Proceedings of the Second Koala Retrovirus Workshop

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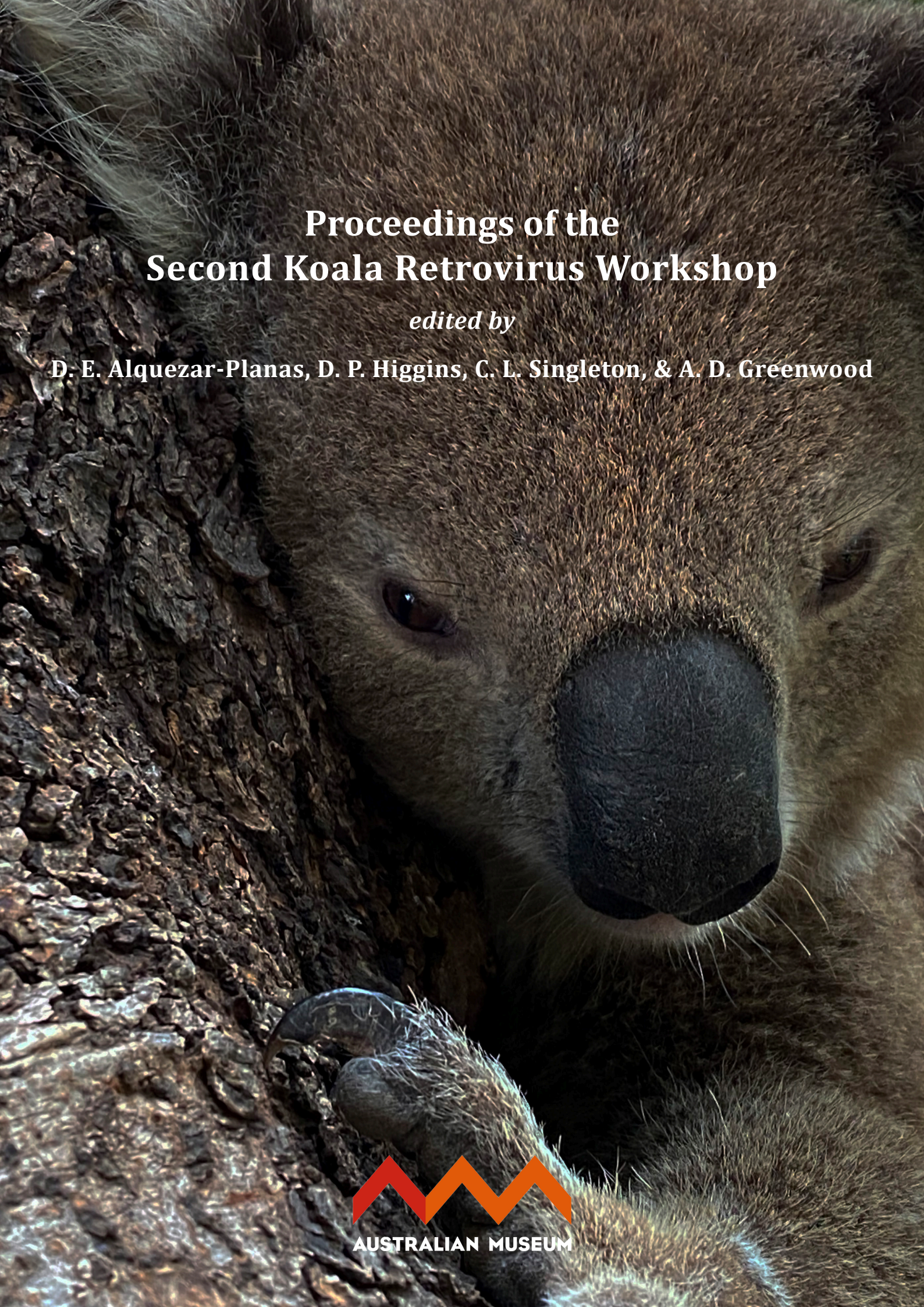

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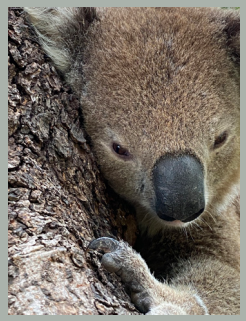

