Current trends and future directions in koala chlamydial disease research

Running head: Koala chlamydial disease research systematic review

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ABSTRACT

Infectious diseases can be key threatening processes for biodiversity conservation. However, establishing the relative importance of disease (among other threatening processes) as a driver of species declines can be challenging. Bias in the directions that a research field may take as it develops – due to factors such as conservation policy, funding, public perception, and available expertise and technology – may exacerbate this difficulty. Chlamydiosis (infection with bacteria in Family *Chlamydiaceae*) is an example of an infectious disease with a poorly understood role in koala (*Phascolarctos cinereus*) population dynamics. The arboreal folivorous koala is an internationally recognized iconic species of high conservation, sociocultural and economic value. To date, no studies have quantitatively examined the breadth and scope of research related to koala chlamydiosis, nor systematically identified the current research gaps. We systematically and quantitatively reviewed a comprehensive database of literature related to koala chlamydiosis, classified and examined the main foci of the research, and evaluated research gaps with the goal of assisting policy planning for funding further koala chlamydiosis research. We examined published literature with regards to journal category, authorship, funding, spatiotemporal scope, study foci and type, chlamydial species examined, methodological design and overall findings. Among the 117 peer-reviewed papers published between 1970 and 2016 that fit our criteria, the most striking finding was the relative lack of population-level disease studies within the last two decades to examine mechanisms of chlamydial infection dynamics. This research gap is of particular concern given the potential role of *Chlamydia* in koala population declines, and the recent dramatic changes in our understanding of pathogen phylogeny and improved diagnostic approaches. Our results demonstrate a pressing need for future *in situ* comprehensive longitudinal population-level studies from diverse geographic regions. These studies must utilize up-to-date diagnostic methods capable of distinguishing chlamydial species and strains to elucidate the role of chlamydial infection in

49 koala population declines and the underlying mechanisms involved. They should also employ
50 rigorous epidemiological methodologies and evaluate co-infection, habitat, climatic and
51 demographic data. Our findings suggest some key research gaps concerning koala chlamydiosis,
52 and are hence important for guiding future research into koala chlamydiosis and conservation.

53 MAIN TEXT

54 **INTRODUCTION**

55
56 Infectious diseases are increasingly recognized as key threatening processes for the conservation of
57 biodiversity (Daszak 2000; Smith et al. 2009; Tompkins et al. 2015). Diseases such as
58 chytridiomycosis in amphibians, West Nile Virus in birds, and white nose syndrome in bats have
59 exerted devastating effects on wildlife species around the globe (George et al. 2015; Skerratt et al.
60 2007; Thogmartin et al. 2012). Microorganisms and parasites are, however, ubiquitous among hosts
61 and in the environment, and many recognized infectious agents persist in reservoirs in the absence
62 of clinical disease (Haydon et al. 2002). Thus, determining the specific role of infectious agents in
63 host population declines can be difficult, particularly if their effects are not readily detected in the
64 form of mass mortalities and/or overt disease (Scheele et al. 2016; Smith et al. 2009).

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66 Chlamydiosis (associated with infection by bacteria in Family *Chlamydiaceae*) is an example of an
67 infectious disease thought to be endemic and ubiquitous in koala populations (*Phascolarctos*
68 *cinereus*, Goldfuss), however, it has a poorly understood role in host population declines
69 (Polkinghorne et al. 2013; Rhodes et al. 2011). The arboreal folivorous koala is the sole extant
70 representative of Family *Phascolarctidae*, and an internationally recognized iconic species of high
71 conservation, sociocultural and economic value (Black et al. 2014; Hundloe and Hamilton 1997;
72 Price 2012). In recent decades widespread population declines have become apparent, particularly
73 in the northern parts of the koala's range (DERM 2009; McAlpine et al. 2015; Melzer et al. 2000b).
74 Koalas are now listed as 'Vulnerable' since 2012 under Australian Federal Government legislation
75 (Environment Protection and Biodiversity Conservation Act 1999; EPBC Act), across two states
76 and one territory (Queensland, New South Wales and the Australian Capital Territory; McAlpine et
77 al. 2015).

78

79 Successfully mitigating disease and managing koala populations to prevent extirpation and reverse
80 declines is a current priority for koala conservation. However, the relative impact of chlamydiosis
81 among other threats (including habitat loss, fragmentation, car collisions and dog attacks) is poorly
82 understood, and decision makers have been unable to effectively guide management policy because
83 there is no consensus regarding the nature and extent of the impact of chlamydial infections on
84 koala populations (McAlpine et al. 2015; Rhodes et al. 2011). Inadequate funding and technological
85 constraints may have limited understanding. Indeed, many fundamental aspects of the disease
86 ecology remain understudied (McCallum et al., under revision). To date, no studies have
87 quantitatively examined the breadth and scope of research related to koala chlamydiosis, nor
88 systematically identified the current research gaps.

