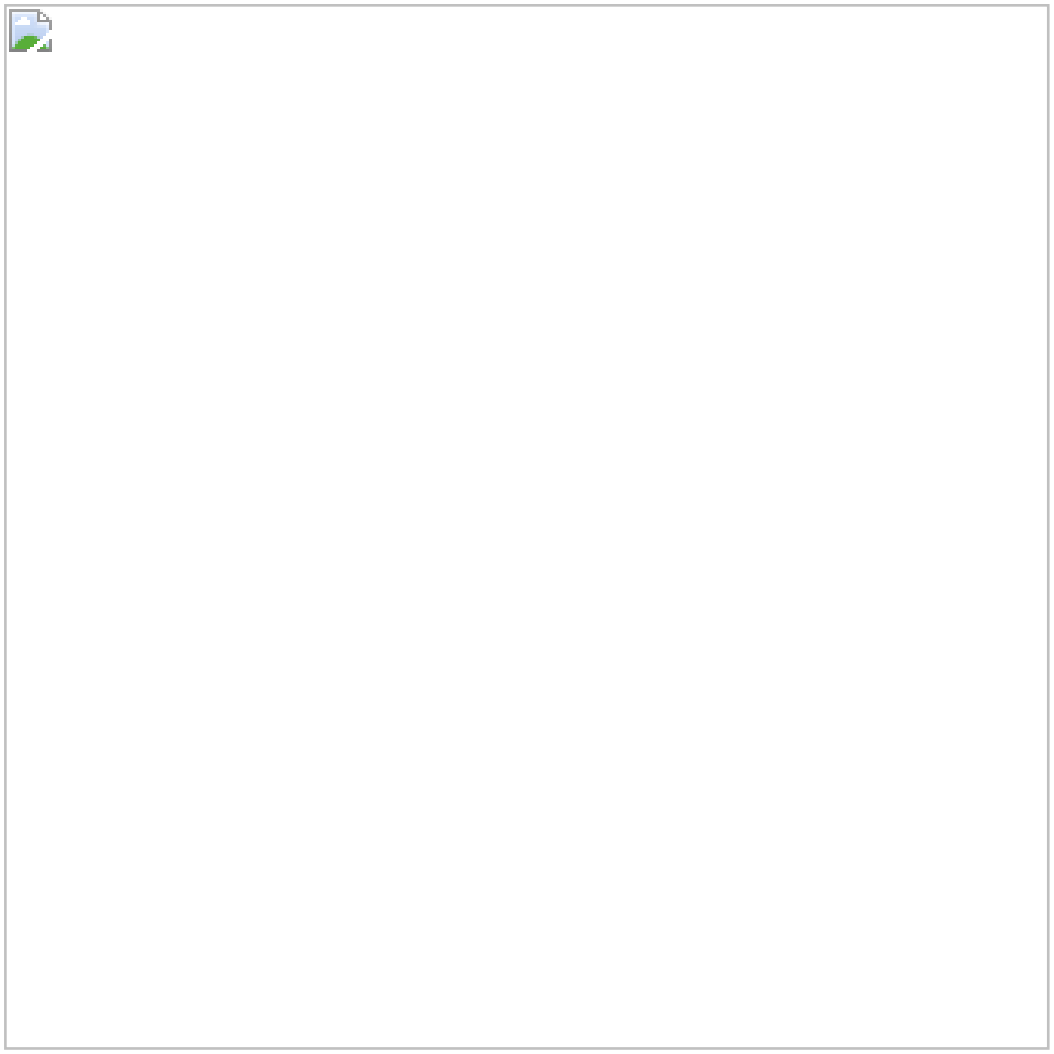

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A proposed roadmap for the control of infections in wildlife using *Chlamydia* vaccine development in koalas *Phascolarctos cinereus* as a template

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Vaccination strategies provide a crucial tool for managements of disease risks in wildlife, but have been utilized mostly for domestic species. However, a significant body of work has now been published describing the successful development of an anti-chlamydial vaccine for the koala *Phascolarctos cinereus*, Goldfuss, 1817. As such, vaccinations against these infections in the koala, can provide important insights into the use of vaccines for wildlife. *Chlamydia* infections in the koala have been intensively studied for over 30 years. Infections cause severe disease states, such as kerato-conjunctivitis (blindness) and reproductive tract disease (infertility), and/or mortality; and are contributing significantly to population declines. We aim to use the plethora of data available from koala chlamydial studies as a template to propose a roadmap for the development of vaccines for other wildlife species, especially in this era of antibiotic resistance. As such we have outlined the important steps that have led to significant milestones resulting in the successful development of a vaccine against an infectious disease in a non-domestic species. We hope to thus provide, not only a timely review on *Chlamydia* vaccines in koalas, but also an important conservation and management roadmap to help guide future researchers that are considering the development of a vaccine for a wild species.

Keywords: conservation, disease, management strategies, marsupial, Phascolarctidae, protocol, wildlife management

In the past two decades there has been an increase in the emergence of infectious diseases in wildlife. Human population expansion has been a significant driving force due to an encroachment into wildlife habitat (Daszak et al. 2000). Anthropogenic global climate change is also causing major changes to the geographic range and incidence of arthropod-borne infectious diseases (Daszak et al. 2000), and also affects the variability of temperature and precipitation, which modulates host–pathogen interactions (Rollins-Smith 2017). The transmission of pathogens from reservoir domestic species to sympatric wildlife (i.e. spill-over) further contributes to the emergence of a range of wildlife infectious diseases (Daszak et al. 2000). For example, wild dogs in the Serengeti became extinct in 1991 due to an epizootic of canine distemper in sympatric domestic dogs (Alexander et al. 1996). Ongoing studies into the molecular epidemiology of *Chlamydia pecorum* in koalas continue to provide genetic evidence that at least some koala *C. pecorum* strains share a common ancestor with those occurring in Australian livestock, evidence of the possibility that cross-host transmission has occurred (Bachmann et al. 2015, Waugh et al. 2016b).

People have benefited from vaccines for more than two centuries. The frequency and magnitude of epidemics of disease increased during the 18th and 19th centuries, principally as a result of changing social patterns and the growth of large population centers in industrializing societies (Anderson and May 1982). This trend has seen reversal during the 20th century and into the 21st century, and is largely due to the development and widespread use of vaccines to immunize susceptible populations against infectious diseases (Anderson and May 1982, Rappuoli et al. 2011). While attempts have been made to protect threatened mammal populations by vaccination (Woodroffe 1999), the use of vaccines in wildlife is underutilized. Due to the 'crisis management' nature of many of these studies it has been difficult to determine the success of such trials, often because no animals were left unvaccinated to serve as experimental controls (Woodroffe 1999). In addition, some vaccination programs have failed to provide protection, for example,