Cover photo
by Damien P. Higgins

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Koala Retrovirus Genetic Diversity and Transmission: Advice for Breeders

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Abstract. The rapid spread of koala retrovirus (KoRV) across Australia and international zoo populations has necessitated appropriate control measures. Along with pathogenicity, the genetic diversity of the virus and how it transmits between animals also needs to be considered when deciding the most suitable measures. Next generation sequencing has become the gold standard approach for KoRV diversity studies due to the high sensitivity, accuracy, and throughput. This approach has identified a large proportion of known KoRV diversity and has provided a broader understanding of KoRV prevalence and abundance within koala (*Phascolarctos cinereus*) populations, specifically identifying individuals with low diversity. Recent evidence has demonstrated that exogenous KoRV transmits from mother to joey, likely through the ingestion of milk and/or pap, and that koalas are not likely to acquire additional KoRV subtypes/sequences later in life. This finding strongly indicates that breeding with KoRV negative or endogenous KoRV-A positive only females is the best chance at alleviating exogenous KoRV from koala populations worldwide. Captive breeders are therefore urged to determine the KoRV profile of all animals included in their breeding program through deep sequencing methods (where feasible) and use this to inform their future breeding regimes.

Introduction

Koala retrovirus (KoRV) is a gammaretrovirus discovered in 2000, closely related to feline leukaemia virus (FeLV) and gibbon ape leukaemia virus (GaLV) (Hanger *et al*., 2000). Alike other retroviruses, KoRV is putatively associated with the onset of neoplasia and other associated cancers in koalas (*Phascolarctos cinereus*) (including leukaemia and lymphoma) and is suspected to cause immunodeficiency and opportunistic disease in this species (Tarlinton *et al*., 2005; Fabijan *et al*., 2020). Whilst habitat destruction and fragmentation, domestic dog attacks and vehicle collisions are among the greatest threats that wild koalas face, the putative KoRV-associated diseases are currently the major contributor towards captive koala mortality. Initially established from wild koala gene pools, captive koala breeding programs are now commonplace in zoos around Australia and internationally. These animals are often exchanged between institutions and, in some cases, exported overseas to increase genetic diversity within colonies. Occasionally, wild koalas are also incorporated into the captive setting and either used for display or as part of the breeding program. Animals approved for this integration are often hand raised and show no wild instincts or have sustained significant injuries, making them unfit to return to the wild. Understanding how to effectively manage these captive populations to reduce the impact from this virus is therefore crucial. The current advice based on recent publications will be addressed in this manuscript.

KoRV genetic diversity

KoRV was first discovered by Hanger *et al*. (2000) in koala genomic DNA through PCR with degenerate primers. This prototypic sequence was later classified as KoRV-A. Since its discovery, more than 10 additional subtypes (B-M) have been identified across multiple institutions around the world (Xu *et al*., 2013; Shojima *et al*., 2013; Xu *et al*., 2015; Chappell *et al*., 2017; Joyce *et al*., 2021; Blyton *et al*., 2021), each with a unique amino acid signature within the receptor binding

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domain of the KoRV envelope protein. It is hypothesized that this variation allows the subtypes to utilize different host cell receptors in attempt to overcome superinfection interference. However, this has only yet been explored for subtypes A and B, which use the sodium-dependent phosphate transporter (PiT1) and thiamine transporter 1 (THTR1) receptors, respectively (Oliveira *et al*., 2006; Xu *et al*., 2013; Shojima *et al*., 2013). Initial investigations into KoRV diversity focused primarily on PCR-based detection methods using subtype-specific primers. Whilst this approach led to the discovery of KoRV subtypes B-E (Xu *et al*., 2013; Shojima *et al*., 2013; Xu *et al*., 2015), it wasn't sensitive or high throughput enough to capture all the KoRV diversity within samples (Legione *et al*., 2017). This prompted the shift to next generation sequencing to allow greater detection of KoRV diversity. This method was first employed by Chappell *et al*. (2017) who detected 108 novel KoRV sequences and four new subtypes (F-I) in 18 wild koalas. This deep sequencing approach is now used as the gold standard for KoRV genetic diversity analyses and has helped detect well over 800 different KoRV sequences (Quigley *et al*., 2019; Sarker *et al*., 2019; Quigley *et al*., 2021; Joyce *et al*., 2021; Blyton *et al*., 2021). The magnitude of this is exemplified in the study recently conducted by our group which detected 421 unique KoRV sequences from 109 captive Australian koalas, the most diversity detected in a single study to date (Joyce *et al*., 2021). This dataset also revealed a novel KoRV subtype, KoRV-K.

