

Chlamydia sp. in free-ranging populations of hawks (*Buteo sp.*) in Northern California

By

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B.M.V. (National San Marcos University) 2008

Med. Vet. (National San Marcos University) 2011

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

Avian Sciences

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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2015

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DEDICATION

A Dios
por guiarme hasta aquí y cumplir una de mis añoradas metas a pesar de los obstáculos

A mis padres: Carlos y Mercedes
por dejarme volar y por brindarme todo su amor y apoyo durante toda mi vida, gracias

A mi familia: los Luján y los Vega
quienes me han brindado su apoyo y cariño a pesar de la distancia

A la Sra. María Elena Vega
por ser la mejor amiga que pude encontrar en Davis

A Omar por guiarme hacia UC Davis

ACKNOWLEDGEMENTS

I would like to extend my sincere gratitude to the following persons and organizations for their continual support and contribution in making this study possible.

First I would like to thank **PRONABEC** from the Peruvian government because of their support through the **Beca Presidente de la República** which gave me the opportunity to earn the Masters of Avian Sciences in the United States. Thank you for believing in young Peruvians giving them opportunities that we did not have before.

Dr. Michelle Hawkins, thank you so much for accepting me as your first master student and advising me through this study, it would not have been possible without your valuable comments. Also thanks for guide me through these two academic years at University of California, Davis.

Thank you, **Dr. Kirk Klasing** and **Dr. Josh Hull** for your support and advice as my thesis committee. Also, I am so glad that I can say that you were my professors in a variety of courses. I really learned so much about avian physiology, avian ecology and raptors. Furthermore, thank you so much Dr. Hull for helping me in the sample collection.

I need to specially recognize the contribution of **Dr. Branson Ritchie** from the Infectious Disease Laboratory of University of Georgia for his valuable help with the laboratory testing of EBA, PCR and sequencing, and for guidance since the beginning of this study.

Many thanks to **Dr. Carolyn Cray** from the Avian and Wildlife Laboratory, University of Miami who promptly and clearly resolved my doubts in serology.

I would also thank **Dr. Christine Johnson** for the statistical design and data interpretation support.

Dr. Pete Bloom and **Chris Vennum**, thank you for your enthusiasm, collaboration and support with the sample collection. Thanks also for answering me so quickly my field doubts.

In addition, I would like thank **Dr. Chris Briggs** from the **Golden Gate Raptor Observatory** and all **volunteers and interns** for their valuable help with the sample collection. Also, I would like to thank **Laura Young** who made possible that I could have a field experience with free-ranging and migratory raptors. Thank you so much Laura.

I thank the **Wildlife Health Center, School of Veterinary Medicine University of California, Davis** for its financial support through a fellowship and the **Association of American and Zoo Veterinarians** who contributed to my study by the Wild Animal Health Fund. Thank you.

Finally, I would like to thank **Dr. Birgit Puschner** who introduced me to a new view of Pharmacology and Toxicology, and whose enthusiasm for teaching has had a lasting effect in my career. As well as to **Cara Harwood Theisen** who inspired me to do the best for my future students.

Chlamydia sp. in free-ranging populations of hawks (*Buteo* sp.) in Northern California

ABSTRACT

Chlamydiaceae bacteria include a large range of vertebrate hosts. Chlamydial organisms in raptors have been poorly studied. Previous reports have shown controversial prevalence in free-ranging raptorial populations. In addition, avian chlamydiosis by *Chlamydia psittaci* strain G has been reported in *Buteo* species in Northern California. The objective of this study was to estimate the prevalence of *Chlamydia* sp. in free-ranging populations of hawks (*Buteo* sp.) from Northern California. We also compared *Chlamydia* sp. status with species (red-tailed hawks and Swainson's hawks), age (juvenile and adult), sex (male and female), season (summer, fall, winter, spring), and bird geographic range (resident, medium-distance migrant, long-distance migrant). A total of 297 free-ranging *Buteo* sp. were sampled for swabs and whole blood. Conjunctiva, choana and cloaca were sampled with a single swab for qPCR analysis. Positive samples were sequenced based on the *ompA* gene and compared by high resolution melt analysis with all known *C. psittaci* strains (A-G, E/B, M56, WC), and *C. pneumoniae*. Aliquots from the plasma samples were sent to commercial laboratories in order to perform serology for IgM using the elementary body agglutination test (EBA; n=78) and for IgY using the indirect immunofluorescent assay (IFA; n=51). The overall prevalence of Chlamydiaceae in free-ranging *Buteo* species sampled in nine counties of Northern California was 1.37% (4/291) via qPCR analysis of multi-mucosal site swabs. An atypical chlamydial organism was detected by qPCR which was not identical to any *C. psittaci* known strain or *C. pneumoniae*. However, the most similar sequence was to the mammalian *C. psittaci* strain M56. None of the samples were positive for either serologic test. Due to the lack of antibody titers in any sample, it is possible that this chlamydial sequence is from a mammalian prey item passing through the gastrointestinal tract. Further studies will be required to identify this bacterium and its impact in the health of wild *Buteo* populations in Northern California.