in African wild dogs, rabies vaccinations seem not to have averted population extinction because the protocol for vaccine delivery was ineffective (Woodroffe 1997). The vaccines were licensed to protect domestic dogs after a single dose, but subsequent trials showed that this protocol does not provoke a strong or sustained antibody response (Woodroffe 1997). Another such example includes vaccination of black-footed ferrets, *Mustela nigripes*, against canine distemper virus (CDV). The vaccine did not induce a protective antibody response (Williams et al. 1988), and resulted in the death of the vaccinated animals due to CVD (Carpenter et al. 1976). This vaccine was known to be protective in various carnivore species (Montali et al. 1983), and the same was incorrectly assumed for ferrets (Thorne and Williams 1988). These particular attempts at disease control have been hampered by a lack of information, and the lack of a roadmap to follow when the need to make decisions under 'crisis' scenarios arises. When certain steps are followed the outcome is more likely to be successful. For example the development of RABORAL V-RG (an oral rabies vaccine) underwent extensive safety and immunogenicity and efficacy steps before use and has now contributed to the elimination of wildlife rabies from three European countries (Belgium, France and Luxemborg) and of the dog/coyote rabies virus variant from the United States of America (USA; see Maki et al. 2017 for a comprehensive review on this system). In this paper we will focus on another successful example, the koala, *Phascolarctos cinereus*, Goldfuss, 1817, and its pathogen *Chlamydia pecorum*. The steps used in the development of the vaccine against *C. pecorum* infections in the koala can be utilized to provide important insights into the use of vaccines for the control of wildlife disease, and as a roadmap for future studies for infectious diseases in wildlife. Though there are other successful examples of vaccines for wildlife (Monica et al. 2019 for a review of vaccines against viruses in wildlife), we have chosen this system for the following reasons: 1) *C. pecorum* infections in the koala have been intensively studied for more than 30 years and a significant body of work has been published in the past seven years (2010–2017) describing chlamydial vaccine trials; 2) no reviews of this system currently exist and 3) it provides a clear and simple system of one host and one pathogen, allowing for the extrapolation of the most important steps in a clear and concise manner. Here we use the body of work available on anti-chlamydia vaccines for the koala to develop a roadmap that can be a starting point for wildlife researchers attempting to develop a vaccine. In the age of antimicrobial resistance, it is going to become more important to focus on strategies such as vaccine development, and thereby a starting point is required. Thus, we have developed and provided a roadmap for vaccination of wildlife against infectious diseases (Fig. 1).

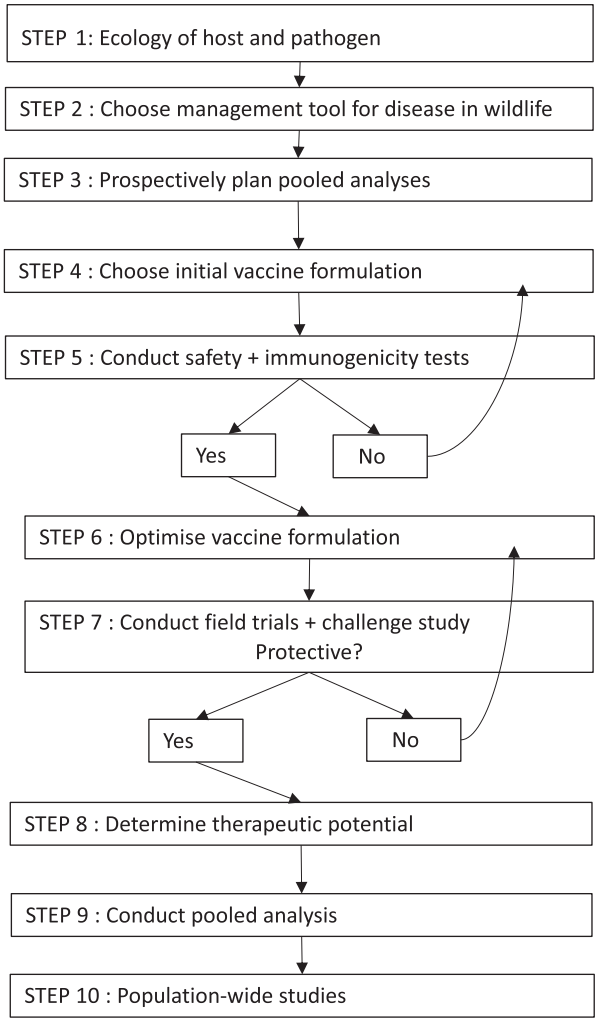

Figure 1. A step by step roadmap for the development of a vaccine for koalas *Phascolarctos cinereus* against *Chlamydia pecorum*, that can potentially be utilised as a roadmap for the development of vaccines in other species of wildlife.

Step 1. Understanding the ecology of the host and the pathogen

Past attempts at disease control have been hampered by a lack of information. An understanding of the host and its disease will greatly enhance the success of a vaccine schedule. Firstly it is important to understand what drives epizootics in the host. For example, epizootics of *C. pecorum* in wild koala populations occurs mostly in northern populations (Queensland, New South Wales and Victoria; (Kollipara et al. 2013a, Polkinghorne et al. 2013)). With less disease occurring in the southern populations (e.g. Victoria), and in these areas it may be prudent to focus on other conservation solutions, rather than vaccines. The reason for this difference is currently unknown. However, koalas co-infected with a koala retrovirus exogenous variant (KoRV-B) were more likely to progress to chlamydial disease when infected than koalas that were KoRV-B negative (Waugh et al. 2017). This can have implications for vaccine design, if for example, an anti-chlamydia vaccine was unsuccessful in producing an immune response, a vaccine could be directed against KoRV-B instead.

Secondly, understanding variant strains will lead to a more successful vaccine formulation, mostly in relation to the correct antigen (Step 4). For the koala, significant diversity (10–30%) of the *C. pecorum* major outer membrane protein (MOMP) has been found amongst wild koala populations (Devereaux et al. 2003, Marsh et al. 2011,

Kollipara et al. 2013a). The nucleotide sequence of *C. pecorum ompA*, which has four variable domains, has been used frequently to genotype *C. pecorum* samples collected from koalas, leading to the description of 11 koala-associated genotypes, named A–K (Kollipara et al. 2013a). Therefore, it is important that a vaccine provide an immune response against the correct genotype, otherwise a vaccine may not be useful.

Lastly, a limiting factor in our ability to manage and control infections in wildlife is an inadequate understanding of their immune response. In the case of the koala, a large body of research on chlamydial infections and subsequent progression to disease of genital infections had already been conducted utilizing the murine model, since the early 1990s (Farris and Morrison 2011). During this time there has been a host of antigens and adjuvants used to evaluate the efficacy and response of the immune system with varying results. What has been notable from these trials is the effectiveness of MOMP as an antigen (Berry et al. 2004, Pal et al. 2005, 2015, Farris et al. 2010) and the continual reports that $CD4^+$ T cells play a dominant role in the protective immunity against *Chlamydia* genital tract infections (Morrison et al. 1995, Farris et al. 2010). Several small animal studies have validated the protective role of IFN-γ secreting $CD4^+$ T cells in chlamydial infection (Loomis and Starnbach 2002). There is also re-emerging evidence that supports the role that B cells play in producing protective anti-*Chlamydia* antibodies (Li and McSorley 2013, Khan et al. 2016b). Neutralizing antibodies have been shown to reduce the initial infectious burden and prevent secondary bacterial infections (Batteiger et al. 2010). Further, in the koala, preliminary work has shown that certain cytokines, such as Interleukin 17A (IL17A), tumor necrosis factor alpha and IL 10 have significantly higher gene expression in animals with outward signs of chlamydial disease (Mathew et al. 2013, 2014). Studies, such as these, will help us understand the role played by cytokines in pathology and protection against infectious disease threats in the koala. The continued analysis of cytokine profiles may also help us understand why some animals experience only asymptomatic infections prior to clearance, compared to those that develop debilitating immunopathology. The immune response mounted by asymptomatic animals may inform us as to what adjuvant is required for the vaccine (Carey et al. 2010). Different adjuvants produce different immune response, so a greater understanding of which aspect of the immune response is important in combating the pathogen in question can thereby inform the choice of an adjuvant.

Step 2. Options for disease management

Management of disease in wildlife can occur via several methods; for example, some bacterial infections can be treated with antibiotics (Govendir et al. 2012), other diseases have been theorized to be able to be controlled by culling (Laddomada 2000), and others with vaccines (Waugh et al. 2016a). Thereby Step 2 (Fig. 1) should consist of a review of the management options available for each species and its pathogen. Below we have provided an example of the options for the koala and how this justified the use of a vaccine.