Analysing KoRV subtype prevalence, abundance, and diversity is pivotal in understanding KoRV evolution within and among koala populations. KoRV-A is ubiquitous among the northern Australian populations of Queensland (QLD) and New South Wales (NSW), where it accounts for 94% of an animal's KoRV sequence reads on average (Joyce *et al*., 2021). However, the distribution and abundance of the remaining subtypes varies considerably among different populations. This is evident in our study where significant subtype differences were observed between two QLD koala colonies, despite the frequent sharing of animals and geographic proximity (Joyce *et al*., 2021). These differences in subtype prevalence and abundance are markedly greater among different regions (Joyce *et al*., 2022). Due to this high variability, the KoRV profile of all koalas housed in captive institutions should be established through deep sequencing methods. This information is pivotal for ensuring the appropriate management of these animals, especially when considering the transmission dynamics of this virus.

Potentially exogenous KoRV transmits from mother to joey

An aspect of KoRV biology is that it transmits via endogenous and potentially exogenous routes. At present, KoRV-A is the only subtype known to have endogenized into the koala genome, having been detected in koala sperm by Tarlinton *et al*. (2006) using fluorescence *in situ* hybridization. Similar work has not been conducted for the remaining KoRV subtypes so there has been no reported evidence of endogenization of these to date, and consequently, these variants are believed to only transmit via exogenous routes. However, many variants are defective, so an exogenous transmission mechanism is not clear. Based on recent studies, we know that if exogenous transmission occurs, it is primarily between mother and joey (Joyce *et al*., 2021).

Mother to joey transmission of KoRV-B has been noted in a few studies conducted worldwide since 2013 (Xu *et al*., 2013; Quigley *et al*., 2018; Zheng *et al*., 2020). However, the first substantial and statistically significant evidence of this transmission, observed for several KoRV subtypes, is from the recent study carried out by our team (Joyce *et al*., 2021). In this study, we conducted a large-scale sequence sharing analysis to track the transmission of KoRV sequences among captive koalas with known pedigree. Overall, we found very strong evidence of mother-joey transmission for all analysed subtypes (A, B, D, H–K), including non-endogenized KoRV-A. Interestingly, we found no evidence of father-joey or sexual transmission of this virus. Analysis of animals over time also revealed that KoRV infection occurs in the early stages of life and that koalas are less likely to acquire additional KoRV sequences or subtypes later in life. Notably, provirus re-integration can still occur within the animal, where substantial accumulation is associated with neoplasia (McEwen *et al*., 2021). Together, these findings highlight that KoRV transmission requires close contact—as seen between a mother and joey (Fig. 1)—and suggest that KoRV transmits through the ingestion of infected fluids. However, alternative scenarios remain possible such as excess integration on the X chromosome which would similarly skew integration site ratios to look like mother-joey transmission.

Whist the exact route of mother-joey transmission is yet to be investigated for KoRV, there are several postulations based on the various fluids shared between the two individuals. The most likely source of KoRV transmission is through the ingestion of infected milk and/or pap (semi-fluid faecal matter). Whilst no active virus has been recovered, KoRV sequences and peptides have been previously discovered in koala lactation milk (Morris *et al*., 2016). Exogenous viral transmission in both milk and faeces is seen to occur for other closely related retroviruses including FeLV, GaLV and mouse mammary tumour virus (Kawakami *et al*., 1977; Pacitti *et al*., 1986; Petropoulos, 1997; Gomes-Keller *et al*., 2008). Detection of KoRV-D in a neonate that failed to make it into the pouch due to consuming amniotic fluid also raises the possibility of viral transmission occurring *in utero* or during parturition (Joyce *et al*., 2022). This form of transmission has also been documented for GaLV (Kawakami *et al*., 1978). It should be noted that GaLV and FeLV contain the CETAG motif and KoRV contains CETTG, which drastically reduces KoRV infectivity, which may limit exogenous transmission. Investigation into whether and which koala excretions carry infectious virus is therefore required and crucial for our understanding of KoRV viral transmission.