Key words: *Chlamydia* sp., *Buteo* sp., qPCR, EBA, IFA, Northern California

CHAPTER I. LITERATURE REVIEW

Bacteria from the family Chlamydiaceae (order Chlamydiales, phylum Chlamydiae) are obligate, intracellular, gram-negative microorganisms that can cause disease in humans and a variety of vertebrates. Currently, this family has been subject to changes in taxonomy and discoveries of new avian Chlamydiaceae species (Sachse et al., 2015a; Sachse et al., 2014) which has led researchers to develop new insights into the epidemiology of avian chlamydiosis. This chapter provides a review of the most relevant and updated chlamydial literature. The first section is a review of the current chlamydial taxonomy. The second and third parts are focused on a description of recent reports in vertebrates (humans, mammals, reptiles, amphibians and birds) with non-avian and avian chlamydial infections. These parts describe the species in which chlamydial organisms have been identified, and whether there was evidence of disease associated with the organism. There is debate as to whether certain animal species harbor *Chlamydia* spp. as a commensal organism, meaning that these bacteria co-evolved with the host without causing pathologic changes under optimal conditions (Reinhold et al., 2011; Tlaskalová-Hogenová et al., 2004). But some authors suggest that these bacteria do cause harmful effects even though the animals have subclinical infections (Reinhold et al., 2011). The last section covers disease caused by avian chlamydial organisms divided in two parts: a brief description of psittacosis (human disease), and a detailed description of avian chlamydiosis (bird disease) which includes the strains, transmission, pathogenesis, clinical signs, diagnostic methods, and treatment.

1.1. Family Chlamydiaceae

Chlamydiaceae is a family that is currently represented by a single genus, *Chlamydia* (Horn, 2011; Sachse et al., 2015a; Stephens et al., 2009). In the past, the family Chlamydiaceae was composed of two genera *Chlamydophila* and *Chlamydia* (Everett et al., 1999); however, due to current molecular studies based on 16S rRNA genes, it was demonstrated that the family Chlamydiaceae has only a monotype genus (Sachse et al., 2015a). A total of 11 species are currently recognized in the genus *Chlamydia* including: *Chlamydia* (*C.*) *trachomatis*, *C. pneumoniae*, *C. pecorum*, *C. abortus*, *C. felis*, *C. caviae*, *C. muridarum*, *C. suis*, *C. psittaci*, *C. avium*, and *C. gallinacea* (Bachmann et al., 2014; Sachse et al., 2015a). Natural hosts include a wide range of vertebrates such as humans, birds, koalas, mice, reptiles, and amphibians (Bachmann et al., 2014). A brief description of *Chlamydia* infections in these vertebrates is addressed below divided by non-avian and avian chlamydial species. Reports in fishes refer to chlamydial-like organisms (Fryer and Lannan, 1994) and to date specific species have not been identified; therefore they are not reviewed.

1.2. Non-avian chlamydial infections

1.2.1. Humans

The two principal *Chlamydia* species that infect humans are *Chlamydia trachomatis* and *Chlamydia pneumoniae* (Bodetti et al., 2002). *C. trachomatis* causes the most frequently reported sexually transmitted disease in the United States which may produce sequelae especially in women such as infertility, ectopic pregnancy or pelvic inflammatory disease (Mishori et al., 2012; Workowski and Berman, 2010). In addition, it causes conjunctivitis that may lead to blindness in some patients (Mishori et al., 2012; Sulis et al., 2014). *C. pneumoniae* causes a

respiratory disease sometimes associated with cardiovascular disorders (Bodetti et al., 2002). The third chlamydial species with zoonotic importance to humans is *C. psittaci*; however, the infection in humans by this microorganism will be discussed in section 1.4.1.

Other non-avian chlamydial species that have lower zoonotic potential include: *Chlamydia abortus* which causes abortion in humans (Corsaro and Venditti, 2004; Longbottom and Coulter, 2003), and *Chlamydia felis* that causes conjunctivitis (Hartley et al., 2001b). Currently, the potential of *C. pecorum*, *C. suis*, *C. caviae*, and *C. muridarum* to be transmitted to humans and cause disease is unknown (Bachmann et al., 2014; Nunes and Gomes, 2014). However, there is a recent report that found *C. suis* in ocular swabs from humans without clinical signs (De Puyseleyn et al., 2014).

1.2.2. Non-human mammals

The range of hosts for chlamydial species increases constantly. Domestic and wild mammals are infected by a variety of *Chlamydia* and may or may not cause disease. Chlamydial organisms have been identified causing clinical identifiable disease in sheep, goats, cattle, pigs, mice, hamsters and cats. In domestic ruminants, *C. abortus* and *C. pecorum* cause negative economic impacts (Corsaro and Venditti, 2004; Poudel et al., 2012). Enzootic abortion caused by *C. abortus* tremendously affects ovine flocks in Europe; however, infections in goats, cattle, pigs, and horses occur less frequently (Longbottom and Coulter, 2003). *C. pecorum* causes keratoconjunctivitis and arthritis in sheep (Jelocnik et al., 2014), and reduced growth rates in cattle (Poudel et al., 2012) while *C. suis* was found in swine causing intestinal disease (Englund et al., 2012).

In laboratory rodents, *C. muridarum* causes respiratory symptoms in mice and hamsters (Ramsey et al., 2009) while *C. caviae* causes conjunctivitis in guinea pigs (Lacy et al., 2011). Both pathogens in their respective natural hosts have been widely used as models for respiratory and ocular infections of *C. trachomatis* studies for humans (Lacy et al., 2011; Ramsey et al., 2009). In companion animals, *C. felis* causes conjunctivitis in cats (Hillström et al., 2012), but while exposure by indirect hemagglutination has been found there is no evidence that *C. felis* causes disease in dogs (Wu et al., 2013). In addition, *C. pneumoniae* has been identified in the respiratory tract of a horse without report of clinical signs (Storey et al., 1993).