Early stages of chlamydial infection can be treated with antibiotics, however, the currently published regime is quite extensive (60 mg kg^{-1} of chloramphenicol daily for 45 days, Govendir et al. 2012). While antibiotics generally control mild chlamydial infections, they do not adequately control severe urogenital (UGT) tract disease, and prevention of long-term recrudescence of infection is yet to be confirmed (Govendir et al. 2012). The asymptomatic nature of some infections means that outwardly healthy koalas are not treated and continue to shed high loads of organisms (Wan et al. 2011), thereby acting as reservoirs. There are two additional reasons why antibiotic treatment of chlamydial infections in koalas is not ideal. Firstly, koalas have an expanded hepatic metabolic capability to enable them to detoxify the components of their eucalypt leaf diet and this results in the breakdown and clearance of many administered antibiotics, especially those metabolised by oxidation (Govendir et al. 2012). This process in the koala is more rapid than reported for eutherian species and often means that adequate plasma levels of the antibiotic needed for clearance of infection, are not reached (Govendir et al. 2012). Secondly, koalas have a unique microbiota composition in their caecum and gut, designed to metabolise their eucalypt leaf diet (Govendir et al. 2012). Antibiotics can have a severe adverse effect on this unique microflora and subsequently, the general health of the koala (Dahlhausen et al. 2018).

Culling has recently been proposed for koalas in northern Australia as option for disease control (Wilson et al. 2015). This report suggested that if heavily infected, terminally diseased and sterile female koalas were removed from the population (euthanized/culled), and other infected koalas were given antibiotics, then chlamydial infections could be significantly reduced and even eliminated, and subsequently positive population growth could be restored (Wilson et al. 2015). While culling is currently being used as a control for diseases of wildlife (e.g. chronic wasting disease in wild reindeer in Norway, (Stokstad 2017), there is increasing evidence that in practice it may not always be effective in eradicating pathogens from wild populations (Bolzoni and De Leo 2013). In fact, a counterintuitive result can occur, such that disease abundance and prevalence may increase with culling (Bolzoni and De Leo 2013). This can be due to the effect of culling on virulence evolution (Bolzoni and De Leo 2013), where increased host mortality may select for less virulent strains being able to establish in sparser populations. Selective pressures on pathogen virulence by changing the ecological conditions were not taken into account by Wilson et al. (2015). Either way, the moral and ethical standings of the majority on culling a nation's most iconic species would likely prevent this strategy from becoming a reality. The Norwegian reindeer cull further highlights the difficulties involved in culling by emphasizing that the effectiveness for disease control depends not only on epidemiology and ecology, but also on social and practical factors (Mysterud and Rolandsen 2018). Another example is culling of badgers to control tuberculosis, where the disruption of stable family groups led to dispersal rather than containment (Ham et al. 2019).

In the case of the koala, the shortcomings of other measures of disease control meant that vaccination was the most

logical approach for containing chlamydial infection and disease in wild koala populations.

Step 3. Prospectively plan experiments for later pooled analyses of data

The nature of vaccine development will be that of a step by step process. Thereby, multiple, independent studies will be required in order to reach the final goal of a vaccine. A pooled analysis of these independent studies will be crucial in determining the final vaccine formulation (see Step 9), and thereby prospectively planning for this is key for success. By planning data pooling during the design phase of epidemiologic studies, combined analysis are easier to conduct since the studies being combined will have similar designs and standardized methods (see Friedenreich 1993 for a full explanation of methods).

Step 4. Choosing the vaccine formulation

As chlamydial MOMP remains the leading vaccine candidate in chlamydial vaccine research in other species, the development of a prototype vaccine for koalas has been developed around the use of this antigen. Thus, the literature concerning the same/similar pathogen in other species, e.g. the mouse model, can often be a starting point for a vaccine for wildlife. However, care must be taken to assess the chosen antigen in the chosen species before a wide roll out of the vaccine (Step 4 and 5). We would suggest based on our experience and the practicality of experimentation, at least three antigens should be initially chosen and tested within one study to determine the most effective antigen for future studies.

Adjuvants enhance immunity to vaccines and experimental antigens by a variety of mechanisms. Adjuvants can be used to influence the magnitude and alter the quality of the adaptive response in order to provide maximum protection against specific pathogens (Coffman et al. 2010). We suggest trialing at least three separate adjuvants within one study to determine which adjuvant will provide the most adequate immune response for your species. For a review of adjuvants please refer to Coffman et al. 2010).

Step 5. Initial safety trials and immune responses

Once the initial vaccine formulation has been selected, the study can move onto the initial safety trials. The first safety trial should vaccinate healthy individuals of the species of interest. For the koala, the first anti-*Chlamydia* vaccine trial was conducted in 2010 (Carey et al. 2010) on 18 captive, healthy female koalas. This first vaccine consisted of a combination of three recombinant chlamydial antigens fused to the expression carrier protein, maltose binding protein. The three chlamydial proteins that were evaluated were derived from the mouse model pathogen, *C. muridarum* and included: 1) chlamydial MOMP, 50 µg; 2) chlamydial ribonucleotide reductase small chain protein (NrdB; ribonucleotide reductase small chain protein, highly conserved chlamydial protein with an essential role in the replication of chlamydiae), 50 µg and; 3) TC0512 (Omp85), 50 µg. The selected antigens had previously shown an ability to provide partial protection against different chlamydial species in the mouse model (Berry et al. 2004, McNeilly et al. 2007, Barker et al. 2008) and were an attempt to provide cross-protection to other chlamydial species for the koala. Three separate adjuvants, immunostimulating complex (ISC), alhydrogel and TiterMax Gold were trialed. The vaccine was administered sub-cutaneously via a three-dose regime (0, 1 and 3 months). In the koala, TiterMax Gold was not a suitable adjuvant, due to adverse reactions at the site of injection. By comparison, ISC was safe and resulted in the highest lymphocyte proliferative response, which was sustained for the duration of the trial (270 days) as well as showing the highest genital IgG antibody response. This initial trial clearly established that koalas could mount a strong and sustained immune response to foreign antigens and resulted in the selection of ISC as a safe and effective adjuvant.

The next trial should demonstrate the safety of using chlamydial vaccine in both healthy and diseased individuals. Thus, for the koala, the second safety trial was then conducted. The risk of inducing an inflammatory response by vaccinating animals that had previously been infected with *Chlamydia* was unknown, and previous mouse studies suggested that this might be an issue if the wrong chlamydial antigens were used (Brunham and Rey-Ladino 2005). Kollipara et al. (2012) vaccinated 10 koalas that were not only infected with *Chlamydia* but were showing clinical signs of disease at the time of vaccination. This study evaluated two antigens, the koala chlamydial recombinant MOMP (rMOMP) G strain, as well as NrdB. The antigens were adjuvanted with ISC, and were administered via three doses (0, 1 and 3 months) via the subcutaneous route. Importantly, none of the animals demonstrated any adverse reactions or worsening of the clinical signs following vaccination.

Not only was vaccination of diseased animals safe, but it resulted in increased immune responses. Both healthy and infected animals showed an increased and sustained (140 days) level of *Chlamydia*-specific IgG antibodies post vaccination, including the development of in vitro neutralizing antibodies. The vaccinated koalas also developed a cell mediated immune response as measured by antigen-specific lymphocyte proliferation. This study confirmed good immune responses to rMOMP and has resulted in rMOMP becoming the preferred vaccine antigen for *C. pecorum* vaccine development.

