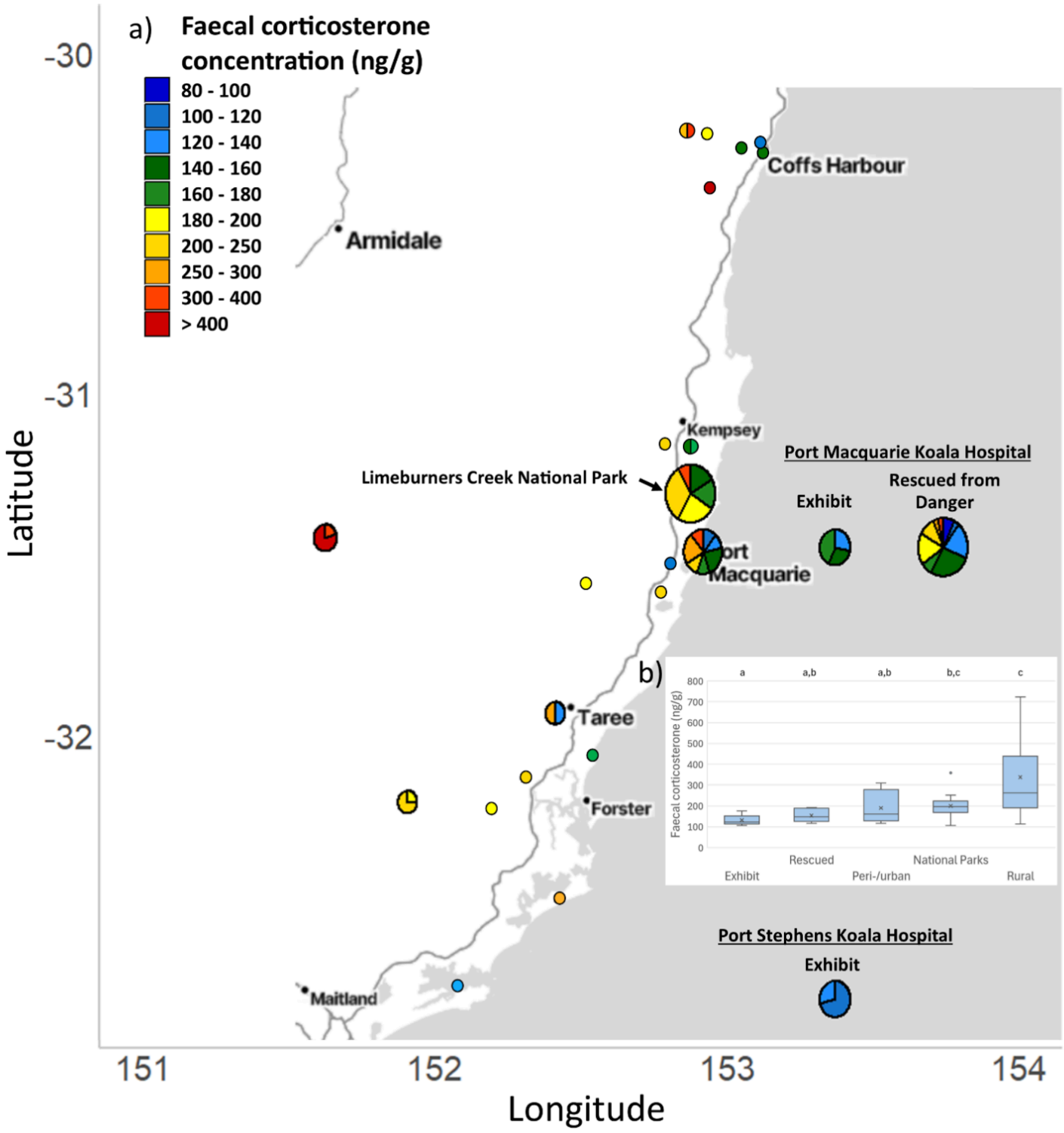


Figure 1

a) Faecal corticosterone metabolite concentrations (ng/g) across the study region. The size of the pie charts is indicative of the sample size at each study site. b) Box plots of faecal corticosterone metabolite concentrations (ng/g) by Cohort. X indicates the mean for each cohort.