The koala (*Phascolarctos cinereus*) is a wild animal species that has been most severely affected by chlamydiosis because some Australian wild populations can have up to 100% prevalence with clinical cases having keratoconjunctivitis, urogenital disease and a rhinitis/pneumonia complex (Polkinghorne et al., 2013). Koalas usually show a mixed infection of *C. pecorum* and *C. pneumoniae* with *C. pecorum* commonly the dominant species involved (Polkinghorne et al., 2013). Severe keratoconjunctivitis was associated with chlamydial infection in other wild mammals such as wild boars (*Sus scrofa*) associated with *C. suis* (Risco et al., 2013), and bighorn sheep (*Ovis canadensis*) with an unidentified *Chlamydia* sp. (Meagher et al., 1992). Furthermore, purulent ocular discharge and conjunctivitis were found in western barred bandicoots (*Perameles bougainville*) caused by *C. pecorum* and other chlamydia-like organisms (Warren et al., 2005).

Asymptomatic wild mammals have also been identified positive to chlamydial organisms. For example, red deer (*Cervus elaphus*) had *C. suis* antibodies and *C. pecorum* was detected in feces by polymerase chain reaction (PCR) and multi-locus sequence typing (MLST) in this cervid species (Di Francesco et al., 2012; Jelocnik et al., 2015). Unidentified chlamydial DNA

sequences were also detected in conjunctival swabs of healthy opossums (*Didelphis virginianus*) and raccoons (*Procyon lotor*) (Pinard et al., 2002) while in alpine ibex (*Capra ibex*), *C. pecorum* DNA sequences were detected in conjunctival swabs (Jelocnik et al., 2015). In the Serengeti, the African buffalo (*Syncerus caffer*) and the spotted hyena (*Crocuta crocuta*) were positive in tissues to *C. abortus* by ArrayTube® microarray; however, it was unclear whether *C. abortus* caused the pathological findings in the tissues or it was related to other pathogens (Pospischil et al., 2012).

Chlamydia sp. has been identified in the majority of reports in mammals by conjunctival swabs with clinical signs related to eye disease (Englund et al., 2012; Hillström et al., 2012; Jelocnik et al., 2014; Poudel et al., 2012; Warren et al., 2005). These bacteria have also been identified without evidence of disease in multiple sample types such as feces, plasma, tissues and conjunctival swabs (Di Francesco et al., 2012; Jelocnik et al., 2015; Pinard et al., 2002; Pospischil et al., 2012). Although this microorganism seems to commonly cause ocular disease in mammals, it cannot be ruled out that it can affect other organs.

1.2.3. Reptiles and amphibians

Poikilothermic species are susceptible to *C. pneumoniae* infections. The first report that involved a poikilotherm with a *C. pneumoniae* infection was a giant barred frog (*Mixophyes iteratus*) in Australia (Berger et al., 1999). After that this organism was also identified in African clawed frogs (*Xenopus tropicalis*), blue mountain tree frogs (*Litoria citropa*), puff adders (*Bitis arietans*), green sea turtles (*Chelonia mydas*), flap-necked chameleons (*Chameleo dilepis*), green iguanas (*Iguana iguana*), Burmese pythons (*Python molurus bivittatus*), emerald tree boas (*Corallus caninus*) using PCR assays based different target genes such as ompA (outer

membrane protein A), 16S rDNA, and 23S rRNA (Bodetti et al., 2002; Hotzel et al., 2001; Jacobson et al., 2004; Reed et al., 2000). In all of these reports, reptiles and amphibians showed clinical signs and tissue samples such as liver, heart, spleen, lung and kidney were collected (Berger et al., 1999; Bodetti et al., 2002; Honeyman et al., 1992; Reed et al., 2000). In general, reptiles tend to have general symptoms such as lethargy or anorexia or more specific such as pneumonia, regurgitation and cardiovascular lesions (Bodetti et al., 2002; Homer et al., 1994; Jacobson et al., 2004) while in amphibians these bacteria can be opportunistic (Berger et al., 1999; Honeyman et al., 1992; Reed et al., 2000) or cause lethal systemic infections (Wilcke et al., 1983). In addition, chlamydial organisms have been identified in intestinal tissues of emerald tree boas (*Corallus caninus*) causing clinically a chronic regurgitation syndrome, and in Siamese crocodiles (*Crocodylus siamensis*) causing lethargy, anorexia, conjunctivitis, and diarrhea (Lock et al., 2003; Sariya et al., 2015). Another chlamydial microorganism closely related to *C. pecorum* has been isolated from tortoises (*Testudo* spp.) with respiratory clinical signs (Cope et al., 2015). However, *C. pneumoniae* is the chlamydial organism which has been extensively identified as a pathogen in reptiles and amphibians.

