Is disease a major causal factor in declines? An Evidence Framework and case study on koala chlamydiosis

Running head: Disease as a causal factor in declines

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ABSTRACT

Determining the role of an infectious agent in contributing to wildlife population declines is a pervasive problem in the field of conservation biology. We expand on a recently proposed broad investigative approach for disease, with a systematic framework outlining the specific types of individual- and population-scale empirical evidence required to demonstrate whether a pathogen is a component cause of declines in wild animal populations. Using koala (*Phascolarctos cinereus*) population declines and their putative association with the bacterial disease chlamydiosis (Family *Chlamydiaceae*) as a case study, we review the relevant published literature and synthesize a logical conceptual argument based on our suggested framework. Available empirical evidence supports a role for chlamydiosis contributing to host mortality and sterility, and cannot rule out a role of chlamydiosis as a component cause of koala population declines. However, the relative importance of chlamydiosis (among other threatening processes) as a driver of changes in koala demography and autecology may differ depending on the particular population or system examined, and this has yet to be elucidated over the koala's distributional range. Our approach allows us to highlight current research gaps in order to assist with future policy planning and conservation strategy. We recommend that a similar approach will assist in the evaluation of the role of disease in population declines in other ecological systems.

MAIN TEXT

INTRODUCTION

Does a particular pathogen or disease cause declines of wild animal populations? This crucial question pervades the interface between the fields of disease ecology and conservation biology, across numerous ecosystems globally (Preece et al. 2017). Infectious diseases can have dramatic, widespread and long-term effects on ecological communities, and are increasingly being recognised as a substantial challenge in endangered species' conservation (Daszak 2000; Fisher et al. 2012; Jones et al. 2008; Scheele et al. 2017; Tompkins et al. 2015). Diseases that have been associated with vertebrate declines and extinctions globally include, for example, white nose syndrome in bats, West Nile Virus and avian malaria in birds, chytridiomycosis in amphibians, and chronic wasting disease in ungulates (Edmunds et al. 2016; George et al. 2015; Samuel et al. 2015; Skerratt et al. 2007; Thogmartin et al. 2012; Tompkins et al. 2015). In the applied field of conservation biology, conservation managers, ecologists, veterinarians and policy-makers are regularly being called upon to distinguish between those pathogens or diseases that deserve our attention, funding and research, and the multitude of other potential disease-causing agents. Expanding on the Disease Investigation Framework presented recently by Preece et al. (2017), in this manuscript we:

1. Specifically address pervasive misconceptions concerning the role of infectious diseases in declines
2. Present a detailed Evidence Framework outlining specific veterinary and ecological methodologies available to provide the evidence required to assess the relative role of disease
3. Demonstrate the application of this Evidence Framework with a detailed case study investigating the role of chlamydial disease in koala population declines

Please note that throughout this text we have used epidemiological terms with specific meanings. For definitions concerning our usage of these terms, please refer to the glossary provided by Preece et al. (2017).

CHALLENGES IN ESTABLISHING THE ROLE OF DISEASE

Recent advances have been made in outlining an approach to determining the role of an infectious agent (and/or the disease it causes) in wildlife population decline and extirpation (Preece et al. 2017; Smith et al. 2009). However, substantial challenges (Table 1) and misconceptions (Table 2) remain. Even overt wildlife disease, causing epidemic or pandemic spread and associated with mass mortalities, may still take considerable time to recognise and diagnose (e.g. chytridiomycosis, white nose syndrome and dramatic mortalities of saiga antelope (*Saiga tatarica*) in Kazakhstan; Milner-Gulland 2015). In contrast, our capacity for recognizing disease as a more subtle component cause (Wensink et al. 2014) of population declines, among other threatening processes, is currently limited (e.g. the sudden disappearance of the woylie (*Bettongia penicillata*) in Australia; Wayne et al. 2015).

Table 1. Key challenges for determining the importance of disease among other threatening processes

#	Challenge	Description
1	Inadequate knowledge	Most systems are poorly understood; there is typically a poor level of baseline ecological knowledge, and drivers of disease dynamics and population dynamics are not well identified (Smith et al. 2009).
2	Lack of protocols	Effective protocols are lacking for the investigation of (1) the impacts of wildlife diseases on population abundance/distribution, and (2) for comparing the relative importance of disease among other threatening processes (Preece et al. 2017).
3	Methodological limitations	The independent effects of disease on wild animal populations are difficult to measure and evaluate with current approaches. These effects might include reduced survival, fertility/fecundity, fitness, and changes in population structure.

For example, sporadic animal mortalities may be difficult to detect *in situ* due to scavenging and rapid carcass decomposition (Wobeser and Wobeser 1992; Wobeser 2007).

4	Limited resources	Robust conclusions require obtaining sufficient independent sample sizes with appropriately-scaled variables at replicated sites. However, these large scale studies are expensive and difficult to achieve without cooperation between industry, government and university sectors.
5	Time-delayed response	Impacts of disease are challenging to predict, manage and mitigate, typically leading to a temporal delay between initial population declines, recognition of disease as a threat, and development of sufficient understanding for implementation of mitigation measures (i.e., consider the case with chytridiomycosis in amphibians; see Grogan et al. 2014; Muths and Hero 2010; Skerratt et al. 2011a).
6	Variable priorities	Research is often limited by sociopolitical and economic constraints associated with species of varying public appeal, inconsistent funding, and the relative lack of appropriate expertise (Grogan et al. 2014). Furthermore, policy implications of research findings may unduly influence interpretation and reporting of results.