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90 With the goal of assisting policy planning for funding future koala chlamydial research, we have
91 used the well-established systematic quantitative review technique (Petticrew 2001; Pickering and
92 Byrne 2014; Rochman et al. 2016) to examine the extent of the peer-reviewed literature on koala
93 chlamydiosis. Our review examined general characteristics of papers/chapters included (such as
94 journal category, authorship, funding and spatiotemporal scope), their major foci and study types,
95 the breadth of chlamydial species covered, as well as methodological design (including data types,
96 sampling and analysis details). In the following sections we critically analyse the results, consider
97 study caveats, and identify research gaps that should be addressed in future studies.

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METHODS

We used systematic search and compilation methodology common to quantitative systematic literature reviews to provide a comprehensive and reproducible overview of the current status of literature on koala chlamydiosis (Pickering and Byrne 2014; Rochman et al. 2016). Our purpose was to evaluate the breadth and scope of the peer-reviewed literature available since 1970 (encompassing the first paper reporting aetiological diagnosis of chlamydiosis in koalas), including identifying the presence of research trends and weaknesses, in order to highlight gaps to inform policy and funding for future research agendas.

We searched *Web of Science: All Databases* (WoS; Thomson Reuters) and *Scopus* (Elsevier) literature databases using ‘title’ or ‘title, abstract, keyword’ searches respectively, with date range from 1 January 1970 to 28 April 2016. We initially examined all English-language literature pertaining to the host species of interest, using the phrase “(koala* OR *Phascolarct**)” (808 articles from WoS, 1151 articles from *Scopus*), then narrowed these lists by including pathogen or disease-associated terms “(diseas* or chlam* or cystit* or “wet bottom” or “dirty tail” or conjunctiv* or keratoconjunc*)” (103 articles from WoS, 350 articles from *Scopus*). We assessed each of these entries individually based on title and abstract (and where necessary, a more detailed assessment of the paper) for relevance and inclusion in our database. We then modified this combined list with (i) the addition of any relevant literature cited by the existing articles that was not originally included in the database results, and (ii) specific inclusion and exclusion criteria (see Table 1).

Table 1. List of criteria governing the inclusion or exclusion of literature for the systematic database.

Criteria	Publication characteristics	Study focus characteristics
Inclusion	<ul style="list-style-type: none"> • Peer-reviewed primary literature • Peer-reviewed 'conference proceedings' if full papers • Edited book sections/chapters 	<ul style="list-style-type: none"> • There must be reference to bacteria of the Family <i>Chlamydiaceae</i>, and infection of koalas (<i>Phascolarctos cinereus</i>) OR • There must be reference to clinical signs and/or pathology of the koala consistent with chlamydiosis
Exclusion	<ul style="list-style-type: none"> • Non-English language publications • Abstract-only publications/proceedings or editorial pieces • Study/paper duplicates • Grey literature (popular articles, theses, governmental reports) • Literature not published between the year 1970 and 28 April 2016 • Peer-reviewed review and modelling papers if they do not present new empirical data in a robust form 	<ul style="list-style-type: none"> • The reference to koala chlamydial infection or disease must not be 'in passing' (brief mention in introduction/discussion), and must instead be a main focus of the literature • We excluded those studies that did not provide empirical results with either quantitative or qualitative data of relevance to chlamydial infection or disease in koalas • We excluded studies of <i>C. pecorum</i> or <i>C. pneumoniae</i> if empirical data did not include isolates from koalas

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127 In this study we define koala chlamydiosis as any infection (or co-infection) of the host species *P.*
128 *cinereus* with bacteria of Family *Chlamydiaceae*. Importantly, this definition includes infection
129 with both the main species of culturable *Chlamydia* bacteria that have been identified as infecting
130 koalas to date (*Chlamydia pecorum* and *C. pneumoniae*), despite their putatively differing host
131 pathogenicity, as well as phylogenetic and geographic origins (Polkinghorne et al. 2013). It also
132 includes the potential infection with a suite of as yet uncharacterized and potentially unculturable
133 chlamydial species or strains that have been detected via genetic sequencing methodologies (Burach
134 et al. 2014; Devereaux et al. 2003). We have been deliberately inclusive and have not distinguished
135 between infections causing subclinical and clinical disease because a thorough recognition of the
136 transmission and infection dynamics with various chlamydial species will likely be crucial for
137 understanding the expression of clinical disease in koalas, and subsequent population-level impacts.

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139 In addition, we recognize four broadly inclusive disease syndromes related to the clinical signs and
140 gross pathology of chlamydiosis in the koala, including ocular keratoconjunctivitis, urinary tract
141 disease, reproductive tract disease, and respiratory disease (including rhinitis-pneumonia complex)
142 as described by Vogelnest et al. (2008). Development and widespread implementation of
143 appropriate diagnostic tests for atypical intracellular pathogens such as those in Family
144 *Chlamydiaceae* was a gradual process (for example, see Hammerschlag 1996). This constitutes a
145 bottleneck in the study of atypical emerging infectious diseases and poses challenges for defining
146 sufficiently comprehensive inclusion criteria when specific diagnostic testing is not yet consistently
147 performed. We thus chose to include the many relevant studies with a major focus on investigating
148 clinical signs and pathology that were highly consistent with our chlamydiosis disease syndromes
149 (see above), despite the absence of a clear aetiological diagnosis or pathogen isolation and
150 characterization. Our broad definition allows us to capture the variety of studies that have been
151 performed on chlamydial infections in koalas.