Step 6. Optimization of the vaccine regime and formulation

Once the initial safety and immune response trials have been successful, the study can then move onto optimization. This for the most part means optimization of the vaccine formula. Some examples are highlighted below.

Table 1. Description of the koala, *Phascolarctos cinereus*, *Chlamydia pecorum* vaccine studies used in the current meta analyses. IgG: immunoglobulin G titers in plasma; IVN: in vitro neutralization capacity of plasma; LP: lymphocyte proliferation ability of peripheral blood mononuclear cells; MOMP: major outer membrane protein (MOMP), NrdB, chlamydial ribonucleotide reductase small chain protein; ISC: immunostimulating complex (Pfizer); TriAdj: triadjuvant (VIDO-InterVac); SC: subcutaneous; IN: intranasal.

Study	Parameters measured	Antigen	Adjuvant	Route
Kollipara et al. 2012	IgG, IVN, LP	MOMPG + NrdB	ISC	SC
Kollipara et al. 2013a	IgG, IVN, LP	MOMPA, MOMPF, MOMPG, MOMPAF	ISC	SC
Khan et al. 2014	IgG, IVN, LP	MOMPAFG	TriAdj	SC
Waugh et al. 2015	IgG, IVN, LP	MOMPAFG	ISC	SC, IN
Khan et al. 2016a	IgG, IVN	MOMPAFG	ISC	SC
Khan et al. 2016b	IgG, IVN	MOMPAFG	TriAdj	SC

Optimization of the antigenic properties

One of the major decisions in the development of vaccines is the choice of antigen. Chlamydial studies in the mouse model had previously shown that the chlamydial MOMP was a major component of the chlamydial surface, highly immunogenic and could provide a level of immunity against live challenge (Brunham and Rey-Ladino 2005). The chlamydial MOMP is a 40 kDa highly disulphide cross-linked surface-exposed protein, comprising around 60% of the chlamydial outer membrane (Caldwell 1982). One disadvantage of chlamydial MOMP however is that it varies between species and strains of *Chlamydia*. Kollipara et al. (2013a) investigated the variation of the MOMP (coded by the *ompA* gene) in koala strains of *C. pecorum*, from 11 locations across Australia (based on eight wild koala populations and admissions to three wildlife hospitals). Of the 10 amino types reported, five amino types (A, E′, F, G and H) were present in the southeast Queensland koala populations and eight amino types (A, B, F, F′, G, I, J and K) were present in the NSW and South Australian locations. Further, MOMP aminotypes B, F′, I, J and K were only found in koalas admitted to southern wildlife hospitals, suggesting geographical restriction of some strains. Two major clades were found. The smallest (E′, F, F′) were widespread across the locations. The more recently diverged amino types (A, B, G, H, I, J and K) clustered to form a larger clade and was restricted to a smaller range of geographical locations. Although there was evidence that a single MOMP genotype could produce cross-reacting antibodies against other MOMP proteins (Kollipara et al. 2012), the degree of cross-reactivity achievable throughout different locations was obviously essential for developing an optimal *Chlamydia* vaccine capable of producing widespread protective immunity.

Despite the diversity seen between strains of koala *C. pecorum*, rMOMP was chosen by Kollipara et al. (2012) as the vaccinating antigen and then evaluated in a series of trials. Initially, the single 'G' variant of rMOMP was used (Kollipara et al. 2012), but subsequently a multiple subunit vaccine containing a combination of rMOMPs e.g. A, F and G (the most prevalent variants found in circulation in wild koalas) was evaluated. Kollipara et al. (2013b) assessed the cross-protective ability of three *C. pecorum* rMOMP genotypes (A, F and G), combined with ISC adjuvant, in a vaccine trial of healthy, captive female koalas. The vaccine was administered in three doses (0, 1 and 3 months) via the subcutaneous route. In this study, koalas were vaccinated with either a single rMOMP genotype (A, F or G) or, for the first time a combined rMOMP A plus F genotype vaccine. It was demonstrated that circulating plasma antibodies in the combined rMOMP were capable of not only neutralizing the homologous *C. pecorum* infection in vitro but also heterogeneous *C. pecorum* infections, and a significant cross-strain lymphocyte proliferation ability was demonstrated. A formulation of three rMOMPs (3rMOMP) A, F and G, was selected for downstream evaluations.

Sex differences and route of administration

These first vaccine trials had focused on female koalas. As *C. pecorum* is a sexually transmitted disease, it was of equal importance to understand the immune response in male koalas. The differences between sexes, in relation to the immune response, can have an impact on the pathogenesis of infectious diseases (Oertelt-Prigione 2012, Pennell et al. 2012). To evaluate the efficacy of a chlamydial vaccine and the immune response in male koalas, Waugh et al. (2015) conducted a trial on 12 healthy, captive male koalas. To find the most effective delivery system, they also evaluated the differences between administering the vaccine subcutaneously and via the intranasal route. Two groups of male koalas were vaccinated at the three intervals (0, 1 and 3 months) with the 3rMOMP (A, F and G) formulation, combined with the

Table 2. Response and explanatory variables utilized in the general linear model to provide a meta-analysis of the available data on the anti-chlamydia, *Chlamydia pecorum*, koala *Phascolarctos cinereus* vaccine. LP: lymphocyte proliferation ability of peripheral blood mononuclear cells; IgG: immunoglobulin G titers in plasma; IVN: in vitro neutralization capacity of plasma.

Variable	Description	Type
Immune response (LP, IgG or IVN)	Measurement of immune response	Continuous response variable
Vaccine status	Vaccination status of the koala (vaccinated or not vaccinated)	Binary explanatory variable 0 = no vaccination, 1 = vaccinated
Sex	Sex of the koala	Two level categorical variable 1 = female, 2 = male
Infection status	Current infection detected or not	Two level categorical variable 1 = no, 2 = yes
Wild or captive	Captive status of the koala	Two level categorical variable 1 = no, 2 = yes
Disease status	Disease status of the koala	Two level categorical variable 1 = no, 2 = yes

ISC adjuvant. One group had the vaccine administered subcutaneously while the other received an intranasal version. Male koalas from both groups developed humoral and cellular immune responses, sustained for up to 52 weeks, regardless of the route of administration. Comparing the route of administration more closely showed that a higher and more sustained level of rMOMP-specific IgG antibodies was observed in plasma from the subcutaneous group, compared to the intranasal group. This study also examined the IgG titres in mucosal sections of the urogenitial tract and ocular secretions, which showed a higher IgG level in the intranasal group. The neutralisation capacity of plasma samples as well as ocular and UGT secretions showed no statistically significant differences between the subcutaneous and intranasal groups at 52 weeks post vaccination. The lymphocyte proliferative response showed a statistically significant difference over time between the groups, with the subcutaneous group providing a stronger and more sustained response. As the intranasal route proved to be logistically difficult to administer in the koala, the ease of the subcutaneous route was preferred for ongoing studies.