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89 Addressing these challenges in practice requires a transdisciplinary approach bridging
 90 veterinary science, epidemiology/disease ecology, general ecology, and conservation biology
 91 fields. It requires not only recognizing and appreciating the tools and expertise utilized by the
 92 various disciplines in solving these problems, but respecting the validity of these various
 93 methodologies. Discipline-specific jargon and research silos can result in ineffective
 94 transdisciplinary communication and interpretation of data and results, as well as
 95 misconceptions surrounding the potential role of pathogens and disease in host population
 96 declines (Table 2).

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Table 2. Correcting some pervasive misconceptions regarding infectious diseases and their potential or realized role in population declines

#	Misconceptions	Most correct or plausible understanding	Explanation, examples and references
1	Detection of infectious agent = disease and population impact	On its own, the detection of an infectious agent tells you nothing about its ability to cause disease or population declines	An infectious agent may be capable of infecting a particular species without causing any disease or ill-effects. In this case, infected individuals may be called carriers, reservoirs or subclinically infected hosts. An infectious agent that causes disease may be called a pathogen. Infected individuals may transition from subclinical infection to a diseased state with more obvious clinical signs. Depending on the nature and severity of disease, it may or may not result in concomitant effects on host fitness (Murphy 2012). Even if host fitness is compromised, populations may avoid decline through other compensatory mechanisms (such as increased recruitment). Given multiple factors that might contribute to population declines, the role of infectious disease at the individual and population scale must be evaluated independently of the presence of a pathogen (Smith et al. 2009).
2	Infection load correlates with time since infection	Quantitative measures of infection burden are not necessarily linearly or monotonically associated with time since infection or the degree of clinical disease manifestation	Infection load may fluctuate with time and tissue location within a single host. Many factors in addition to simple geometric growth, such as host immune mechanisms, may play an important role in altering the trajectory of infectious burden and progression of clinical disease (Murphy 2012; Rockwood 2006). For example, chronic infections may become latent, sometimes for many years, as in the case of human immunodeficiency virus (HIV; Pantaleo et al. 1993). It is also important to take sampling factors into account (e.g., mucosally-shed pathogen DNA concentration may be significantly higher or lower than absolute tissue DNA load, depending on the tissue sampled).
3	Endemic = benign	Endemic pathogens are not necessarily benign	Endemic pathogens are those constantly present in a population through time (Porta et al. 2008), and presence through time does not necessarily require the organism to be benign (avirulent). The virulence-transmission trade-off hypothesis (Alizon et al. 2009) suggests that intermediate virulence (rather than avirulence) should be most favoured in long-term host-pathogen co-evolutionary associations, although examples may differ (Brunner and Collins 2009).

- 4 Infection and disease are static through time and space
Pathogen transmission, host infection and the manifestation of disease are dynamic processes both at the individual host level, and at the population scale
Infections may or may not progress to clinical disease over time, and infection prevalence within a population typically changes over time due to a number of factors. For example, the classic epidemic curve scenario observed in the recent Zaire Ebola Virus outbreak demonstrates a change in the incidence of cases through time (Baize et al. 2014). Longitudinal data (repeated sampling of individuals through time) are preferable to cross-sectional data (taken from populations at one point in time) for examining causation or dynamics of infection and disease (Caruana et al. 2015).
- 5 Prevalence = impact on the population
Neither infection prevalence nor disease prevalence are reliable correlates for disease impact at the population level
A benign host-pathogen association is not implicit in (1) dissimilarity between infection and clinical disease prevalence levels, (2) comparatively high infection prevalence, or (3) comparatively low clinical disease prevalence. High infection prevalence may be associated with both highly pathogenic and relatively benign infectious agents (Botzler and Brown 2014; Brown et al. 2003). Low observed clinical disease prevalence may be a consequence of both sampling biases and infection dynamics (Wobeser 2007). For example, a chronic infectious disease (such as HIV) might have a long subclinical period and short clinical period (AIDS) leading to relatively high infection prevalence and comparatively low clinical disease prevalence (Whiteside et al. 2003).
- 6 Disease impacts populations only through mortality
Disease can have a multitude of detrimental effects on a population, in addition to causing morbidity and mortality.
Non-mortality effects of disease might include (1) reducing recruitment, (2) changing population structure, or (3) altering fundamental demographic parameters. Examples of the latter include changes in sex ratio, secondary sex ratios, age structure, population sub-structures, basic autecology, behavioural traits, and timing of breeding season (Kankova et al. 2007; Knowles et al. 2010; Kolby et al. 2010; McDonald et al. 2014; Pioz et al. 2008; Reiner et al. 2009; Scheele et al. 2016; Scott 1988). Pathogens (or parasites) that prevent their hosts reproducing have particularly severe effects on host populations (Lafferty and Kuris 2009).
- 7 Population viability = population size
Population abundance and distribution are not necessarily the only appropriate measures of population viability where
A number of factors may contribute to the long-term viability of a population, and it is important to understand the balance of mechanisms driving population dynamics in addition to stochastic effects (Caughley 1994; Rockwood 2006). Small variations in the interaction of disease dynamics and population demography may lead to

	disease is a potential threatening process.	dramatic differences in population size and trajectory, as exemplified by the variety of amphibian population responses to chytridiomycosis (Tobler and Schmidt 2010).
8	Only a single major driver of declines is important	Multiple threatening processes including disease can interact in contributing to detrimental population effects (Smith et al. 2009). Disease may be having negative effects at the population scale even in the absence of decline by impacting demographic parameters or population structure. For example, frog populations may have increased vulnerability to other threatening processes such as drought due to age structure truncation caused by the disease chytridiomycosis, in the absence of overt declines (Scheele et al. 2016).