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153 We were particularly interested in peer-reviewed original empirical data papers describing *in situ*
154 population-level chlamydial infection or disease dynamics in koalas. In general, we excluded
155 population-level demographic studies that made no specific mention of chlamydial infection or
156 disease signs in the results. Despite this focus, we recognize that key understanding has been
157 developed through studies predominantly focused on the pathogen in terms of phylogeny and
158 characterization, so these were included. We excluded both general review and modelling papers
159 from the database to eliminate overlapping data reporting, and ‘unpublished data’ from which their
160 results sometimes derive. Data were extracted from all publications included in the systematic
161 database by a single investigator for consistency, and were cross-checked and evaluated by all co-
162 authors.

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164 For each paper that met our inclusion criteria we thoroughly reviewed their content over several
165 occasions and constructed a database, recording data under the following headings: (i) literature
166 details, (ii) study focus, (iii) chlamydial species, (iv) study type, (v) koala sampling details, (vi)
167 koala source location, (vii) diagnostic methods, (viii) data type, and (ix) funding details. We
168 summarized and analysed the resulting database to detect patterns and also examined trends within
169 the groups defined by major study focus where this was relevant. However, we did not explicitly
170 apply weighting criteria to the various studies due to the breadth of research covered and lack of an
171 appropriately broad and inclusive rating scheme.

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173 Under the 'Study focus' heading, we assessed papers for their main focus of either 'Koala' or
174 'Pathogen', and after examining the complete database, categorized studies into the following sub-
175 foci that best described their major purpose or aim. Under the 'Koala' category: (i) population-level,
176 (ii) pathology, (iii) immunology, (iv) pharmacology, (v) other pathogen, (vi) diagnosis, and (vii)
177 experimental infection; and under the 'Pathogen' category: (i) *in vitro* response, and (ii) phylogeny.
178 Several of these categories had to be relatively broad to encompass the diversity in the koala
179 chlamydial literature, and it is important to note that papers often reported data that were somewhat
180 peripheral to their research question or main focus.

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182 The 'Koala' focused category included studies where koalas were directly or indirectly sampled or
183 observed for signs of chlamydial infection, disease, or diagnostic samples. This category included
184 studies advancing the diagnosis of koala chlamydiosis (and frequently included prevalence
185 reporting), but excluded studies predominantly characterizing the pathogen. The 'Pathogen' focused
186 category included papers where the samples being investigated involved koala-specific
187 *Chlamydiaceae*, but were mainly concerned with characterizing the pathogen, without consideration

188 of responses of koalas from which the samples or isolates were derived. To avoid paper overlap
189 between categories, where a paper focused on more than one major aim or purpose, we selected the
190 category that fit most closely.

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RESULTS

Overall summary, disciplinary scope, authorship and funding

From our comprehensive systematic review of the koala research literature between 1970 and 2016 (constituting 808 articles identified via *WoS* versus 1151 identified via *Scopus*), we identified a total of 117 original peer-reviewed research papers that met our inclusion criteria (Table 1) pertaining to koala chlamydial infection and disease (see Appendix S1 for complete list of studies examined and details recorded). These papers were published in a total of 36 different journals and two books (Fig. 1i and ii). The two journals individually publishing the highest number of papers were the *Australian Veterinary Journal* (20 published papers), and the *Journal of Wildlife Diseases* (11 published papers).

Figure 1. Bar graph demonstrating the proportion of (i) journals grouped according to category, and papers grouped by (ii) journal category, (iii) publication decade, (iv) chlamydial species examined, (v) data type explored, (vi) major study foci, and (vii) minor study foci. Please note that Journal categories used in (i) and (ii) above are not mutually exclusive (a journal may have more than one category allocated according to Thomson-Reuters™ InCites™ Journal Citation Reports®). Major and minor study foci were mutually exclusive (only one major and one minor study focus allocated per paper, please see supplementary information for more details).

These 117 papers represent the collective work of a total of 207 authors, who each contributed to between 1 and 46 papers. The distribution of number of papers co-authored by specific researchers was highly positively skewed (Fig. 2). The most highly represented authors (≥ 10 papers) included P. Timms (46 papers), A. Polkinghorne (23 papers), K. Beagley (17 papers), P. J. Canfield (15 papers), D. P. Higgins (13 papers), F. N. Carrick (12 papers), A. A. Girjes (12 papers), J. Hanger (11 papers), and M. F. Lavin (11 papers). The profile of first-authorship varied from that of all co-authors combined, with a range from between 1 and 10 papers first-authored by a single individual. The authors with the highest first-author representation (≥ 4 papers) included P. J. Canfield (10 papers), A. A. Girjes (7 papers), A. Kollipara (4 papers), and R. W. Martin (4 papers). The most highly represented co-authors were not typically highly represented as first-authors: P. Timms (first-author on 1 paper), A. Polkinghorne (0 papers), K. Beagley (0 papers), P. J. Canfield (10 papers), D. P. Higgins (3 papers), F. N. Carrick (0 papers), A. A. Girjes (7 papers), J. Hanger (1 paper) and M. F. Lavin (1 paper).