Evaluation of a novel one-dose adjuvant (Tri-Adj)

The results of these initial *C. pecorum* vaccine trials had shown that a vaccine consisting of 3rMOMP (A, F and G) proteins combined with ISC adjuvant, given sub-cutaneously as a three-dose vaccination schedule, was: 1) safe to administer to both healthy and clinically diseased koalas; 2) led to the development of specific humoral and cell-mediated immune responses, and 3) elicited a therapeutic effect on animals already infected (Nyari et al. 2019). Whilst this formulation of the vaccine was showing promising results, the logistics of the three-dose vaccination regime remained challenging for administering in wild animals. Khan et al. (2014) addressed this by trialing the 3rMOMP (A, F and G) with a novel adjuvant which only required one dose. This was a tri-adjuvant (TriAdj) comprised of polyphosphazine (PCEP), polyinosinic polycytidylic acid (poly I:C) and a host defense peptide (HDP) HH2, that has previously been used successfully in laboratory animal models (Dar et al. 2012). In Khan et al. (2014) six captive, healthy and sero-negative captive female koalas were used (with six unvaccinated controls). A significant immune response was maintained for 54 weeks, post vaccination. MOMP-specific IgG antibody levels, with neutralization potential, were present in plasma, ocular and urogenital secretions, and a significant lymphocyte proliferation ability was noted. This suggested that the 3rMOMP/Tri-Adj formulation resulted in a strong and long-lasting, cellular and antibody response. The vaccine was then tested in a cohort of *Chlamydia* negative wild koalas (n = 10). In wild koalas this formulation produced strong *Chlamydia*-specific cellular (IFN-γ and IL-17A) responses in circulating PBMCs, as well as MOMP-specific and in vitro neutralizing antibodies

Step 7. Field trials and a challenge study

For a vaccine to be effective, it must not only produce and immune response, but it must also provide protection to the individual when exposed to the pathogen. A well-controlled infection study is required where the individuals are

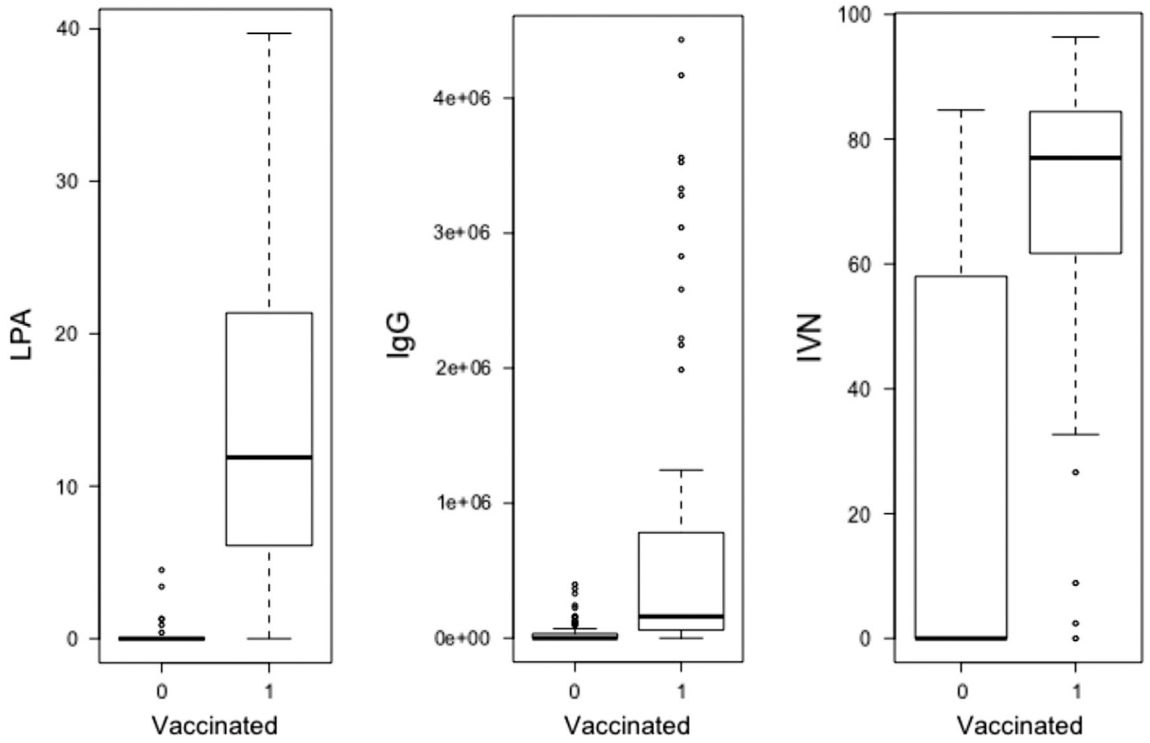

Figure 2. Modelling of the estimated probability that vaccination of a koala *Phascolarctos cinereus* predicts immune response. Vaccination status (0: not vaccinated; 1: vaccinated) against different immune parameters (LPA: lymphocyte proliferation Ability in percentage %; IgG: immunglobulin G titers; IVN: in vitro neutralization potential in percentage %).

vaccinated before being purposely exposed to the pathogen. However, in wildlife studies, ethical, moral and logistical restrictions often prevent this type of study from taking place (Waugh and Monamy 2016, Aske and Waugh 2017). Thus, we suggest that semi-controlled field trials could replace traditional controlled challenge studies (Waugh et al. 2016c).

For the koala example, Waugh et al. (2016c) conducted the first field trial that vaccinated and followed wild koalas in their natural habitat. The aim of this study was to determine the protective value of vaccination, following natural exposure of koalas to *Chlamydia* in the wild. Sixty wild koalas were separated into two groups of 30, each containing a mix of males and females, as well as a mixture of animals that were either *Chlamydia* PCR positive or negative at the time of vaccination. One group received no vaccine (control) while the other received a vaccine containing 3rMOMP (A, F and G; 50 µg of each) combined with ISC adjuvant (three doses were given at 0, 3 and 6 months). All koalas were fitted with radio collars for tracking and recapturing. The results of this trial were very promising, and indicated a protective effect of the vaccine. Importantly, it was noted that although some *Chlamydia* negative vaccinated koalas contracted a new infection post vaccination (12–20%), only one koala (4%) went on to develop disease. This contrasts with animals in the control unvaccinated group, where a significant proportion contracted new infections (20–25%), and then progressed to severe disease pathology (15%). In vaccines the protectiveness of a vaccine often has to be close to 100% in order for it to be considered for medical use, even though a lower percentage could also benefit the population. However, in wildlife, we do not have the same restrictions thus a protective effect, less than 100%, can be useful for conservation and management.

Further studies can then be conducted on wild population to further understand the characteristics of the immune response to vaccination. For example, Khan et al. (2016b) compared the production and specificity of antibodies following natural infection to the response following vaccination with the 3rMOMP plus ISC vaccine. They looked at a population of 20 wild koalas separated into four groups: 1) *Chlamydia* positive naturally but no vaccine (n = 5); 2) *Chlamydia* positive naturally plus vaccinated (n = 5); 3) *Chlamydia* negative no vaccine (n = 5); and 4) *Chlamydia* negative plus vaccinated (n = 5). Antibody responses to rMOMP, as well as chlamydial elementary bodies, were observed in all *C. pecorum* positive and/or vaccinated koalas. However, they found a significantly higher antibody level was reached in those animals vaccinated and combined with a pre-existing chlamydial infection. Khan et al. (2016b) also identified unique sets of MOMP epitopes, recognized by antibodies that reflected the koala's state of disease, infection or vaccination status. This highlights that studies of the response to vaccination can inform on strategies to improve vaccine development. For example, even though this MOMP formulation has many strengths, this information from the above mentioned study (Khan et al. (2016b) is currently being utilised to develop a synthetic peptide based vaccine (Nyari et al. 2018). A synthetic based vaccine would be more time and cost effective, as well as logistically easier to transport (e.g. cold trail not required).

Step 8. Therapeutic potential of a vaccine: a potential bonus effect

Therapeutic vaccines are a promising new approach to enhance immunogenicity and reduce viral and bacterial load. We suggest that once a vaccine has been deemed protective, that it should then also be assessed for its therapeutic potential. Vaccines can provide an important alternative to the use of antibiotics in humans, wildlife and domestic animals.