There is a long history of the development of criteria for causal inference, from the early work of David Hume (Hume 1739-1740) to the well-known Henle-Koch Postulates (Box 1; Evans 1976), to more recent applications (Plowright et al. 2008). While these methods for ‘testing’ the plausibility of causal associations are essential and extremely valuable, no single set of ‘rules’ will be suitable for all scenarios, and all have their exceptions. For example, the Henle-Koch Postulates (Box 1), widely used for identifying and confirming the causative agent of an infectious disease within an individual, are inappropriate criteria for non-infectious, multifactorial, and chronic diseases due to their focus on pathogen isolation (Evans 1976). Furthermore, no single study is able to definitively prove an association between a pathogen and population declines, and thus contemporary scientific inference demands corroboration of the results via multiple studies.

Box 1. Henle-Koch Postulates (Evans 1976)

1. The pathogen is present in all cases of disease
2. The pathogen is not present as a commensal or colonist in unaffected hosts
3. Inoculation to a naïve host after isolation of the pathogen from a diseased host and growth in pure culture, induces disease in the new host

EVIDENCE FRAMEWORK

The Disease Investigation Framework provided by Preece et al. (2017) (see Box 2) outlines a series of steps for investigating cases of wildlife population decline where disease is a putative cause. Here, we expand on the three components of this process (steps 6, 7 and 9 in Box 2) that are most challenging in subtle cases of disease-associated decline (i.e. where pathogens are present, but mass mortalities are not observed). Our Evidence Framework (Fig. 1) provides a structure for identifying disease-associated wildlife declines where disease cannot be clearly ruled out as a causal factor.

Box 2. Disease Investigation Framework (reprinted with modification from Preece et al. (2017))

1. Case definition: Define the outcome of interest at individual or population level
2. Verify that the decline is real, incorporating best understanding of species' ecology and demography
3. Determine the spatial, temporal and demographic characteristics of the observed cases
4. List hypotheses concerning potential threatening processes, risk factors or component causes for population decline
5. Determine risk factors
6. Undertake health and pathological examinations of individuals
7. Formulate a tentative diagnosis (most likely hypothesis)
8. Implement control of the decline based on the above (management actions)
9. Test and examine multiple competing hypotheses. Assess each disease hypothesis against causal criteria.
10. Refine ongoing management within an active adaptive management framework based on step 9.

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124 In our Evidence Framework (Fig. 1) we separate disease processes into two **scales** – the
 125 individual host scale (Fig. 1A) and the host population scale (Fig. 1B). To confirm that a
 126 pathogen may potentially contribute to population declines, it should be demonstrated to be
 127 having an effect at both of these scales. In the figure columns we outline the main **effects** that
 128 pathogens can have on individual fitness (Fig. 1AI) and population size (Fig. 1BI), together with
 129 **evidence** required to demonstrate these effects (Fig. 1AII and 1BII), and a suite of veterinary
 130 and ecological **methods** by which this evidence can be provided (Fig. 1AIII, 1BIII and
 131 examples provided in Figs. 1AIV, 1BIV). We define 'individual fitness' following Barrows

(2011) as ‘an individual’s relative contribution to the gene pool of its next and future generations’.

To use the Evidence Framework, at the individual host scale (Fig. 1A), it is important that a pathogen of interest should both be proven to be present (Fig. 1AII), and demonstrated as causally associated with one or more of the listed effects (Fig. 1AIIb-d) on individual hosts. It should be noted that lack of proof of pathogen presence does not confirm its absence without appropriate test sensitivity, sample sizes and study power (Skerratt et al. 2011b). Veterinary pathologists are well situated to collect appropriate evidence (Fig. 1AII) for causally associating tissue or organ dysfunction (Fig. 1AIIc) with pathogen presence (Fig. 1AIIa) and consistent clinical signs (Fig. 1AIIb). Their expertise may be called upon to confirm lack of a more plausible cause. Specific laboratory diagnostic testing is usually required to provide evidence for consistent host physiological and immune responses. Ecologists may be best suited to provide evidence concerning other forms of reduced fitness (Fig. 1AIIc) and behavioural changes (Fig. 1AIIId) in individuals with evidence of disease. While evidence for these latter effects is supportive, it is not in itself sufficient, and veterinary examinations are still required.

At the host population scale (Fig. 1B), the effects of negative population growth (Fig. 1BIa) and/or increased population vulnerability (Fig. 1BIb) should be demonstrated in association with the presence of disease in individuals (Fig. 1BIIa). Although not essential for confirming disease as a potential component cause of declines, identifying and examining the relative importance of other threatening processes putatively contributing to population declines can help elucidate the relative role of disease, and/or the presence of any synergisms or interactions between threatening processes and their effects on populations. It is also important to recognise that population decline need not be occurring for manifestation of negative impacts at the population scale, and that disease may increase population vulnerability in the absence of continued declines (Scheele et al. 2016). Ecologists and conservation biologists have a suite of

159 methods (Fig. 1BIII) that can be used to provide evidence (Fig. 1BII) relating individual effects
160 of disease with changes in demographic rates (Fig. 1BIIb-c) or population structures, processes
161 and distributions (Fig. 1BIIId-f). With regards to reduced recruitment rates, it is particularly
162 valuable to identify whether this is the result of adult reproductive fitness, population sex ratios
163 and contact networks, breeding behaviours, or neonatal/juvenile fitness.

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165 While we focus here on infectious diseases (i.e. where a pathogen is involved as a causative
166 agent), we do not intend to limit this approach, as there are many cases where relevant
167 pathogens may not yet have been identified, or where the disease is multifactorial with non-
168 infectious synergistic drivers. To apply the Evidence Framework to non-infectious diseases,
169 simply remove the requirement to associate pathogen presence (Fig. 1AIIa) with the other forms
170 of evidence (Fig. 1AII), and follow the remaining strategy outlined above, relying on veterinary
171 confirmation of disease presence in individuals in the first instance.