Other chlamydial species have been identified in reptiles using a *Chlamydia* genus-specific 16SrRNA assay using similarities of genome sequence such as *C. abortus* in puff adders, and Burmese pythons, and *C. felis* in green iguanas; however, they were concomitant with *C. pneumoniae*, thus there is not enough evidence to confirm that they really caused the clinical signs (Bodetti et al., 2002). In addition, *C. muridarum* was identified in a healthy Bismarck ringed python (*Bothrochilus boa*) by ArrayTube® microarray (Taylor-Brown et al., 2015).

1.2.4. Birds

Although there are three recognized *Chlamydia* species that infect mainly birds, other *Chlamydia* spp. can also infect birds. For example, in addition to *C. psittaci* feral pigeons can harbor *C. trachomatis*, *C. abortus* (Sachse et al., 2012), and *C. pecorum* (Sachse et al., 2012; Tanaka et al., 2005). In contrast, five species of asymptomatic wild-caught passerines in Argentina were positive to *C. pecorum* but none of them were positive to *C. psittaci* (Frutos et al., 2012). *C. abortus* was detected in a Brown skua (*Catharacta antarctica lonnbergi*) (Herrmann et al., 2000), common kestrels (*Falco tinnunculus*) and lesser kestrels (*Falco naumanni*) (Lemus et al., 2010) as well as *C. suis* and *C. muridarum* were identified for the first time in birds in the two kestrel species mentioned above (Lemus et al., 2010).

1.3. Avian chlamydial infections

1.3.1. Non-avian species

Chlamydia psittaci has been found also in non-avian species causing disease. Dogs have been identified with *C. psittaci* genotype C with keratoconjunctivitis and severe respiratory distress where the source of the infection seemed to be from pet birds (Sprague et al., 2009). Rodents are also affected. Septicemia was caused in laboratory rats by *C. psittaci* (Henning et al., 2008). In addition, wild rodents are suspected to be a potential source of *C. psittaci* for other animals because 12.9% seroprevalence was found in 8 wild rodent species (Cisláková et al., 2003). *C. psittaci* genotypes WC (from cattle) and M56 (from muskrats and snowshoe hares), have been isolated once each during mammalian epizootics, but are believed to have crossed from an avian host (Page, 1967; Spalatin et al., 1971). In the case of reptiles, crocodiles also have been identified as positive to *C. psittaci*; however clear information was not described if these positive

animals really presented with clinical signs (Robertson et al., 2010) while pneumonia was associated with *C. psittaci* in Moorish tortoises (*Testudo graeca*) (Vanrompay et al., 1994).

1.3.2. Birds

Birds are the principal natural hosts of *C. avium*, *C. gallinacea*, and *C. psittaci*, where the first two *Chlamydia* species were only very recently identified by phylogenetic analysis of multiple genes (16S rRNA, enolase A, ompA genes), as well as multi-locus sequence analysis (Sachse et al., 2014; Zocevic et al., 2012; Zocevic et al., 2013). In addition, there is another potential candidate species, *Chlamydia ibidis* (Sachse et al., 2015a). This proposed *Chlamydia* species was identified from an asymptomatic feral sacred ibis (*Threskiornis aethiopicus*) in France (Vorimore et al., 2013).

Chlamydia psittaci affects domestic, companion and wild birds. A total of 467 avian species belonging to 30 avian orders have been reported as *Chlamydia* spp. positive by multiple diagnostic tests such as bacterial isolation, antigen detection and/or serology (Kaleta and Taday, 2003). Psittaciformes is the avian order with the highest number of chlamydia-positive species (Kaleta and Taday, 2003); other orders in which *C. psittaci* positive species are commonly reported, and are important to mention due to their proximity to human beings are Columbiformes, Passeriformes, and Galliformes. For example, feral pigeons and passerines that frequently visit gardens and parks are free-ranging birds with constant opportunities to be near humans (Beckmann et al., 2014; Magnino et al., 2009) while domestic poultry in captivity are also able to be a source of infection of these bacteria to farm workers (Dickx and Vanrompay, 2011) and owners of backyard flocks.

Another group of birds that is frequently in contact with people and has not been deeply studied are raptors. Wildlife rehabilitation centers, zoos and falconers across the United States and Europe commonly keep these kinds of bird species. For example in Europe, a long-eared owl (*Asio otus*) showing clinical signs associated with *C. psittaci* genotype B was suspected to be the source of psittacosis in a worker of an avian refuge (Kalmar et al., 2014). Also avian chlamydiosis has been described in rehabilitation centers in the United States in *Buteo* sp. (Hawkins, 2010; Johns et al., 2009; Mirande et al., 1992; Zabka et al., 2004).

1.3.2.1. Psittacines

A total of 153 psittacine species have been identified as positive for *C. psittaci* (Kaleta and Taday, 2003). Birds kept in captivity as pets or in aviaries, birds that are sold in live bird markets, and free-ranging psittacines are susceptible to *C. psittaci* infection and disease.

A multistate study revealed that *C. psittaci* was widespread in apparently healthy Amazon parrots in aviaries of Brazil (Raso et al., 2002). The overall *C. psittaci* prevalence in this study was 35.8% (34/95) by direct immunofluorescence of cloacal smears and seroprevalence was 77.3% (34/44) by enzyme-linked immunosorbent assay (ELISA) (Raso et al., 2002). However, the *C. psittaci* prevalence in psittacines kept as pets was lower in Brazil (Santos et al., 2014). This prevalence was 3.4% (5/148) by using oropharyngeal and cloacal swabs for semi-nested PCR. Sensitivity and specificity of the tests may influence the variations of the prevalence in these studies. In Costa Rica, a study revealed 3.4% (4/117) prevalence of *C. psittaci* by conventional PCR in captive psittacine populations that included unmanaged populations, shelters and rescue centers (Sheleby-Elias et al., 2013). All positive birds were from veterinary clinics (Sheleby-Elias et al., 2013). In the United States, the prevalence of *C. psittaci* identified

via qPCR was approximately 0.3% in managed populations of psittacines whereas in unmanaged populations the prevalence was approximately 2.5% (Ritchie, 2015).