Figure 2. Histogram of the frequency of authors represented by the number of papers they co-authored.

The majority (97) of studies specifically acknowledged funding agencies, organizations or schemes for financial support, and the funders listed were diverse (and included a number of international funders from USA, Japan, UK, Iraq, etc.). A total of 37 papers reported funding from the Australian Research Council (ARC; with 20 specifically acknowledging ARC Linkage grants and 7 acknowledging ARC Discovery Projects). The next most prolific funders are listed in Table 2, with the remaining funders attributed in four or fewer papers.

Table 2. Funding agencies or schemes most frequently attributed for financial support (those attributed by five or more papers; funder attribution was not mutually exclusive).

Major funding agencies or schemes attributed	Frequency
Australian Research Council (ARC)	37
Australian Koala Foundation (AKF)	15
Lone Pine Koala Sanctuary	13
National Parks and Wildlife Service (NPWS)	11
The University of Queensland Koala Fund (UQKF)	9
Queensland Government National and International Research Alliances Program Scheme (NIRAP)	7
Sun Herald Koala Appeal Fund	7
Queensland Department of Environment and Heritage Protection Koala Research Grant (QDEHP)	5
Koala Preservation Society of New South Wales (KPSNSW)	5

Spatiotemporal breadth and key koala source locations

We purposely selected the temporal range of our review (1 January 1970 to 28 April 2016) to include the earliest peer-reviewed publication that reported an aetiological diagnosis of chlamydial infection in koalas (Cockram and Jackson 1974; published in the *Australian Veterinary Journal*). Two papers were published in 1970s, 23 papers in 1980s, 36 papers in 1990s, only 13 papers were published in 2000s, and 43 papers were published from 2010-2016 (Figs. 1iii and 2).

Figure 3. Number of papers primarily based on data from each state/territory by decade (not mutually exclusive – some papers used koala or chlamydial isolate data collected from multiple states).

Seventy-four studies contained koala samples, data or chlamydial isolates from Queensland (59 of which were solely QLD data), 37 with data from New South Wales and/or the Australian Capital Territory (21 of which were solely NSW/ACT data), 22 with data from Victoria (15 solely VIC),

and nine studies with data from South Australia (only 2 solely SA) (Fig. 3). Four studies contained insufficient detail on the koala *Chlamydia* isolates used to determine their geographic origin.

Several key study locations (captive sanctuary or hospital situations) were identified as sources for koalas, samples, or chlamydial isolates in many studies (Fig. 4). These included most frequently Lone Pine Koala Sanctuary, QLD (approximately 15 studies), Port Macquarie Koala Hospital, NSW (15), Australia Zoo Wildlife Hospital, QLD (10), Queensland Parks and Wildlife Service Koala Hospital Moggill, QLD (7), Featherdale Wildlife Park (4), Dreamworld, QLD (2) and Currumbin Sanctuary, QLD (2) (several studies were unclear, and some included many sources).

Figure 4. Map of the key source locations for sampled koalas in Australia (red crosses = populations sampled in the 16 population-level studies; orange crosses = key captive or hospital *ex situ* sites), plotted against the probable maximum extent of koala distribution (diagonal shading), modified from Threatened Species Scientific Committee (2012). States are shaded according to the relative proportion of the total papers (117) that reported on data from koalas or chlamydial isolates sourced from each state (see text for further details). The inset shows the south-east Queensland region surrounding Brisbane in greater detail. QPWS = Queensland Parks and Wildlife Service.

Koala subjects – context, sampling size and sampling robustness

The distribution of the number of koalas examined per paper was strongly positively skewed (Fig. 5). Data from a total of up to 11,027 koalas were reported in the studies identified (assuming no overlap between individual koalas and koala samples), with an average of 94 koalas sampled or examined per paper. However, the majority of studies examined data from 27 (median) or fewer koalas (18 studies examined chlamydial isolates, samples, or data from three or fewer koalas), with only four studies examining more than 500 koalas. The maximum number of koalas reported in a single paper was 2,674 in a 30 year retrospective analysis of hospital admission data (Griffith et al. 2013). Examining only the 16 population-level studies, this average was 177 (median = 99, total across these studies = 2,837). Overall, 41 papers involved captive koalas, while 87 involved free-ranging koalas (25 studies involved both), the majority of which were brought in opportunistically through passive surveillance for care at koala hospitals by members of the community rather than being sampled in the field. Fourteen studies were unclear on whether the koalas from which isolates were used were free-ranging or captive.

Figure 5. Frequency histogram of the frequency of papers associated with the number of koalas sampled per paper (bin width = 20 koalas).

We assessed 107 (91%) papers as presenting data from opportunistically sampled koalas. This category included studies involving koalas admitted to hospitals, captive koala populations, archived sample specimens, individual chlamydial isolates, or poorly reported field-collected samples. While 33 studies mentioned field-sampled animals, only 10 of these reported robust field sampling methodologies, design and epidemiological analysis.