Implications for koala breeding programs

The evidence collected thus far strongly indicates that the KoRV status of female koalas is important. Captive breeders are urged to preferentially breed with female koalas that are KoRV negative or positive for KoRV-A only. Where this is not possible/feasible, breeders should opt for females with the least KoRV genetic diversity. This has been shown to be effective in a southeast Queensland (SE QLD) zoo that actively removed KoRV-B positive individuals from their breeding program several years ago. This population is found to have drastically reduced KoRV diversity compared to all other populations analysed by our group (Joyce *et al*., 2022), in particular the two SE QLD populations from our recent publication that reside in the same geographic area (Joyce *et al*., 2021). Instigating this change across all captive institutions should therefore help alleviate the transmission of subtypes with unknown health risk within captive koala populations.

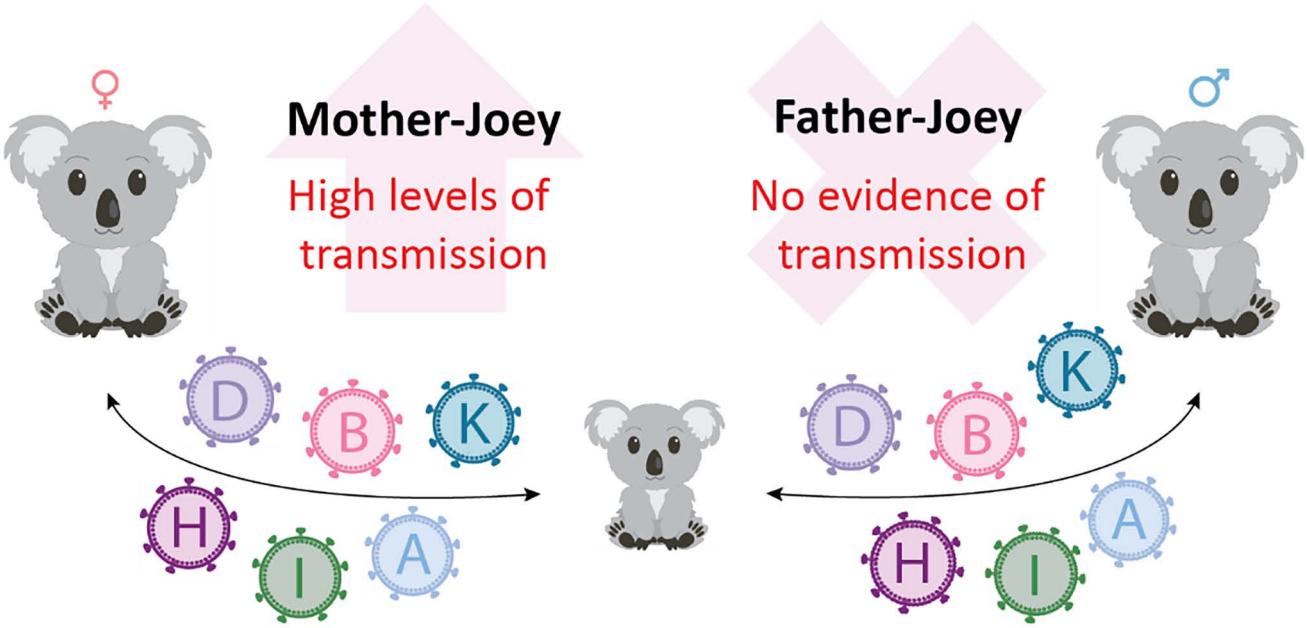

Figure 1. Schematic depicting key exogenous transmission dynamics of koala retrovirus. Letters refer to respective KoRV subtypes.

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