Figure 1. Evidence Framework for identifying disease-associated wildlife declines. A pathogen should be demonstrated to be having an effect at both the (A) individual and (B) population *scales*, in order to confirm its potential to contribute to declines. At the (A) individual host scale, the infectious agent (pathogen) should be demonstrated as causally associated with one or more of the listed (I) *effects* on individual hosts. This may be achieved by demonstrating (II) *evidence* by way of certain (III) *methods*, with some of the (IV) *examples* provided in the final column. At the (B) host population scale, the (I) *effects* of negative population growth and/or increased population vulnerability should be demonstrated in association with the presence of disease in individuals. These effects can be demonstrated via (II) *evidence* related to demographic rates or population structures and processes, provided by a variety of (III) *methods*, including those (IV) *examples* provided.

KOALA CHLAMYDIOSIS CASE STUDY

Background

To demonstrate the application of the Evidence Framework detailed above, we use koala (*Phascolarctos cinereus*, Goldfuss) population declines and their putative association with the disease chlamydiosis (infection by bacteria in Family *Chlamydiaceae*; Fig. 2) as a case study, in light of a recent comprehensive evaluation of the koala chlamydial literature (Grogan et al. 2017). Throughout this case study we make reference to sections of the Evidence Framework in parentheses (i.e. Fig. 1A and 1B). Chlamydiosis is an example of an infectious disease with a putative but poorly understood role in koala population declines (DERM 2009; Polkinghorne et al. 2013; Rhodes et al. 2011). As an example of these declines, two key southeast Queensland populations were shown to have declined by 80.3% and 54.3% respectively between 1996 and 2014 (Rhodes et al. 2015). Box 3 provides a brief overview of koala chlamydiosis. We consider the breadth of research performed on koala chlamydiosis, and follow the Evidence Framework to synthesize a conceptual argument supported by empirical evidence at both the individual host

and population scales. We discuss methodological caveats and identify current research gaps relevant to policy planning and conservation/research funding.

Figure 2. Photos of koala chlamydiosis. (A) Uninfected koala, (B) ‘wet-bottom’ as a clinical sign of cystitis (indicative of urogenital tract disease), (C) ‘pink-eye’ (ocular keratoconjunctivitis). To assist with orientation in this latter photo, we are viewing the koala’s right eye, and the koala’s nose and mouth are directed towards the top right of the image. Photo credits: (A) L. Grogan, (B) W. Ellis, (C) Koala Ecology Group, University of Queensland.

Box 3. Koala chlamydiosis – pathogen, infection and disease basics

- The *Chlamydiaceae* are a family of bacteria with a unique biphasic obligate-intracellular life-cycle (Bastidas and Valdivia 2016). They infect many vertebrate host species including humans, domestic animals and wildlife.
- Infections typically manifest as chronic and sometimes fatal urogenital tract disease (often causing infertility), ocular keratoconjunctivitis, pneumonia/rhinitis, polyarthrititis, or encephalomyelitis (the former three are found in koalas; Newman et al. 2015; Vogelnest et al. 2008; Walker et al. 2015).
- Two main chlamydial species, *Chlamydia pecorum* and *C. pneumoniae*, are implicated in koala chlamydiosis infections, however there is also evidence

suggesting possible infection with a suite of unculturable and currently largely uncharacterized *Chlamydiaceae* (Burach et al. 2014; Devereaux et al. 2003).

- Of the two main species, *C. pecorum* has been demonstrated to be the more pathogenic (Jackson et al. 1999), and evidence suggests this species may have a more recent origin in Australian koalas, coinciding with cross-species transmission from livestock (Bachmann et al. 2015; Jackson et al. 1997; Jelocnik et al. 2013).
- Infection and disease prevalence vary markedly between populations across the koala's range (Polkinghorne et al. 2013), however, infection prevalence increases with host age as expected with chronic or latent/persistent infections (Jackson et al. 1999; Obendorf and Handasyde 1990; Patterson et al. 2015; White and Kunst 1990; White and Timms 1994).

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212 **Chlamydiosis causes morbidity, mortality and sterility in individual koalas**

213 *Initial aetiological diagnoses*

214 Cockram and Jackson (1974) provided the earliest aetiological diagnosis of chlamydiosis in

215 koalas (i.e. the first confirmation that chlamydia was associated as a cause of the observed

216 disease signs). In the absence of modern diagnostic methods, they cultured (Fig. 1AIIIa)

217 *Chlamydia* from the eyes of koalas in chick embryo yolk sacs, then tested the yolk sacs

218 serologically for *Chlamydia*. They reported positive results from the eyes of all eight koalas

219 afflicted with keratoconjunctivitis and uni- or bilateral muco-purulent ocular discharge

220 (fulfilling the first Henle-Koch Postulate; Box 1; Evans 1976; Hill 1965). Conversely,

221 *Chlamydia* could not be detected from the eyes of 25 koalas that had no previous history of

222 conjunctivitis, thus providing evidence for causality based on strength of association and the

223 second Henle-Koch Postulate (Box 1; Evans 1976; Hill 1965). Brown and Grice (1986) fulfilled

224 the third Henle-Koch Postulate (Box 1), confirming *Chlamydia* as the primary cause of

225 morbidity and mortality (Fig. 1AIIa) associated with the clinical syndrome (Fig. 1AIIb). Their

226 small-scale captive infection experiment involved first the isolation (Fig. 1AIIIa) of *Chlamydia*

from ocular and urogenital sites of a clinically diseased koala, with subsequent inoculation of four naïve live koalas. All four exposed koalas developed clinical signs (Fig. 1AIIb) consistent with chlamydiosis (conjunctivitis, cystitis, reproductive tract infection and/or rhinitis), were culture-positive for *Chlamydia*, and three of the four died (Fig. 1AIIa) with severe clinical signs at 27, 62 and 126 days post-exposure. Brown and Grice (1986) stated that (pg. 352): “the cause of death was considered due to the deleterious effects of the chlamydial infections”. Four sham-exposed controls showed no clinical signs, no evidence of complement fixing antibodies, and all survived to the end of the experiment at 400 days post-exposure.