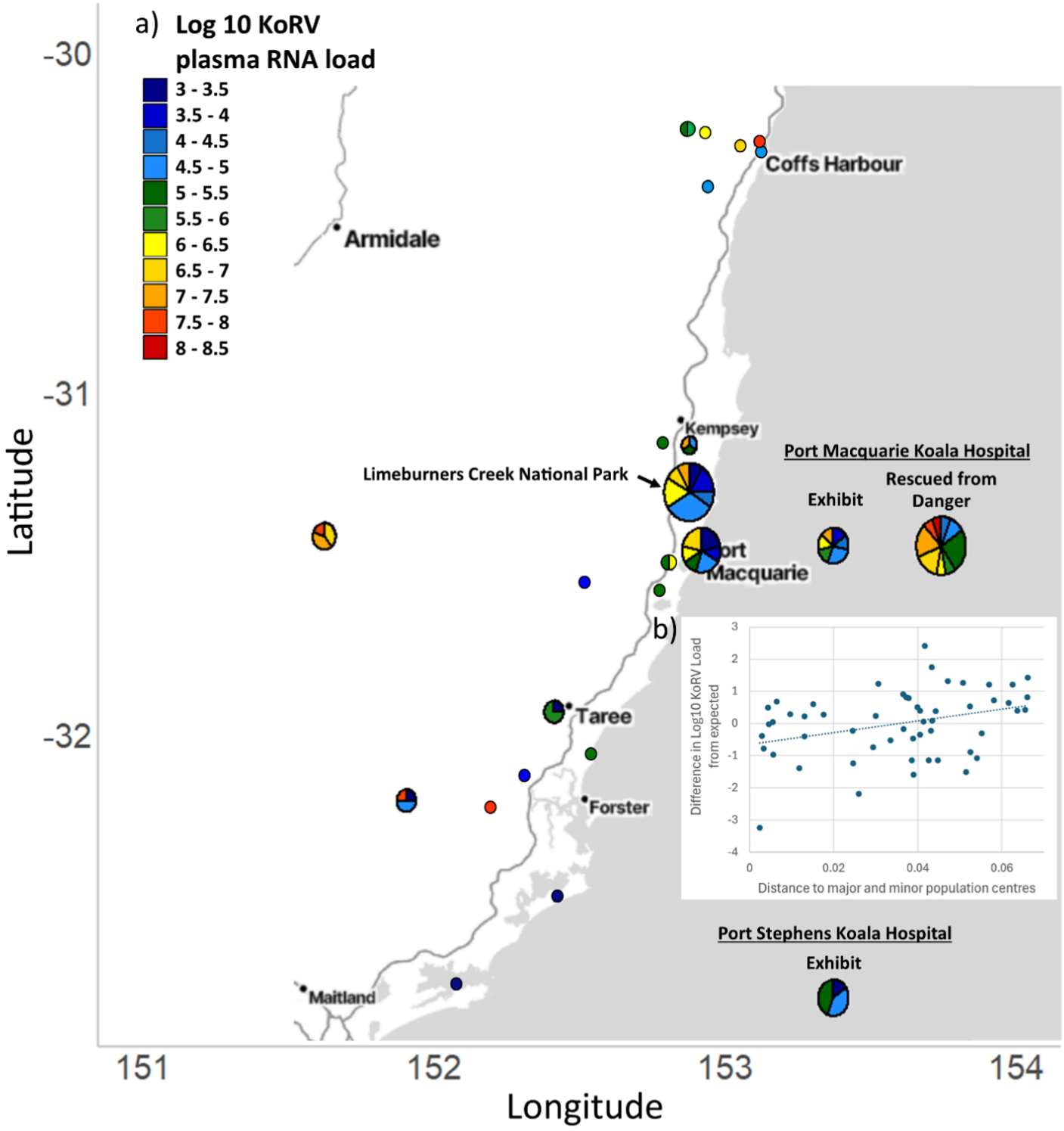


Figure 2

a) KoRV plasma RNA load across the study region. The size of the pie charts is indicative of the sample size at each study site. b) Difference in KoRV load from expected value plotted against the distance to major and minor population centres. The expected value was calculated from the linear regression analysis of KoRV load against the number of KoRV subtypes carried by a koala.