In pet markets the prevalence in Brazil was 17.2% (28/163) by using oropharyngeal and cloacal swabs for semi-nested PCR (Santos et al., 2014). Non-native Brazilian psittacines testing positive included lovebirds (*Agapornis* sp.), budgerigars (*Melopsittacus undulatus*) and cockatiels (*Nymphicus hollandicus*), and the native species blue-fronted Amazon parrots (*Amazona aestiva*) and conures (*Aratinga* sp.) (Santos et al., 2014). However, the prevalence in live bird markets in China was higher. *C. psittaci* seroprevalence was 35.4% (110/311) in psittacines by indirect haemagglutination assay, and suggested that juvenile birds were more susceptible than adult and subadult birds (Zhang et al., 2015). The differing prevalence in these two studies may be due to the different tests that were used for analysis, or due to the different environmental conditions in the two different countries.

Nestling free-ranging psittacines are also susceptible to *C. psittaci* (Raso et al., 2006). For example, two blue-fronted Amazon parrots with the ages of 32 and 45 days old were positive by semi-nest PCR giving a total 6.3% (2/32) prevalence while hyacinth macaws (*Anodorhynchus hyacinthinus*) between the ages of 33 to 88 days old were positive at 37.8% (17/45) prevalence by either semi-nested PCR and/or complement fixation (Raso et al., 2006).

Chlamydia avium has also recently implicated in infection and disease in psittacines. A parrot of the genus *Polytelis* was positive to this new species showing hepatosplenomegaly at necropsy (Sachse et al., 2014).

1.3.2.2. Columbids, passerines and domestic poultry

Host reservoirs including gregarious or migratory species can harbor a pathogen without any clinical signs, as colonial behavior facilitates microbial spread amongst conspecifics and a migratory ecology permits dispersal over a wide geographic region. Among gregarious birds, the Columbiformes are an excellent host reservoir for *C. psittaci*. More than fifty studies in feral pigeon populations from 1966 to 2006 showed seroprevalence ranging from 10% to 95.6% (Magnino et al., 2009). Feral pigeons (*Columbia livia*) are a special concern for public health (Andersen and Franson, 2007) because they have highly dense populations that inhabit larger cities (Magnino et al., 2009). A study in Italy revealed that other columbid species also has a high prevalence of *C. psittaci* (Donati et al., 2015). A 43.4% (33/76) prevalence was found via cloacal samples by real time PCR specific to *C. psittaci* in the Eurasian collared dove (*Streptopelia decaocto*) (Donati et al., 2015). Other columbid species positive for *C. psittaci* include wood pigeons (*Columba palumbus*) (Pennycott et al., 2009; Sharples and Baines, 2009) and white-winged doves (Grimes et al., 1997). The recently discovered *C. avium* detected by the development of a real-time PCR based on the target gene enolase A (enoA) was also found in urban and captive pigeons (*Columba livia*) showing clinical signs (Sachse et al., 2014; Zocevic et al., 2013). Also, in some cases this bacterium was present in combination to *C. psittaci*, showing that pigeons can have more than one chlamydial species infection at a time (Sachse et al., 2014).

Avian chlamydiosis in wild birds is often not detected until large die-offs occur as a result of flock behavior (Andersen and Franson, 2007) such as drinking a unique source of water. Birds infected with *C. psittaci* can contaminate the water by nasal discharges, feces or conjunctival exudates and produce a source of infection for the rest of the flock (Georgopoulou and Tsiouris,

2008). Reports of massive die-offs of birds due to chlamydiosis include gulls, doves, geese and ducks (Andersen and Franson, 2007).

Passerines have been underestimated as a possible source of *C. psittaci* infection (Beckmann et al., 2014). Isolated reports in Europe demonstrated the presence of *C. psittaci* in passerines (Colvile et al., 2012; Pennycott et al., 2009); however, some of these reports assumed that avian chlamydiosis was a concomitant disease (Pennycott et al., 2009). A recent study showed that *C. psittaci* genotype A is circulating between passerines that frequent gardens (dunnocks, *Prunella modularis*; great tits, *Parus major*; robins, *Erithacus rubecula*; and blue tits, *Cyanistes caeruleus*) in Great Britain (Beckmann et al., 2014).