Pathology studies confirm the role of chlamydiosis on individual host fitness

Since these studies, numerous pathology case series’ describing veterinary post-mortem investigations on hundreds of koalas presented to wildlife hospitals over the last four decades have provided consistent evidence for an association between the pathogen (Fig. 1AIIa) and effects on host fitness (Fig. 1AIIa-c). These include studies confirming the presence of *Chlamydia* (via microscopy, antigen detection, PCR, or culture isolation; Fig. 1AIIa-b; Brown and Grice 1984; Canfield and Hemsley 2000; Ellis et al. 1993; Evans and Woodland 1983) in association with the following individual-scale evidence:

1. Inflamed tissues or systemic infection (via light or electron microscopy, or immunohistochemistry; Fig. 1AIIId; Brown and Grice 1984; Burach et al. 2014; Hemsley and Canfield 1996, 1997; Higgins et al. 2005a, b)
2. Host anti-chlamydial antibodies or other immune markers (complement fixation test and other serological and tissue-based assays; Fig. 1AIIIf; Brown and Grice 1984; Mathew et al. 2013b; Mathew et al. 2014; McColl et al. 1984)
3. Organ dysfunction (via gross pathology; Fig. 1AIIId; Hemsley and Canfield 1997)
4. Clinical signs of chlamydiosis (Fig. 1AIIIf; Markey et al. 2007)
5. Organ or systemic pathology sufficient to be the proximate cause of koala mortality or sterility (aspermia, reproductive tract fibrosis or occlusion; Fig. 1AIIId), as determined

through expert veterinary assessment (Canfield et al. 1991; Canfield and Spencer 1993; Higgins et al. 2005b; Johnston et al. 2015; Obendorf 1981; Obendorf and Handasyde 1990).

Furthermore, circumstantial evidence linked female sterility (Fig. 1AIIb) with chronic chlamydiosis through observations of the absence of young in diseased females, or the translocation of females and their subsequent infection and failure to breed (Brown et al. 1984; McColl et al. 1984; Obendorf and Handasyde 1990; White and Timms 1994). Alternative causes for the pathology and clinical signs associated with chlamydiosis have largely been eliminated. For example, consumption of phyto-oestrogens, earlier considered a plausible mechanism leading to reproductive tract disease, was later demonstrated to be unlikely as a cause of the observed disease syndromes (Brown et al. 1984; Handasyde et al. 1990). Mutimer et al. (1990) examined 37 koalas with clinical keratoconjunctivitis, detected chlamydial antigen (Fig. 1AIIIb) from all of them, isolated *Chlamydia* from 33% (Fig. 1AIIIa), and failed to identify any aerobic organism as a more parsimonious pathogen associated with the disease. In an immunohistochemical study (Fig. 1AIIIc), Higgins et al. (2005b) demonstrated that chlamydial infections were frequently accompanied by other opportunistic invaders, however, their numbers were low compared with *Chlamydia*.

Caveats and research gaps at the individual scale

Although the empirical evidence suggesting the pathogenicity of *Chlamydia* for koalas is robust, investigations performed on emerging infectious diseases are often compromised by methodological or technical challenges. For example, early pathology studies of koala chlamydiosis did not recognise all relevant disease syndromes (Fig. 1AIIb), likely leading to an underestimate of the impact of disease-associated effects (Canfield 1990; Canfield 1987; Cockram and Jackson 1974; Obendorf 1981, 1983). Many studies did not confirm clinical diagnoses with specific aetiological diagnostic tests (Fig. 1AIIIa-b and 1AIIIe; Canfield 1989;

Obendorf 1983; Weigler et al. 1987), and until the studies by Girjes et al. (1994) and Glassick et al. (1996), it was not recognised that there were at least two chlamydial species infecting koalas. In the light of variable pathogenicities and likely origins of these chlamydial species (see Box 3), aetiological differentiation is crucial, and represents a research gap in pathology studies that needs to be addressed more thoroughly.

As is common with wildlife disease investigations, the majority of studies collected data and specimens opportunistically via general community surveillance, hence they were not robust to sampling biases. For example, koalas examined in hospital admission and road kill necropsy surveys cannot be considered a representative sample of their respective populations. These samples usually rely on general community surveillance, preferentially involve roadsides and urban areas, and hence are likely to over-estimate car collision trauma cases as a cause of morbidity and mortality within koala populations (Canfield 1991; Griffith et al. 2013). The sample is also likely biased towards younger male koalas due to seasonal dispersal (subpopulation behavioural variation; Griffith et al. 2013; Weigler et al. 1987). These opportunistic data may lead to underestimating the frequency of a chronic-progressive disease such as chlamydiosis. The chronic nature of chlamydiosis also often complicates interpretation of individual-level field-based results. For example, unlike earlier studies (Brown et al. 1984), Obendorf and Handasyde (1990) identified variable degrees of infertility (Fig. 1AIIb) associated with urogenital tract infections (Fig. 1AIIId). They found that infected females continued to breed for several seasons, and attributed the variation to differing extent and severity of existing disease (as has since been supported by other studies; Hemsley and Canfield 1997; Higgins et al. 2005a, b).