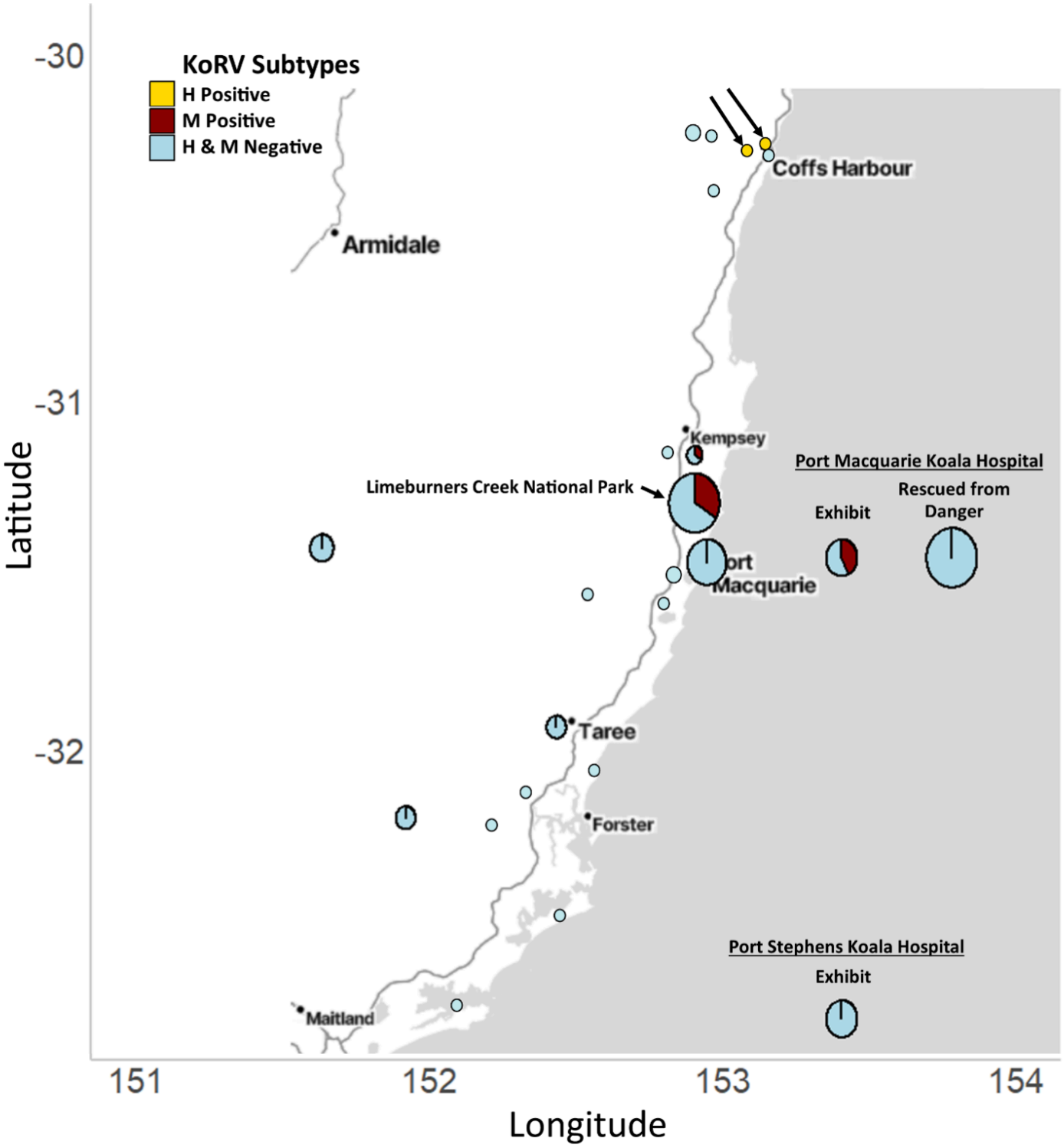


Figure 3

KoRV-H and KoRV-M prevalence across the study region. Note that none of the koalas had both KoRV-H and KoRV-M. The size of the pie charts is indicative of the sample size at each study site.