Avian chlamydiosis in poultry is scarcely reported (Arzey and Arzey, 1990); however, the organism has been widely identified in carcasses and mucosal swab samples creating concerns in human health (Gaede et al., 2008; Laroucau et al., 2009b; Yin et al., 2013). There is great interest in the newly discovered *Chlamydia gallinacea* and the avian species that are affected. A previous report of avian chlamydiosis and ornithosis from domestic poultry and people, respectively, were misunderstood until recently because a report showed the presence of Chlamydiaceae as responsible for infection in birds and people (Laroucau et al., 2009b); however, the agent involved was *C. gallinacea* (Sachse et al., 2014) which was identified by an specific real-time PCR targeting the 16S rRNA gene (Zocevic et al., 2012). Chickens (*Gallus gallus domesticus*), turkeys (*Meleagris gallopavo*) and guinea fowl (*Numididae*) were positive to *C. gallinacea* (Hulin et al., 2015; Sachse et al., 2015b); however no clinical signs or lesions has been described. In contrast, domestic ducks (*Anatidae*) seem to be more susceptible to *C. psittaci* than *C. gallinacea* (Hulin et al., 2015). Furthermore, ducks were shown to excrete the bacteria into soil on farms becoming a source of infection of *C. psittaci* for people (Laroucau et

al., 2009a). An atypical avian chlamydiosis occurred in turkeys where these birds have swollen heads due to nasal gland infection with *C. psittaci* (Shivaprasad et al., 2015). Backyard poultry can be also affected by *C. psittaci* causing a respiratory disease (Prukner-Radovčić et al., 2014); however, it seems that this bacterium is not considered to be a cause of major mortality in backyard flocks in Northern California. Based on one study of 1301 submissions, this bacterium was not identified (Mete et al., 2013). More studies are needed to determine if backyard poultry can asymptotically carry this zoonotic agent.

1.3.2.3. Raptors

Raptors or birds of prey include all the bird species that hunt other animals for feeding. These birds can be diurnal or nocturnal and include the hawks, eagles, falcons, kites and owls. Few studies have investigated the role of raptors as natural reservoirs of *C. psittaci* (Blomqvist et al., 2012; Fowler et al., 1990; Gerbermann and Korbel, 1993; Mirande et al., 1992; Ortega et al., 2012; Schettler et al., 2003; Zabka et al., 2004). Early reports from Germany identified 13.2% positive samples from 152 free-living raptors by examination of cloacal swabs using an antigen-based ELISA assay, and *C. psittaci* antibodies were demonstrated in 85.1% of 121 birds (Gerbermann and Korbel, 1993). Later serological studies from this region found 74% of sampled raptors examined were positive, reflecting a ubiquitous occurrence of *C. psittaci* exposure in raptors in this country (Schettler et al., 2001). A study evaluating cloacal swabs by PCR from wild raptors identified a 1.3% prevalence in 319 vulnerable top-raptorial predators in Sweden (Blomqvist et al., 2012) whereas in a study published the same year from Spain detected *Chlamydia* spp. DNA in 85.7% of adult raptors belonging to 12 species (Ortega et al., 2012). Most of the reports were of asymptomatic raptors; however a recent report showed clinical disease due to chlamydial organisms in raptors. *C. psittaci* genotype B was identified in a long-

eared owl (*Asio otus*) which showed clinical signs associated with avian chlamydiosis (Kalmar et al., 2014) and gammopathies were found to be associated with *C. abortus* infection in common (Falco tinnunculus) and lesser (Falco naumanni) kestrels (Lemus et al., 2010). In addition, ill gyrfalcons (*Falco rusticolus*) and peregrine falcons (*Falco peregrinus*) in captive settings have been positive to chlamydial organisms in tissues; however, concurrent diseases were also identified (Wernery et al., 1998).

Information about *C. psittaci* in North American raptors is scarce, and to our knowledge there have been no published studies evaluating *C. psittaci* infection in any free-ranging North American raptor populations. There are few reports of *C. psittaci* infection in captive raptors in the U.S.; *C. psittaci* was isolated from four red-tailed hawks (*Buteo jamaicensis*) in Northern California (CA) that died suddenly in 1983 (Fowler et al., 1990). One hundred captive raptors representing 14 species were subsequently serologically tested from Northern CA, and 44% were antibody positive using a human *C. pneumoniae* ELISA antibody test. The ELISA was repeated on 54 raptor samples in 1986 and 41% were considered positive, suggesting *C. psittaci* was endemic or constantly present, in this population at that time (Fowler et al., 1990). Avian chlamydiosis has been detected in an outbreak and an isolated case of juvenile red-tailed hawks in Davis, California (Johns et al., 2009; Zabka et al., 2004). Diagnose was performed by real-time PCR from whole blood, choana/cloacal swabs and tissues (Johns et al., 2009; Zabka et al., 2004). The most recent report is an abstract from a retrospective study from the UC Davis-Veterinary Medicine Teaching Hospital (VMTH) medical records (2000-2009) of clinical cases of avian chlamydiosis in raptors. A total of 20 of the 33 described cases were confirmed by histopathologic examination and immunofluorescent antibody staining for *Chlamydia*-species antigen of spleen, liver, air sacs, and lungs (Hawkins, 2010). The species involved include red-

tailed hawks (18/30), a Swainson's hawk (*Buteo swainsoni*) (1/2) and a ferruginous hawk (*Buteo regalis*) (1/1). Clinical signs included lethargy, weight loss, moderate-to-severe leukocytosis and radiographic evidence of splenomegaly (Hawkins, 2010).