***Chlamydia* are implicated in detrimental effects at the population scale**

Evidence for population-scale associations between chlamydiosis and reduced recruitment

In the absence of a single clear demographic effect (Fig. 1AIIa-b and Fig. 1BIIb-c) underlying recently documented koala population declines (Adams-Hosking et al. 2016; DERM 2009; Melzer et al. 2000; Phillips 2000), we next examined the putative population-level effects of disease (Fig. 1BI). Many population-level studies were consistent with a detrimental effect of chlamydiosis on koala populations, through reduced fecundity (Fig. 1BIIC) and population growth rates (Fig. 1BIa). For example, McColl et al. (1984) found a negative association between infection prevalence and female fecundity comparing island populations in Victoria. Lee et al. (1990) reported a substantial drop in female fecundity of tracked individuals approximately two years after translocation to *Chlamydia*-infected populations. Martin and Handasyde (1990) compared the fertility rate (the proportion of females with dependent young) between three presumed *Chlamydia*-free Victorian populations (fertility 59-70%) and six *Chlamydia*-infected Victorian populations (fertility 0-56%), consistent with a marked effect of chlamydiosis on fertility/fecundity. Gordon et al. (1990) found that disease was associated with higher mortality (Fig. 1BIIb) and reduced fecundity (Fig. 1BIIC) using long-term annual data from Oakey, QLD, although the long-term persistence of the population was apparently unaffected. In their study at Mutdapilly, QLD, White and Timms (1994) found a negative association between fecundity and urogenital infection that was more overt after the development of clinical signs. Patterson et al. (2015) also found a negative correlation between chlamydial infection and fecundity at three Victorian sites.

Limited evidence for chlamydiosis affecting survival at the population scale

In contrast, there is comparatively little published data for assessing an effect of chlamydiosis on survival (Fig. 1BIIb) at the population scale, although some studies suggested an effect (Gordon et al. 1990; Lee et al. 1990; Rhodes et al. 2011; White and Kunst 1990). Given evidence for high individual mortality (Fig. 1AIIa) from pathology studies, albeit non-representational samples, this may finding be more likely due to the lack of appropriate *in situ* longitudinal cohort studies (Fig. 1BIId) able to track and detect mortalities (Fig. 1BIIb) in the

field, than to an absence of effect. Indeed, this highlights a common problem with field-based disease ecology studies. It is often difficult to detect animal mortality in the field due to carcass scavenging and rapid autolysis, even when actively searching for carcasses (Wobeser and Wobeser 1992; Wobeser 2007). This is exacerbated by the predominance of cross-sectional (Fig. 1BIIIb) infrequent sampling and short-term studies (especially cohort-based tracking or translocation studies). It is further compromised by the spatiotemporally sporadic mortalities that might be expected with chronic-progressive infections, as opposed to rapidly progressive diseases and relatively synchronous mass mortality events. Thus, one would expect any opportunistically recorded mortalities to be a marked under-representation of the true occurrence of disease-associated mortality in the field (Wobeser 2007). As yet, no robust cohort (mark-recapture and tracking; Fig. 1BIIIId) studies of sufficient length (> several years) and sample size have been performed in the context of koala chlamydiosis (Griffith et al. 2013).

Limited evidence for chlamydiosis affecting population growth rates

Few papers have demonstrated that chlamydiosis affects population growth rates directly (Fig. 1BIa). Martin and Handasyde (1990) found that the intrinsic rate of increase was greater in two uninfected populations than in an infected population. However, the estimated rate of increase was based on two time points only, which were 27 years apart for the infected population and 14 and 12 years apart for the uninfected populations, hampering interpretation of this result. Gordon et al. 1990 found an association between koala *density* and cystitis prevalence the prior year, but an association between *intrinsic rate of increase* and cystitis prevalence in the previous year was not supported (McCallum 2012). Examining overall population growth rates is important for confirming actual population declines as disease-associated demographic changes may be compensated for at the population level by increased recruitment, immigration or compensatory declines in mortality (i.e. compensation between Fig. 1BIb and BIc). For a non-koala example, Twigg et al. (2000) found that it was necessary to sterilize upwards of 60%

of female rabbits to have any impact on population growth, with the decreased fertility being compensated for by increased survivorship.

Two studies reported tree defoliation coincident with low koala reproductive rates (40% fecundity; Fig. 1BIIc) attributed to disease, suggesting that the presence of chlamydiosis was insufficient to limit population growth (Fig. 1BIa) on its own (Lee et al. 1990; Mitchell et al. 1988). However, suggestions of over-browsing were not substantiated in these studies as an appropriate proxy for increased population abundance, and other demographic rates and other potentially threatening processes were not elucidated. Disease may still contribute to increasing population vulnerability (Fig. 1BIb) irrespective of whether a population is actually declining, however, these studies are largely lacking for koala chlamydiosis. For example, in a non-declining but disease-affected population, the addition of one or more non-disease threats that increase mortality may exceed the population's recruitment buffering capacity, resulting in declines (Gordon et al. 1990).

Caveats and research gaps at the population scale

The quality and quantity of population-level evidence regarding the effect of chlamydiosis on koala demographic rates is comparatively poor. Although an effect of chlamydiosis on fecundity (Fig. 1BIIc) has been demonstrated repeatedly, particularly in Victorian studies, lack of appropriate data has hindered adequate assessment of disease-associated effects on survival rates (Fig. 1BIIb) and overall population growth rates (Fig. 1BIa). White and Timms (1994) suggested that high levels of chlamydial infection prevalence and low expression of clinical disease indicated that disease alone was unlikely to limit population growth, however, see Table 2, # 5 for an explanation of why this may not necessarily be the case.