Diagnostic testing performed and chlamydial species examined

While most papers (98) included some form of *Chlamydia* aetiological diagnostic testing (including a suite of tests from the earliest yolk sac isolation tests, to the current best practice methods of quantitative polymerase chain reaction (qPCR) for chlamydial antigens, and enzyme-linked immunosorbent assay (ELISA) for serum antibodies; Carey et al. 2010; Markey et al. 2007), 15 papers only contained diagnosis of chlamydial infection or disease on the basis of consistent clinical signs, pathological evidence (gross or histological), or radiographic/ultrasonographic evidence for signs of reproductive tract disease. These papers were included because appropriate aetiological diagnostic tests were not routinely available in the early years of chlamydial investigations. A diverse range of methodologies was reported for diagnosis, comparison or investigation (Table 3).

Examination of clinical signs of disease (either physically captured/handled animals, or distance observations in situ) was the most commonly reported diagnostic method.

Table 3. Diagnostic methods and other specific laboratory techniques, and the total number of times they were reported in the full set of papers (not mutually exclusive).

General category	Specific technique	Frequency	Total
Routine veterinary diagnostics	Clinical signs	36	83
	Gross pathology	23	
	Haematology	9	
	Ultrasonography	5	
	Radiography	4	
	Blood biochemistry	4	
	Ectoparasite examination	1	
	Urinalysis	1	
Culture or isolation methods	Cell culture	21	36
	Aerobic culture	7	
	Chick embryo yolk sac isolation	4	
	Anaerobic culture	3	
	Fungal culture	1	
Microscopy	Histopathology	25	47
	Immunohistochemistry	8	
	Cytology on smears or wet mounts	6	
	Electron microscopy	4	
	Direct immunofluorescence	4	
PCR and genetic methods	Polymerase chain reaction (PCR)	18	75
	Quantitative PCR (qPCR)	18	
	Gene, genome or DNA sequencing	18	
	Gene and DNA probe or cross-hybridization	11	
	Restriction enzyme analysis	4	
	High resolution melt curve analysis	2	
	Culture-independent DNA capture	1	
	Microarray	1	
	PCR amplicon sequencing	1	
	<i>Chlamydia</i> multi-locus sequence analysis	1	
Serology and specific tests	Complement fixation test (CFT)	22	
	Enzyme-linked immunosorbent assay (ELISA)	21	
	Lymphocyte stimulation assay	9	

	Western blotting	8	
	DNA or immuno dot or slot blot	7	
	<i>In vitro Chlamydia</i> neutralization	6	
	Pepscan methodology	2	
	CFT with whole blood sampling paper	1	
	Flow cytometry	1	77
	Other	7	7

Four papers did not contain any individual-level aetiological, clinical, pathological, radiographic or ultrasonographic diagnosis of *Chlamydia* (Hernandez-Sanchez et al. 2015; Martin and Handasyde 1990; Martin 1985b; McLean and Handasyde 2006). These papers mostly reported chlamydial infection or disease at the population level, including either unpublished data summaries, or unsubstantiated claims of particular populations being ‘*Chlamydia*-free’. Hernandez-Sanchez et al. (2015) reported a retrospective vaccine survival study with no chlamydial diagnostic testing performed (although the vaccine was produced based on sequenced rMOMP of *C. pecorum*).

We grouped papers by the major chlamydial species studied (Fig. 1iv). *Chlamydia pecorum* was the major focus of 35 papers, *C. pneumoniae* in 7 papers, *C. psittaci* in 39 papers, and ‘*non-specific, multiple or other chlamydial species*’ in 36 papers. In addition to non-culturable *Chlamydiaceae*, this latter category included all studies where chlamydiosis was diagnosed on the basis of clinical signs of disease or histopathology of infection, in the absence of any specific aetiological diagnostic test for chlamydial species. The *C. psittaci* category contained all early studies, prior to the separate identification of the two main infecting chlamydial species (*C. pecorum* and *C. pneumoniae*; Glassick et al. 1996). Due to earlier reporting, infections were assumed to be caused by a single *C. psittaci* pathogen species. Here we have included any reports of *Chlamydophila* species together with the respective *Chlamydia* species, as the change in taxonomic naming that occurred in the early 2000s was not widely accepted and has since been abandoned (Devereaux et al. 2003; Polkinghorne et al. 2013).

Study and data types

We examined papers for the type of study performed, including field, hospital/captive, experimental laboratory, and computer analysis components (not mutually exclusive) (Fig. 1v). The majority of studies involved laboratory experimental analyses *in vitro* (either performed on samples or isolates; 74), and approximately half of all studies utilized hospital or captive population koala samples (61). There was only one experimental infection study undertaken in a captive environment (Brown 1986). Ten studies involved the use of some form of developmental chlamydial vaccine, and hence additionally constituted experimental manipulation of animals, with or without natural infection in the wild. There were five studies involving treatment trials, however the majority of hospital/captive population studies involved necropsy case studies (28) or sample collection (37). Less than a third of studies involved a field component (33), and most of these reported sample collection (27) with minimal corresponding ecological data or collection methodology (such as mark-recapture, which was reported in 8 papers; or radio-tracking, reported in 5). There were five studies that involved some form of field koala translocation (Khan et al. 2016; Lee et al. 1990; McColl et al. 1984; Obendorf and Handasyde 1990; Waugh et al. 2016). A computer-based bioinformatics genetic analysis component was performed in 24 studies (phylogenetic pathogen-focused studies), while there were four studies based on retrospective computer records.