1.4. Disease caused by avian chlamydial organisms

1.4.1. Psittacosis: Disease in humans

The terms psittacosis and parrot fever are used to describe the disease caused by *C. psittaci* in humans transmitted by psittacine species, and the term ornithosis was the disease caused by non-psittacine birds (Andersen and Franson, 2007; Smith et al., 2011). Transmission from birds to humans occurs by inhalation or ingestion of contaminated ocular secretions, nasal discharges, dried fecal dust or fresh feces or urine (Beeckman and Vanrompay, 2009; Corsaro and Venditti, 2004; Harkinezhad et al., 2009). A typical risk factor is the close contact of pet owners with their birds, specifically mouth-to-beak contact (Corsaro and Venditti, 2004) or of veterinarians infected from shedding birds (Heddema et al., 2006a). In urban areas, pigeons seem to be a great source of infection (Corsaro and Venditti, 2004) due to their high density populations around high human populated areas. Human-to-human transmission of *C. psittaci* has also been reported by family members and workers in a hospital; however in all cases the suspected bird source was not confirmed (Ito et al., 2002; Raso et al., 2014; Wallensten et al., 2014). The incubation period in humans is from one to two weeks, or longer (Smith et al., 2011). This disease causes mild to moderate flu-like symptoms such as fever, headache, cough, muscle and joint pain and photophobia in humans but can progress to severe pneumonia (Corsaro and Venditti, 2004). If diagnosis is performed in time, patients can recover completely (Corsaro and Venditti, 2004).

1.4.2. Avian chlamydiosis: Disease in birds

Traditionally, *C. psittaci* has been studied as the unique agent causing avian chlamydiosis in a variety of bird species. However, recent studies suggest that *C. psittaci* is not a unique bacterium species involved in this disease (Sachse and Laroucau, 2014) because of the recent discovery of *Chlamydia avium* and *Chlamydia gallinacea* (Sachse et al., 2014). *C. psittaci* strains, transmission, pathogenesis, clinical signs and diagnostic methods are reviewed based on the existing *Chlamydia psittaci* literature.

1.4.2.1. *Chlamydia psittaci* strains

Avian strains are clustered by serotypes using monoclonal antibodies or genotypes based on the outer membrane protein A (*ompA*) gene. Both terms for strains are shown to be equivalent (Vanrompay et al., 1997). Currently, ten genotypes are recognized and differ in virulence and host susceptibility (Read et al., 2013; Van Lent et al., 2012; Wolff et al., 2015). Avian *C. psittaci* genotypes are eight (A–G; E/B), and the remaining two genotypes are mammalian (M56, WC) (Wolff et al., 2015). Genotype A is a highly virulent strain commonly identified among psittacines while genotype B is endemic and less virulent, and it is usually identified in pigeons (Sachse et al., 2015b). All can be transmitted to humans; however, genotype A is most commonly associated with psittacosis (Heddema et al., 2006a; Vanrompay et al., 2007; Verminnen et al., 2008). *C. psittaci* G strain has caused systemic disease in red-tailed hawks (Read et al., 2013; Zabka et al., 2004) and this strain has been identified closely related to the mammalian *C. psittaci* M56 strain (Zabka et al., 2004). In a comparison study of all *C. psittaci* strains, both strains (G and M56) have shown a particular divergence compared to the other *C. psittaci* strains (Read et al., 2013). In addition, *C. psittaci* M56 strain has been previously

identified to belong to a separate branch from other *C. psittaci* strains (Pannekoek et al., 2010). Furthermore, there are six additional proposed genotypes: 1V, 6N, Mat116, R54, YP84 and CPX0308 found in psittacines and wild birds (Sachse et al., 2008), and two provisional genotypes I and J (Madani and Peighambari, 2013). Genotype J seems to be an intermediate from *C. psittaci* and *C. abortus* (Madani and Peighambari, 2013).

1.4.2.2. Transmission among birds

Among birds there a horizontal transmission occurs within a species or among different species by ingestion or inhalation of ocular or nasal droplets, dried fecal dust, fresh feces or urine (Beeckman and Vanrompay, 2009; Corsaro and Venditti, 2004; Harkinezhad et al., 2009). It is known that chlamydial organisms can survive in dried feces for weeks to months (Corsaro and Venditti, 2004). In addition, horizontal trans-shell transmission has been identified experimentally in poultry (Ahmed et al., 2015).

1.4.2.3. Pathogenesis

This pathogen has a biphasic life cycle which includes the extracellular form, the elementary body (EB), and the intracellular form, the reticulate body (RB) (Corsaro and Venditti, 2004; Wyrick and Richmond, 1989). Infection occurs from bacterial uptake across epithelial mucosal surfaces, especially respiratory, urinary, ocular and gastrointestinal systems and shedding occurs commonly in secretions from these systems (Harkinezhad et al., 2009). The development of a systemic infection depends on the host susceptibility (Sachse et al., 2015b). *In vitro* studies have showed that *C. psittaci* can invade many cell-types including epithelial cells, fibroblasts, macrophages, and dendritic cells which gives this bacterium the ability to infect a

variety of tissues while it is transported in the bloodstream easily causing a septicemia (Sachse et al., 2015b).

The incubation period for *C. psittaci* in birds is approximately 3-21 days (Harkinezhad et al., 2009). The *C. psittaci* life cycle consists of five phases: (1) EB attachment and penetration; (2) transformation of EB to RB; (3) growth and binary fission of RB; (4) maturation of the RB that has been produced to the infectious EB; and (5) EB releasing from the host cell to the extracellular space (Wyrick and Richmond, 1989).