Waugh et al. (2016c) assessed the therapeutic value of the vaccine in free-ranging wild koalas as described above. After a 12-month period, vaccinated koalas that were *Chlamydia* positive at the time of vaccination, were significantly more likely to have a reduced or similar chlamydial load (i.e. no increase in chlamydial infection load). By comparison, *Chlamydia* positive control koalas (not vaccinated) were significantly more likely to increase their chlamydial load in the subsequent 6–12 month period.

The therapeutic value of the koala vaccine was then tested in a more controlled environment (Waugh et al. 2016a). Six wild caught male koalas were recruited into the trial. This trial was unique in that it attempted to show a protective effect from vaccinating diseased koalas. Four of the koalas were vaccinated with the 3rMOMP (50 µg, A, F and G) plus TriAdj (one dose at 0 months) vaccine, while two of the koalas were not vaccinated, but instead treated with the currently recommended antibiotic treatment regime as a

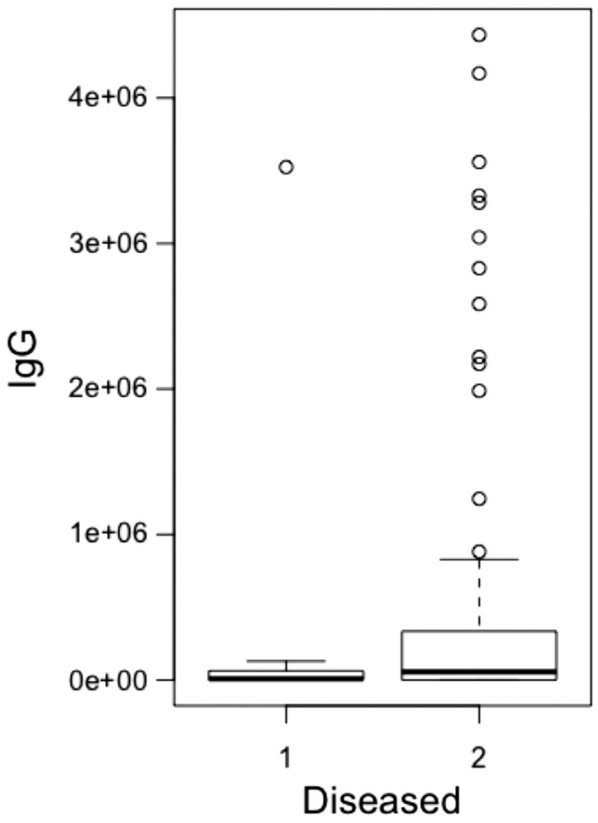

Figure 3. IgG (immunoglobulin G titer) in *Chlamydial pecorum* diseased koalas *Phascolarctos cinereus* when vaccinated with an anti-chlamydial vaccine formulation. Diseased, 1: no signs of clinical chlamydial disease; 2: clinical signs of chlamydial disease present in koala preceding vaccination.

control. All six koalas eliminated their chlamydial infections regardless of treatment. Two of the four vaccinated koalas showed decreased signs of chlamydial disease (i.e. decreased inflammation and discharge at the conjunctival site) over the six weeks following vaccination. The study demonstrated that the use of a vaccine can have a positive effect in koalas already with clinical signs of ocular disease, suggesting a possible therapeutic effect and an alternative to antibiotic therapy.

This study was then expanded upon (Nyari et al. 2019). Seven koalas with ocular disease were vaccinated (3rMOMP, 50 µg, A, F and G plus TriAdj, one dose at 0 months) and assessed for six weeks, evaluating any changes to the conjunctival tissue and discharge. The results clearly showed an improvement in the clinical ocular disease state of all seven koalas, post-vaccination (Nyari et al. 2019).

However, there are potential risks in such a strategy. If a host is immunocompromised, for example, disease can be a complication of vaccination, e.g. bacilli Calmette–Guérin (BCG) vaccine that is administered to prevent tuberculosis can cause BCG disease after revaccination of individuals who were anergic following the initial vaccinated with BCG vaccine (Talbot et al. 1997).

Step 9. Pooled analysis of all vaccine studies

To assist with determining an optimal formulation for a wildlife vaccine, we suggest the use of a pooled-analysis to: determine if the antigen, adjuvant and/or route of administration plays a significant role in the level or type of immune response. We have conducted such an analysis with the available koala data for two purposes: 1) to provide a statistical protocol for users to follow and 2) to provide the optimal formulation for the koala vaccine for future studies and use.

Primary data was provided by the authors from the following to be published articles included in the pooled analyses (Table 1) (Kollipara et al. 2012, 2013b, Khan et al. 2014, 2016a, b, Waugh et al. 2015). We followed standard procedures for data exploration (Friedenreich 1993, Zuur et al. 2010) and ensured there were no outlying observations, nor collinearity between any explanatory variables (Supplementary material Appendix 1). There was, however, heterogeneity between studies (as often found with pooled analyses; Friendenreich 1993), thus we utilized linear mixed-effects models using R ver. 3.2.3 (package lme4).

Immune response to koala vaccinations

To assess the immune response following vaccination of koalas, lymphocyte proliferation (LP), total serum antibody production (IgG) and plasma in vitro neutralization (IVN) potential were the endpoints with the most data that could be analysed statistically. We modelled the relationships as a linear mixed-effect model, using LME4 package. We selected explanatory variables (vaccine status (yes or no), sex (male and female), prior chlamydial infections status (yes