The majority of studies used mixed quantitative/qualitative results reporting (95 mixed, 22 qualitative only), although most of these only presented summary (descriptive) statistics and didn't perform hypothesis testing statistics or model evaluations. We found 39 studies (excluding phylogenetic analyses, sensitivity/specificity testing, and qPCR results reporting) that performed and reported on specific statistical testing procedures.

Major and minor study foci

While the majority of included studies (85) focused predominantly on koalas (Fig. 1vi) (compared with only 32 focused on specific koala chlamydial pathogens), most of these involved outcomes at the individual-host level (Figs. 1vii and 5): pathology (23 papers), diagnosis (21), immunity (17), pharmacology (5), other pathogen (3), and experimental infection (1). Only 16 studies (14% of all studies) focused on population-level disease dynamics or impacts. Of papers/chapters that focused on the pathogens, most investigated phylogeny (25) compared with only six that characterized *in vitro* responses.

Figure 6. Number of papers published in each of the minor study foci by decade (minor study foci categories are mutually exclusive – a paper is only allocated to one minor study focus).

We classified papers into the ‘Pathology’ category (23 papers) if they investigated the impact of infection and disease on the individual host using predominantly veterinary diagnostic pathology

techniques (reported as case studies or series). These were proportionally over-represented in the early years of koala chlamydial investigations and mildly under-represented in later years (9 papers in 1980s, 7 in 1990s, 1 in 2000s and 6 in 2010s) (Fig. 6). Most pathology studies originated from NSW (14), with fewer from the other states (5 from QLD, 4 from VIC, 2 from SA).

Other studies that aimed to develop and evaluate methods of diagnosis for koala chlamydial infection/disease were grouped into the ‘Diagnosis’ category (21 papers). The frequency of publication of these studies peaked in the 1990s (8 papers) around the time of reclassification of koala chlamydial species from *C. psittaci* firstly to *C. psittaci* type I and II, then to *C. pneumoniae* and *C. pecorum* respectively (Girjes et al. 1994; Girjes et al. 1988; Glassick et al. 1996), with fewer papers focused solely on diagnostic methods since the introduction of PCR techniques. Most of the koalas sampled for these studies were obtained opportunistically through wildlife hospitals, zoos and captive koala populations from QLD (15 papers), with only four studies from NSW and one from VIC.

Studies examining mechanisms of immunity or immune-based mitigation techniques (vaccination) fell within the ‘Immunity’ category (17 papers). Since 2010 these studies were mostly performed in the northern parts of the koala’s range (15 papers with koalas sourced from QLD, 2 papers with koalas from NSW).

We only identified 16 ‘Population-level’ studies that focused on population-level data to assess the extent and impact of chlamydiosis on free-ranging koala populations and other epidemiologic parameters. These were proportionally over-represented in the early years (4 studies published in 1980s, 8 papers in 1990s, one paper in 2006 and one paper published in 2015). The geographic spread of the studied populations was also limited, with most studies performed on Victorian

populations (8), followed by seven studies on QLD populations, and one from NSW. The geographic locations of these studied populations are identified in Fig. 1.

A small number of studies were identified that examined pharmacology related to treatments for koala chlamydial infections (5), or identified correlations between chlamydiosis and other pathogens (including trypanosomes, koala retrovirus or herpesvirus; McInnes et al. 2011; Stalder et al. 2015; Tarlinton et al. 2005). Brown and Grice (1986) presented the only data involving experimental infection in a controlled environment, and no papers presented empirical data on natural routes of infection transmission either *ex situ* or in the field.

From the studies focused predominantly on the chlamydial pathogens, 25 involved phylogenetic characterization, most commonly published in the last decade (11 papers from 2010s), and almost invariably including koala chlamydial samples or isolates from QLD (18 papers), although geographic sources were diverse in many. A further six studies focused on characterizing koala chlamydial pathogens *in vitro*, including morphology, growth, environmental factors, and survival responses to antifungal treatments.

DISCUSSION

The objective of this quantitative systematic review was to consider the breadth of research performed on koala chlamydiosis within the context of recent koala population declines. We thus mapped the development of research in the field with regards to journal category, authorship, funding, spatiotemporal scope, study foci and type, chlamydial species examined, methodological design and overall findings. Here we critically evaluate trends in the existing literature and identify directions for future research in order to assist with policy planning and to guide research funding.

Limited population-level disease data for koala chlamydiosis

In a research field motivated largely by sociopolitical concerns for both the conservation of an iconic species and the welfare of its individuals (McAlpine et al. 2015), it was striking to find comparatively few studies reporting empirical data that were appropriate for evaluating population-level impacts of chlamydiosis in the context of population declines. Of the 117 papers included in our review database, only 16 focused on epidemiological population-level data, aside from proportional infection measures from opportunistically sampled individuals. While several other papers reported a field component to their study, these were mostly based on samples collected from field-caught koalas without any indication of sampling methodology or other demographic parameters.