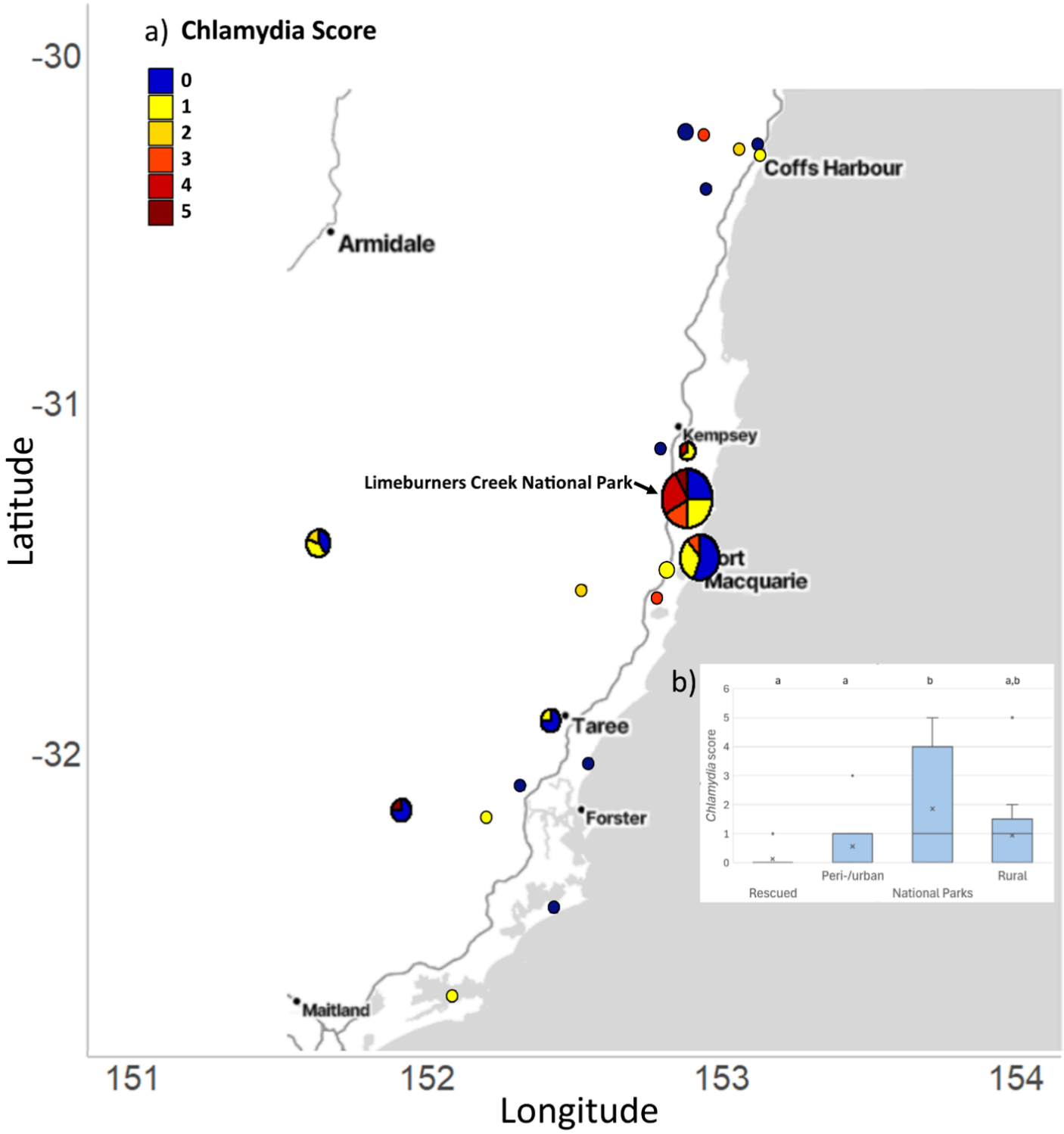


Figure 4

a) *Chlamydia* score across the study region. The size of the pie charts is indicative of the sample size at each study site. b) Box plots of *Chlamydia* scores by Cohort. X indicates the mean for each cohort.