Resource and technological limitations have resulted in inherent sampling and methodological biases in the majority of population-level (Fig. 1B) studies performed to date on koala

chlamydiosis (Grogan et al. 2017). The most important of these caveats was the lack of appropriate diagnostic techniques capable of distinguishing chlamydial species/strains and infection severity (Fig. 1AIIIa-b). Indeed, it was surprising to find that only three population-level disease studies have been published in the last two decades since development of these techniques (Jackson et al. 1999; McLean and Handasyde 2006; Patterson et al. 2015). Differentiating chlamydial species is essential given findings that *C. pecorum* is comparatively more pathogenic than *C. pneumoniae* (Bachmann et al. 2015; Jackson et al. 1999; Jelocnik et al. 2013). A recent study by Patterson et al. (2015), paradoxically found that only two of three Victorian populations demonstrated evidence of chlamydial infection by PCR, despite the presence of consistent clinical disease in all three. Given the possibility for persistent non-shedding chlamydial infections (Burach et al. 2014; Govendir et al. 2011; Griffith et al. 2010; Obendorf and Handasyde 1990), we speculate that their paradox may have been resolved with repeated diagnostic testing, less specific PCRs (Fig. 1AIIIb), and the use of serology for detecting anti-chlamydial antibodies (Fig. 1AIIIe). Another diagnostic difficulty was that several studies used distance observation of clinical signs (Fig. 1AIIIC) as their primary means of determining chlamydial disease status, which is likely to have underestimated disease frequency (Weigler et al. 1988; White and Kunst 1990). We recommend future studies employ diagnostic gold standards (qPCR and antibody-detection via ELISA; Fig. 1AIIIb; outlined in Carey et al. 2010; Markey et al. 2007) to distinguish chlamydial species and strains in individuals sampled as part of population-level studies.

A second pervasive limitation of these studies, and one that relates closely to the chronic-progressive nature of chlamydiosis, was that most studies did not consider population structure (particularly gender and age; Fig. 1BIIIe) in their assessment of the impact of infection and disease. For example, while the study by Weigler et al. (1988) demonstrated high female fertility concurrent with high infection prevalence (82% adult females with young, 67% adult females infected), due to the prevailing diagnostic capabilities they did not explore either the

age structure of the population or the age of females examined. This limits our confidence in their conclusions, given the possibility that their results may have originated from a young-adult-biased sample, prior to the manifestation of clinical reproductive tract disease. Similarly, White and Kunst (1990) did not provide details on age structure, despite their conclusion that high overall fertility (67%) and the presence of ocular damage in otherwise healthy individuals demonstrated that chlamydiosis was not significantly threatening populations. These conclusions may or may not be correct – loss of fertility in older females that have already reproduced may have limited effects on population growth, but some form of age structured model is needed to evaluate the effect of loss of fertility with age (Fig. 1BIIIe-f). At their Springsure study site, Gordon et al. (1990) found no association between disease and population growth parameters. In another study at Springsure, Ellis et al. (1993) suggested that this absence of effect stems from inherently lower alienation pressures. However, it is important to note that high population turnover driven by environmental conditions as noted by Gordon et al. (1990) may inhibit observation of the effect of chronic diseases such as chlamydiosis. Severe clinical signs may take several years to manifest, and if the average age of the population is relatively low due to other mortality factors, then most koalas may never be observed with advanced disease (for an example related to fecundity, see Martin 1981). Evaluation of the age structure (Fig. 1BIIIe) of the population and appropriate modelling (Fig. 1BIII f) may help resolve this issue.

Could chlamydiosis just be a symptom of other threats such as habitat loss?

Speculation regarding the role of other threatening processes in the manifestation of chlamydiosis has been a persistent feature throughout the koala chlamydial literature (Canfield 1989; Canfield et al. 1991; Carey et al. 2010; Cockram and Jackson 1981; Ellis et al. 1993; Patterson et al. 2015; White and Timms 1994). These other threatening processes include habitat loss, climate change and urbanization (McAlpine et al. 2015) and are hypothesized to contribute to increasing koala stress that, in turn, results in the expression of disease. However,

for the observed manifestation of chlamydiosis to *solely* be the result of these other threatening processes, there is an implicit assumption that *Chlamydia* are opportunistic pathogens in koalas (normally innocuous organisms that become pathogenic with host immune compromise; Porta et al. 2008). In the following section we demonstrate that this exclusive hypothesis and implicit assumptions are unlikely to be correct, at least with the demonstrated more pathogenic *C. pecorum* (Jackson et al. 1999). However, potential for synergies between chlamydiosis and other threatening processes should undoubtedly warrant further investigation.

There is currently little data available to investigate an association between habitat loss/urbanization and chlamydial infection and disease. Griffith et al. (2013) showed that admissions of koalas with clinical chlamydiosis did not change over the 30 years of their study, suggesting that urbanization around the Port Macquarie region was not associated with increased incidence of disease. Ellis et al. (1993) and Gordon et al. (1990) investigated non-urban populations and found both chlamydial infection and clinical disease, albeit at relatively low prevalence. Stress hormone levels can now be measured using non-invasive techniques (Fig. 1AIII f; Johnston et al. 2013; McInnes et al. 2011; Narayan et al. 2013). No studies to date have established whether (1) threatening processes such as habitat loss increase measurable stress in individual koalas, or (2) whether an increase in stress is correlated with increased expression of chlamydiosis in koalas. Furthermore, measures of stress hormones can be difficult to interpret, and establishing the direction of causality between stress and disease is particularly challenging (Griffith et al. 2013; Narayan et al. 2013).