Population-level disease studies were spatiotemporally restricted

Of particular concern, only three population-level disease studies were identified as published in the last two decades (Jackson et al. 1999; McLean and Handasyde 2006; Patterson et al. 2015). However, prior to the mid-1990s, diagnostic techniques for differentiating chlamydial species and strains were not available (qPCR; Markey et al. 2007), and further, the two main species of

Chlamydia infecting koalas had not yet been recognized as separate entities (*Chlamydia pecorum* and *C. pneumoniae*; Girjes et al. 1994; Glassick et al. 1996). Additionally, over half of these studies were performed on Victorian populations (including four island sites) that are arguably of lower concern with regard to disease linked declines (Melzer et al. 2000a), with even fewer studies performed in NSW. Consequently, our appreciation of *in situ* infection dynamics for the different chlamydial species across the koala's distributional range is severely limited. Unfortunately, ecological studies that examine population demography independent of reporting infection and disease data are unable to provide the necessary detail.

Population-level disease studies were methodologically limited

Observational cross-sectional and comparative population-level studies were the most common, but unfortunately these methodologies are readily confounded as per the ecological fallacy when used to investigate disease (Clancy et al. 2003; Wobeser 2007). Only eight papers reported some form of mark-recapture/re-sight methodology, and three population-level studies reported radio-tracking individuals. The longest study was performed by Gordon et al. (1990) with annual surveys over 15 years. Other cohort studies ranged from as little as three weeks of observation data (Hirst et al. 1992), to approximately three years (Lee et al. 1990; Martin 1985a, b), with one other study comprising two point-surveys of marked animals five years apart (Mitchell et al. 1988). Most population-level studies reported measures of fecundity in association with disease (for example McColl et al. 1984; Patterson et al. 2015), however, there was little focus on other demographic parameters, such as estimates of population-level survival probabilities and population growth rates in the context of disease (for example Gordon et al. 1990; Martin and Handasyde 1990). Most studies were also deficient in description of koala sampling methods, lacking details of study site, spatio-temporal sampling regimen and protocols, summaries of individual animal data or sub-population structure, and justifications regarding potential bias and confounding (for an example of

a study that does provide these details, see White and Kunst 1990). Without further detail we were unable to confirm robustness to sampling bias and had to assume that their sampling was opportunistic.

Future directions for population-level disease studies on koala chlamydiosis

Appropriate population-level data are crucial for wildlife disease studies for two reasons: (i) they provide ecological validity by elucidating disease mechanisms in the stochastic context, complexity and correct scale of the real-life system, and (ii) they generate supporting ecological data (demography and population-structure) that are essential for identifying causes of heterogeneities of infection and disease at the population scale. The relative lack of population-level koala chlamydiosis studies identified in this review may be due to the following reasons. First, field studies are expensive to perform, particularly at a spatiotemporal scale appropriate for the species' autecology (relatively large distributional ranges and long generational intervals; Schneider 2001), and funding for wildlife disease studies is typically scarce (Grogan et al. 2014). Second, field-based disease studies are logistically complex requiring transdisciplinary expertise and resources in order to ensure representational and ethically appropriate disease sampling (Wobeser 2007).

Strengthening the diversity and currency of population-level disease studies on koala chlamydiosis should be a priority for future research. Greater emphasis should be placed on manipulative population-level studies and longitudinal studies, using methodologies such as mark-recapture and tracking in order to better elucidate infection mechanisms and parameters, as well as improving data on basic koala demographic rates in association with disease (particularly robust measures of infection-state associated survival, recruitment and population growth; Cormack 1964; Pradel 1996). Population-level manipulative experiments, in which pathogens or parasites are managed in some populations with other populations used as controls, are rarely undertaken, largely due to

expense, logistical challenges (for example, see Marcus et al. 2015), and the difficulty of identifying *Chlamydia*-free populations. They are, however, essential to determine whether compensatory effects to parasite-induced impacts on fecundity or survival exist. One of the few examples in the literature is the classic study by Hudson et al. (1998), which demonstrated that population cycles in red grouse are driven by nematodes by treating some populations with anthelmintics whilst keeping others as controls.

Multi-state mark-recapture studies incorporating sampling for qPCR and serology can provide probability estimates for transition to and from infection and disease states (Cooch et al. 2012; Lebreton and Pradel 2002). Such approaches have proved critical in understanding disease threats in wild populations (see, for example Faustino et al. 2004; Lachish et al. 2010; Murray et al. 2009). Mark-recapture data should also be collected in association with information on individual covariates (particularly infection and disease states, age and gender; Bonner et al. 2010). Disease-state occupancy surveys and other spatial techniques may also assist in better understanding the role of source-sink dynamics on the transmission of infection and manifestation of disease across populations in a range of environments and under a range of threatening processes (Adams et al. 2010; Cashins et al. 2015; Mackenzie et al. 2009). Given the generally poor long-term efficacy of chlamydial vaccines in multiple species (Entrican et al. 2012; Rey-Ladino et al. 2014), research into alternative field-based management options may also be beneficial in addition to the current koala vaccine studies (Khan et al. 2016; Waugh et al. 2016). Another area that warrants further investigation is the putative association of chlamydiosis with other diseases (such as trypanosomes, herpesvirus and Koala Retrovirus; McInnes et al. 2011; Stalder et al. 2015; Tarlinton et al. 2005). In particular, longitudinal studies may help tease apart the direction of causality and the nature of association.