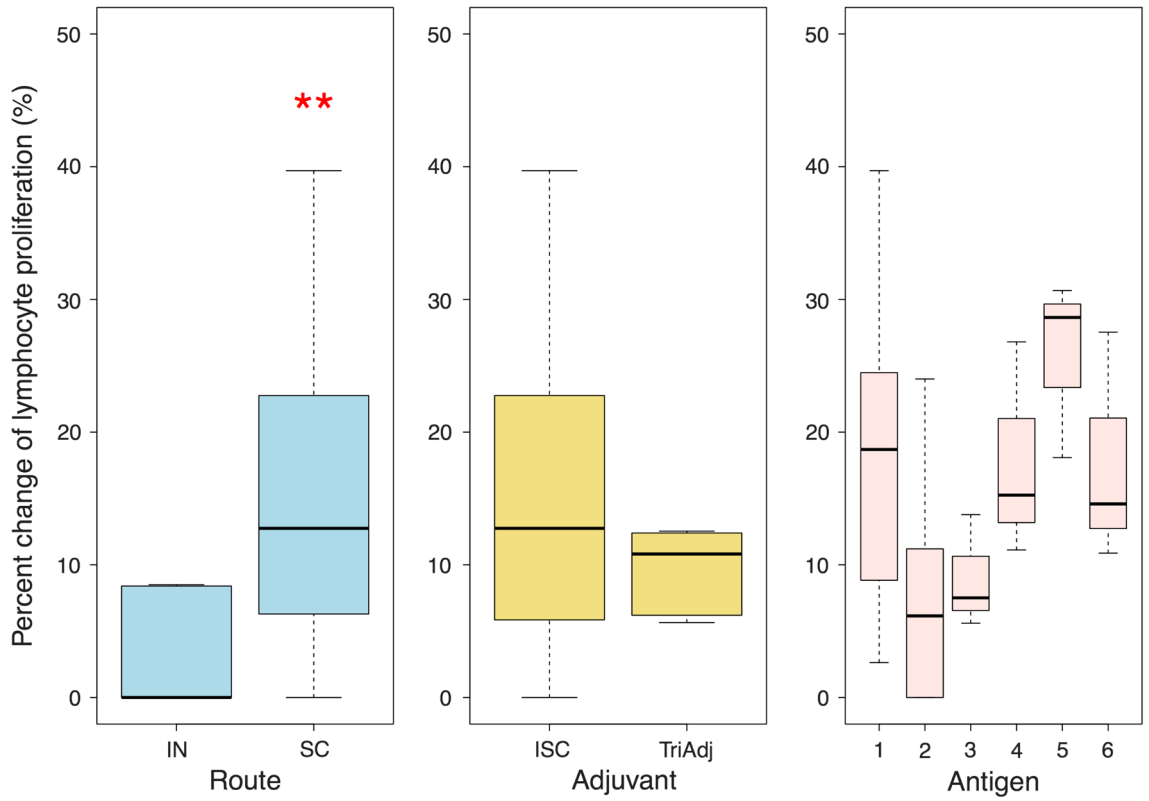

Figure 4. Percent change in lymphocyte proliferation in anti-chlamydial vaccinated koalas *Phascolarctos cinereus*. Error bars represent 95% confidence intervals. Route of vaccination, i.e. IN, intranasal or SC, subcutaneous. Adjuvant consisted of either an immune stimulating complex (ISC) or a Tri-adjuvant formulation (TriAdj). Antigen: 1 = MOMP + NrDB; 2 = 3× MOMP (A, F and G); 3 = MOMP A; 4 = MOMP F; 5 = MOMP G; 6 = 2× MOMP AF.

or no), prior disease status (yes or no), and captivity status (wild or captive; Table 2). that we considered biologically and statistically meaningful; to explain the variation in the immune responses measured (LP, IgG, IVN) as well as We fitted a separate linear mixed-effect model for each immune parameter (Zuur et al. 2010). Our sample size was n = 98 (LP), n = 154 (IgG) and n = 157 (IVN). We used Akaike information (AICc) and an information-theoretic approach (Anderson and Burnham 2002) to identify the minimum adequate model. We assessed the importance of each explanatory variable within the minimum adequate model, using a likelihood ratio test.

In each case, vaccination status was the most significant factor driving the variation in the immune response parameters. Modelling of the estimated probability confirmed that vaccination of a koala predicted a significantly higher immune response than non-vaccinated koalas (Fig. 2). No other considered explanatory variables played a significant role in predicting the immune response of the koala apart from diseased animals having a significantly higher IgG response than non-diseased animals when vaccinated (Fig. 3) (this effect was not apparent within the two other aspects of the immune response measured here. One possible explanation is that some of the wild koalas were previously infected with *C. pecorum*. While these previous infections were not protective, subsequent vaccination has resulted in a strong and beneficial boosting immune response.

Comparison of vaccine route, antigen and adjuvant used to formulate the vaccine

To assist with determining an optimal formulation for a koala chlamydial vaccine, we tested the relationship between the immune response of a vaccinated koala and different explanatory variables. We used linear models to determine what explanatory factors (i.e. antigen, adjuvant, immunization route) might promote a successful vaccination event. Lymphocyte proliferation (LP), serum antibody production (IgG) and plasma in vitro neutralization (IVN) were again the endpoints. Our samples size was n = 46 (LP), n = 69 (IgG) and n = 68 (IVN). Each parameter was converted to % change from 0 (pre vaccinated) to 6 months (post vaccinated). To model the immune response (LP, IgG or IVN) of vaccinated koalas as a function of the covariates, a linear model was used (Eq. 1). Fixed covariates are the antigen (categorical with six levels), adjuvant (categorical with two levels) and the route of administration (categorical with two levels). To select the best model we then used AIC as a measure of goodness of fit (lowest AIC is the best model) using backwards selection.

$$\text{Immune response}_i \sim \alpha + \beta_1 \text{Antigen} + \beta_1 \text{Adjuvant} + \beta_1 \text{Route} + \varepsilon_i \quad (1)$$

The results of the linear model for LP indicated that the route of vaccination (subcutaneous) and the adjuvant

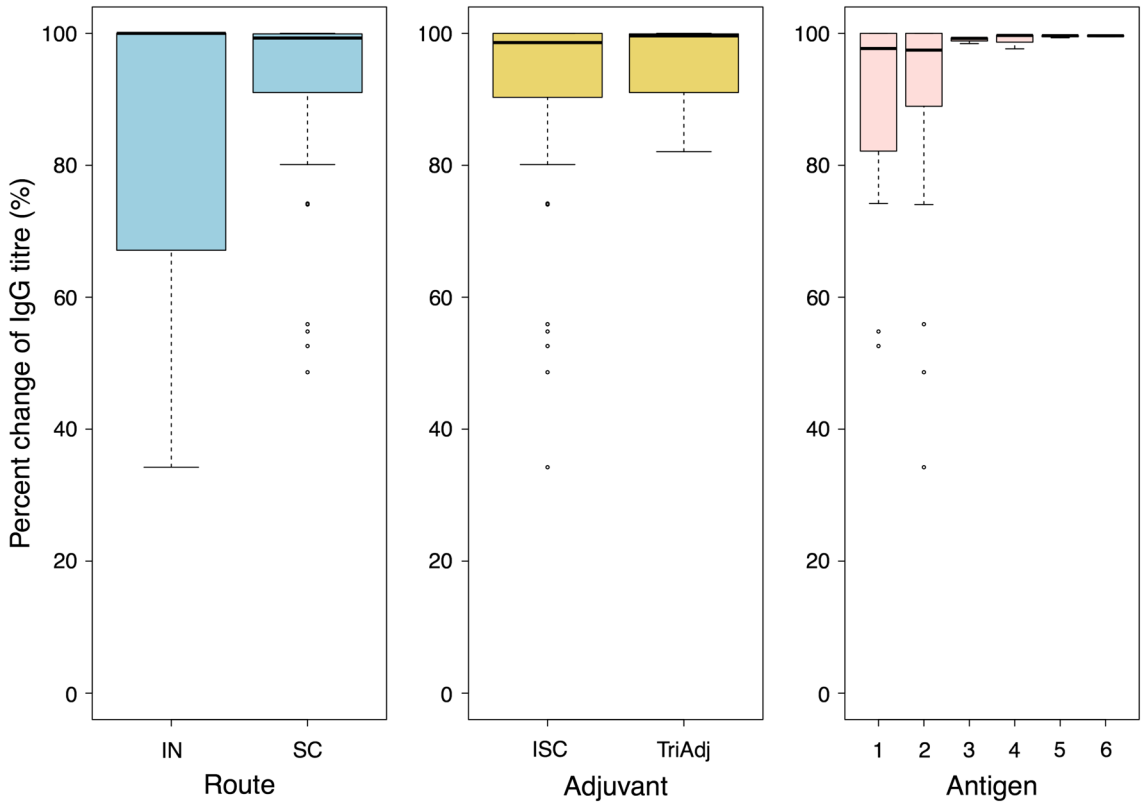

Figure 5. Percent change of Immunoglobulin G titres in anti-chlamydial vaccinated koalas *Phascolarctos cinereus*. Route of vaccination, i.e. IN, intranasal or SC, subcutaneous. Adjuvant consisted of either an immune stimulating complex (ISC) or a Tri-adjuvant formulation (TriAdj). Antigen: 1 = MOMP + NrDB; 2 = 3× MOMP (A, F and G); 3 = MOMP A; 4 = MOMP F; 5 = MOMP G; 6 = 2× MOMP AF. Error bars represent 95% confidence intervals.