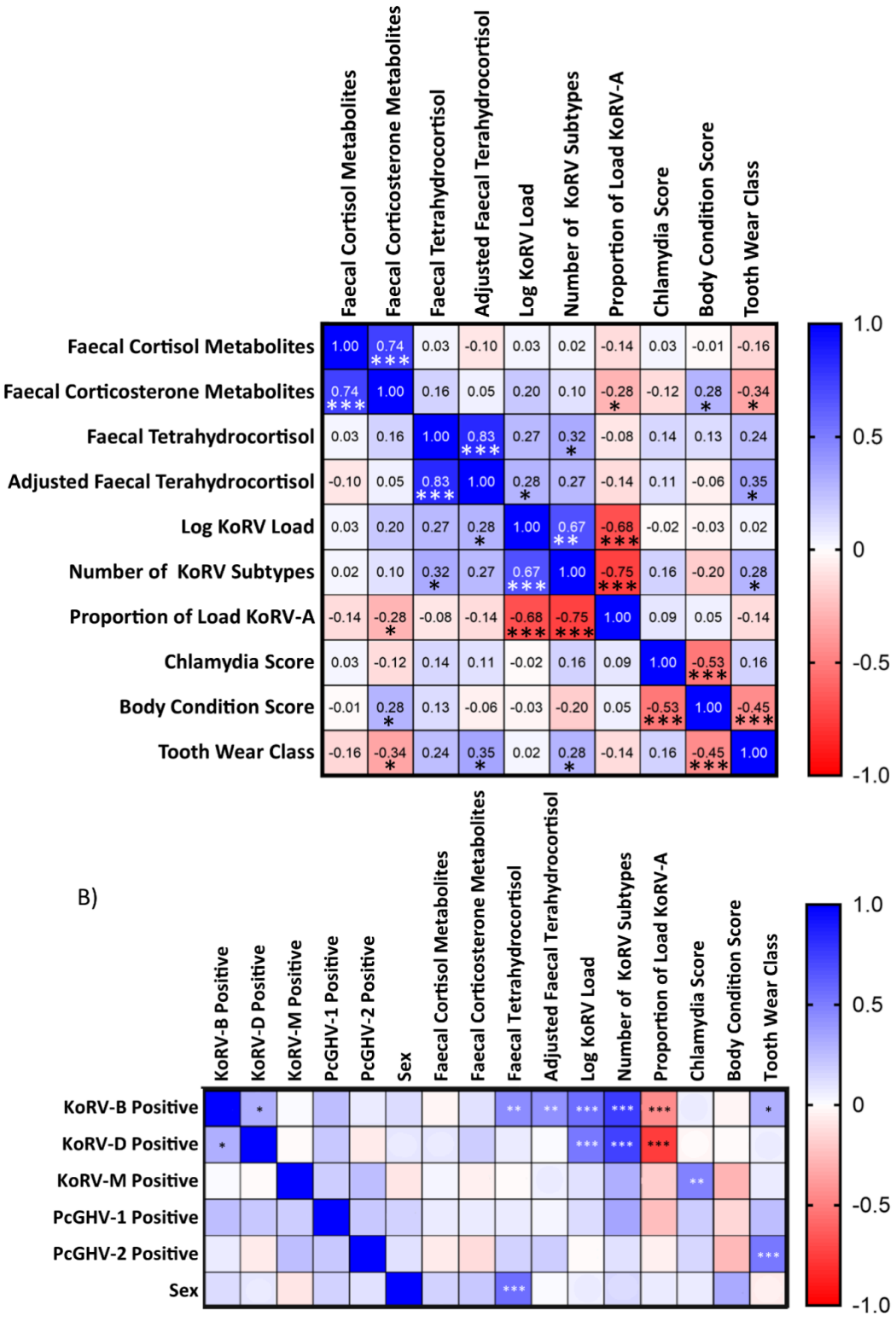


Figure 5

FGM and disease correlation matrices. A) Continuous or ordered factor variables with correlation R values given in the boxes. B) binary variables. Stars indicate significance of association. * < 0.05, ** < 0.01, *** < 0.001

Supplementary Files

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