Koala chlamydiosis research was otherwise diverse and reflects development of the field

Aside from the limited population-level disease studies, the published literature on koala chlamydiosis was diverse. Trends within the work appear to be a likely consequence of either the existing expertise within the field, or the changing needs in the development of the field with improving technologies and diagnostic capabilities. For example, there was an early focus on pathology and diagnosis, and a late focus on immunity and pathogen phylogeny. The number of papers published in the field has generally increased over the last four decades as expected with an emerging field, but there was an unexpected dip after the turn of the century (Fig. 3). This temporal shift may have been associated with a change in funding availability, commencement or progress of long-term studies, or a change in the perceived importance of koala chlamydiosis at that time. Spatially, the source of data was biased towards the northern parts of the koala's range, in accord with indicators of population decline. Several study locations, both private and government funded hospitals and sanctuaries, featured as major sources for koalas and samples. Pathology studies were highly represented by koalas sampled from NSW, and diagnostic and immunity studies were predominantly from QLD, possibly due to the range of hospital and captive sites providing access to koalas (Fig. 4), and the perceived need for mitigation measures in declining populations. While widely distributed geographic sampling was reported in a range of papers, these were predominantly pathogen phylogenetic studies that deliberately sought a geographically diverse range of samples, in a non-systematic fashion for comparative purposes.

A broadly transdisciplinary focus for the included papers and journals was identified, and this is common to the study of wildlife diseases due to the overlap of ecology, veterinary and conservation biology disciplines. Many authors were represented throughout the published literature, but some individuals had a disproportionate role in the field likely due to their involvement in driving particular avenues of research expertise in their respective teams. Funding was diverse, and

interestingly, private organisations and overseas funders were attributed more often than we
 expected. This might suggest that the iconic status of koalas attracts private funding, but possibly
 also contributes to bias in the development and direction of the research field.

The relative lack of hypothesis-testing manipulative experiments with live koalas (including
 treatment trials, translocation studies, vaccine reintroduction studies and infection experiments;
 Brown and Grice 1986; Govendir et al. 2011; Lee et al. 1990; Markey et al. 2007; Waugh et al.
 2016) was surprising, but a likely consequence of the ethical and logistical challenges of working
 with an iconic arboreal specialist species, and a reflection of top-down priorities for funding.

Otherwise, the diversity of study and data types was largely expected although we were surprised
 by the predominance of opportunistically sampled koalas (particularly the large number of studies
 utilizing koalas and koala samples from hospitals), and relatively small sample sizes (median of 27
 koalas per study). We also identified a general absence of substantiation for claims that populations
 are ‘*Chlamydia*-free’. Sampling strategies have been developed in other disease systems for proving
 absence of infection and disease, and these should be adapted where possible (Caporale et al. 2012;
 Skerratt et al. 2008).

Studies investigating diagnostic techniques made up a large proportion of published papers,
 particularly in 1980s-2000s (Fig. 6). This was likely due to the inherent difficulties in accurately
 diagnosing an intracellular bacterial pathogen, the gradual development of technologies, and two
 key events: (i) the identification of two main chlamydial species infecting koalas (*C. pecorum* and
C. pneumoniae), as opposed to the originally identified *C. psittaci* (Cockram and Jackson 1974;
 Glassick et al. 1996), and (ii) taxonomic renaming of the main genus of interest from *Chlamydia* to
Chlamydophila, and then back again (Bavoil et al. 2013; Devereaux et al. 2003; Polkinghorne et al.
 2013). After the differentiation of chlamydial species, the majority of studies (35) focused

exclusively on *C. pecorum*, likely due to its demonstrated higher pathogenicity (Jackson et al. 1999), compared with only seven exclusively focused on *C. pneumoniae* (usually in comparison with human isolates; Mitchell et al. 2010; Myers et al. 2009).

Conclusions and future directions

In this quantitative systematic review we critically analysed trends in the published peer-reviewed English-language literature pertaining to koala chlamydiosis from 1970 to 2016. In particular, we identified a striking lack of recent population-level disease studies, which makes evaluating the role of chlamydiosis in recent population declines difficult. Hypothesis-testing manipulative experiments for elucidating disease dynamics were also comparatively poorly represented. We recommend a greater emphasis in future studies on elucidating population-level disease mechanisms and parameter estimation, particularly through the use of manipulative and longitudinal studies at appropriate spatiotemporal scales (Schneider 2001) and sample sizes (Griffith et al. 2013) relative to the ecology of the koala.

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