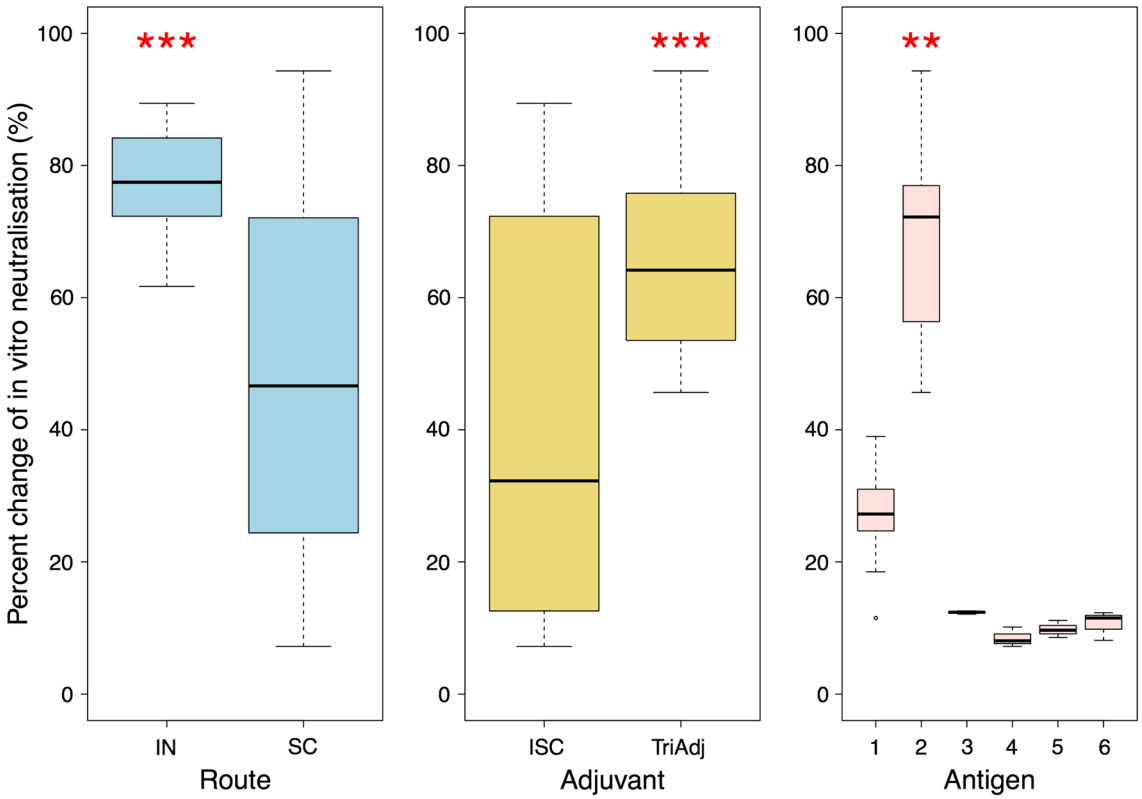

Figure 6. Percent change of in vitro neutralisation in anti-chlamydial vaccinated koalas *Phascolarctos cinereus*. Route of vaccination, i.e. IN, intranasal or SC, subcutaneous. Adjuvant consisted of either an immune stimulating complex (ISC) or a Tri-adjuvant formulation (TriAdj). Antigen: 1 = MOMP + NrDB; 2 = 3× MOMP (A, F and G); 3 = MOMP A; 4 = MOMP F; 5 = MOMP G; 6 = 2× MOMP AF. Error bars represent 95% confidence intervals.

(TriAdj) were the best predictors of a strong LP response, though only the route of vaccination (subcutaneous over intranasal; p = 0.00183) was a significant predictor (Supplementary material Appendix 1 Table A1, Fig. 4). The results of the linear model for plasma IgG titres indicated that the predictors were route of administration and antigen, however neither were significant predictors (Supplementary material Appendix 1 Table A1, Fig. 5). The model that best predicted IVN ability included all three of the covariates, antigen (3rMOMP; p = 0.0034764), route of vaccination (intranasal; p = 0.0001728) and adjuvant (TriAdj; p = 0.0001432; Supplementary material Appendix 1 Table A1, Fig. 6).

In relation to moving towards optimization of a vaccine formula, the results of this meta-analysis (summarized in Fig. 7) show that while the antigen and adjuvant are not of major relevance for total IgG antibody response and LP potential, there is a clear effect on IVN, suggesting that, for greater IVN potential in the plasma, an optimal formulation should include a 3rMOMP vaccine adjuvanted with TriAdj. Route of vaccination (subcutaneous versus intranasal) had strengths and weaknesses depending on the immune response studied. To obtain a stronger LP response a subcutaneous method should be selected, however an intranasal formulation produced a stronger IVN response. Taking the logistic difficul-

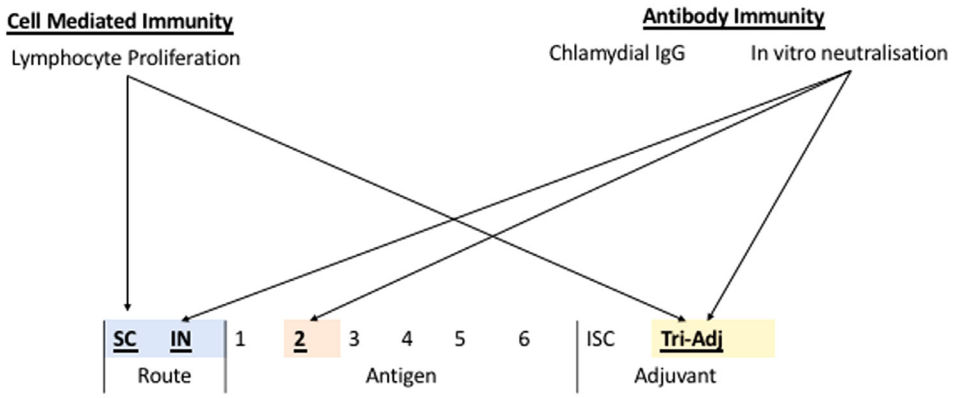

Figure 7. Significant associations between immune response measured and vaccine characteristics. Significant associations are indicated with a linking arrow and highlighted in bold.

ties of an intranasal formulation into consideration, we thus recommend the use of subcutaneous delivery.

In conclusion, the pooled analysis of all available published material on anti-chlamydial vaccines for koalas suggests an optimal formulation of a three rMOMP (A, F and G) and TriAdj version of the vaccine administered subcutaneously to wild koalas. There is also data to suggest that this formulation will result in a decreased chlamydial load, and disease symptoms, in koalas with ongoing infections, and thus provide some therapeutic value.

Step 10. Population wide studies

After the optimal formulation has been developed under semi-controlled conditions, for the vaccine to progress further it will be necessary to implement population wide studies, and comparing vaccinated populations to non-vaccinated populations. This step has yet to occur in the koala. Due to the high public profile of koalas, the studies so far have necessarily been largely opportunistic, as will likely be the case for many wildlife species.

Conclusions

In conclusion, we have provided a roadmap for the development of a vaccine for conservation of wildlife. As an example, we have shown a successful outcome for vaccination of a wildlife species (the koala) against its pathogen *Chlamydia pecorum*. This work can thus provide an important framework for the use of vaccines to manage the continued emergence of infectious diseases in wildlife. It highlights the need for basic research into and understanding of the epidemiology of infections in order to develop safe and effective vaccines. Few vaccines have been tested on non-domestic species (Bittle 1993) and as such, the comprehensive testing on the koala can provide a repository of experience to inform important future management decisions for other wildlife species.

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Supplementary material (available online as Appendix wlb-00627 at <www.wildlifebiology.org/appendix/wlb-00627>). Appendix 1.