There is little evidence of immunosuppression associated with stress in chlamydial-infected koalas (Hemsley and Canfield 1997; Mathew et al. 2013a; Mathew et al. 2013b; Mathew et al. 2014). Indeed, chlamydiosis-associated tissue structural damage (Fig. 1AIII d) appears to be exacerbated by an overactive or unregulated immune system (immune-mediated tissue damage; Fig. 1AII d; Hemsley and Canfield 1997). Interestingly, several studies demonstrated multi-

parasitism involving co-occurrence of chlamydiosis with other pathogens such as koala retrovirus and herpesvirus (Higgins et al. 2005b; Stalder et al. 2015; Tarlinton et al. 2005). This may indicate (1) common risk factors (including possibly underlying immunosuppression), or (2) pathogen-facilitated synergistic co-occurrence (Vaumourin et al. 2015). However, the evidence for these associations is still relatively insubstantial, and considerable work is needed to establish these as causal associations.

Veterinary necropsy surveys present data indicating that chlamydiosis (or clinical signs consistent with chlamydiosis, where aetiological tests were not performed) was diagnosed as the proximate cause of mortality (Fig. 1A1a) in a high proportion of cases presented to wildlife hospitals through general community surveillance (in the range of 19-44%; Canfield 1990; Canfield 1987; Griffith et al. 2013; Obendorf 1983; Weigler et al. 1987). This high proportion of cases would be unexpected if *Chlamydia* caused purely opportunistic infections only when koalas were stressed and immunocompromised. For example, in a necropsy survey (Fig. 1A11d) of 75 free-ranging koalas in south-east Queensland, Weigler et al. (1987) stated that “lesions consistent with a diagnosis of koala chlamydiosis were found in 29 animals, [...] in 19 of these were the lesions deemed severe enough to be the cause of death” (pg. 572). Griffith et al. (2013) found chlamydiosis to be the second most frequent cause for hospital admission (20.4% of admissions) after trauma, and Canfield (1990) identified urogenital disease as the second most common finding in presented koalas (19%) after trauma, although an estimate of chlamydiosis could be as high as 40% from their data when considering other consistent clinical signs. By definition, as obligate intracellular pathogens, *Chlamydia* cannot be considered as opportunistic (i.e. *Chlamydia* are never innocuous commensals and their presence indicates that an infectious process is occurring; Porta et al. 2008). Furthermore, the predominance of disease signs and mortality associated with a *single opportunistic pathogen* over a broad spatiotemporal range would be considered unusual. One would more commonly expect to find a variety of opportunistic infectious agents if presenting animals were immunosuppressed (consider co-

infections associated with HIV/AIDS; Anonymous 2016). However, mass die-offs of saiga antelope have been associated with a single opportunistic bacterium *Pasteurella multocida* (Milner-Gulland 2015; Samuel 2016) in combination with synergistic environmental factors, so the associations may be complex.

The role of chlamydiosis in koala population declines

The weight of published empirical data currently suggests strong evidence for chlamydiosis (particularly caused by *C. pecorum*) as a major cause of morbidity, mortality (Fig. 1AIa) and sterility (Fig. 1AIb) in individual koalas. Necropsy surveys, although subject to sampling biases, have repeatedly implicated chlamydiosis as one of the two major causes of koala mortality (the other being trauma) at least in urban and semi-urban areas. Population-level studies confirm a detrimental impact of chlamydiosis on koala recruitment (Fig. 1BIc) in various populations. Data on population-level impacts of chlamydial infection on overall population growth rates (Fig. 1BIa) are very sparse. In the absence of a field deliverable treatment or prophylaxis for chlamydiosis, the “gold standard” of replicated population level manipulations of infection (Fig. 1BIId) is not yet possible. However, it is disappointing that so few time series (Fig. 1BIId) of koala population dynamics and chlamydial infection, which could shed light on this question, are available for analysis (Grogan et al. 2017).

Conclusions

We have presented a systematic Evidence Framework for demonstrating whether a pathogen is a component cause of declines of wild animal populations. We have also highlighted key challenges and corrected pervasive misconceptions regarding the potential for infectious diseases to cause population declines. Through our koala chlamydiosis case study we have provided an example of how to integrate a plethora of different studies and approaches into the Evidence Framework context, helping identify not only gaps in the existing approaches, but also providing suggestions for avenues for further research. From the results of our case study we

found that available empirical evidence supports a role for chlamydiosis contributing to host mortality and sterility at the individual scale. The evidence for a role of chlamydiosis as a component cause of koala declines at the population scale was less robust, likely due to existing research gaps from predominantly non-representational sampling. In conclusion, chlamydiosis has the potential to be an important contributor to koala population declines among other threatening processes, and hence it cannot be disregarded as a component cause of declines. This does not imply that chlamydiosis is necessarily the main cause or driver of koala population declines, nor that all policy and funding should be directed towards research on koala diseases. It does, however, indicate that chlamydiosis must continue to be considered as an important potential threat in future koala management and conservation strategies. Our koala chlamydiosis case study has provided a practical implementation of the Evidence Framework within a nuanced and complex host-pathogen system. It has also allowed us to highlight the breadth of research that contributes to understanding the role of disease in wildlife population declines, and some of the challenges therein. We believe that our Evidence Framework approach will provide a straightforward way to evaluate the role of disease in wildlife population declines within a broad range of ecological systems.

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