The attachment of the EB to the host cell is via unidentified specialized membrane microdomains on the cell surface (Dautry-Varsat et al., 2004). Then, the bacterium is engulfed by endocytosis or phagocytic vesicles and taken up into the cell. Morphologically, EBs and RBs are composed by cell envelopes containing an inner cytoplasmic membrane and an outer membrane such as those of gram-negative bacteria (Hatch et al., 1984). A major structural component of both EBs and RBs is the chlamydial major outer membrane protein (MOMP) (Bodetti et al., 2002). The MOMP seem to play an active role as adhesion and the ability to form pores (Vanrompay et al., 1995). Reticulate bodies are metabolically active, leading the energy mobilization and the synthesis of proteins, such as cysteine-rich proteins that will be part of the future outer membrane protein of EBs (Hatch et al., 1984). EBs are the infectious particles which are resistant to physical and chemical factors such as osmotic stress in the extracellular matrix due to a cell envelope rigidity (Hatch et al., 1984; Longbottom and Coulter, 2003). After the RB reproduction, they mature to EBs and are released from the host cell via lysis and are then able to infect other cells or to be passed into the environment (Sachse et al., 2015b).

1.4.2.4. Clinical signs

Chlamydia psittaci infection in birds can be asymptomatic, acute or chronic depending of the species, age, life conditions, strain, infective dose, and stress factors (Corsaro and Venditti, 2004; Smith et al., 2011). Many bird species shed the bacterium without clinically apparent disease (Harkinezhad et al., 2009). Persistent, clinically inapparent infections contribute to difficulties encountered in the control of these infections, especially due to intermittent shedding of the organism (Harkinezhad et al., 2009; Vanrompay et al., 1995).

Generalized non-specific clinical signs such as lethargy, anorexia, and weight loss are common with *C. psittaci* infections, but signs can be associated with virtually any organ system (Smith et al., 2011). Mucopurulent or serous nasal or ocular discharge, conjunctivitis, yellow to green urates are also present in clinical cases (Smith et al., 2011). Wild birds are generally reported to show minimal or no clinical signs of *C. psittaci* (Andersen and Franson, 2007).

Smith et. al (2011) defined a confirmed, probable and suspected case of avian chlamydial infection. A confirmed case involves: (1) isolation of *C. psittaci* in a bird with clinical signs; (2) presence of chlamydial antigen in bird's tissues by immunofluorescence; (3) a change in the serologic titer of chlamydia antibodies (fourfold) in a bird in two weeks at the same laboratory and; (4) smears of tissues or secretions with EBs in macrophages. A probable case involves: (1) a high serological titer with clinical signs suspicious for the disease and; (2) Chlamydiaceae antigen detection by enzyme-linked immunosorbent assay, PCR or fluorescent antibody in feces or mucosal swabs in a bird with compatible clinical signs. Finally, a suspect case is: (1) compatible clinical presentation but without laboratory confirmation linked with a confirmed case in a human or other bird; (2) a bird with no clinical signs but a single high serological titer

or detection of *C. psittaci* antigen; (3) compatible disease with positive new or nonstandardized test; and (4) compatible disease that responds to *C. psittaci* antimicrobial therapy (Smith et al., 2011).

1.4.2.5. Diagnostic methods

Diagnosis of *C. psittaci* is challenging, especially in the absence of clinical signs, and single testing methods are most often inadequate. Combinations of antibody-detection and antigen-detection testing modalities are commonly incorporated in multimodal diagnosis. Culture is the most precise way of diagnosing *C. psittaci*, but it must be isolated in tissue culture or embryonated chicken eggs and specialized laboratory facilities and handling are necessary for reliable identification and adequate worker protection (Smith et al., 2011). A brief description of the main ante mortem and post mortem tests are addressed below.

a) Ante mortem testing

The two most common antemortem techniques utilized today are a combined conjunctival, choanal and cloacal swab for quantitative PCR (qPCR) to detect antigen (Andersen, 2004; Madani and Peighambari, 2013; Ortega et al., 2012), and a liver biopsy for qPCR or chlamydial immunohistochemical (IHC) antigen staining (Smith et al., 2011). However, intermittent shedding of the organism may lead to false negative qPCR samples for the swabs. Quantitative PCR can be genus or species specific depending of the target gene that is used. Chlamydial IHC antigen staining is not as sensitive as qPCR because cross reactions may occur with other bacteria and fungi (Andersen and Franson, 2007).

Cytological staining including Giemsa, Gimenez, and Macchiavello stains can be used to detect EBs of chlamydial organisms in exudate, feces, and impressions of liver and spleen inexpensively and rapidly; however, these stains are less sensitive to IHC and require experience with chlamydiae for an accurate interpretation (Andersen and Franson, 2007; Arizmendi and Grimes, 1995). In addition, blood smears are sometimes useful to identify chlamydial organisms (Johns et al., 2009). A routine blood smear evaluation from an injured red-tailed hawk detected small, dark blue inclusions within heterophils compatible with *Chlamydia* or *Ehrlichia*; however, in this case *Chlamydia* sp. was confirmed by IHC in tissues after the bird's death. (Johns et al., 2009).

Genetic sequencing confirms chlamydial species and strains in positive samples. Sequencing of the *C. psittaci* ompA (MOMP) gene allows for differentiation of the 10 known genotypes, including the 2 non-avian genotypes (Geens et al., 2005; Heddema et al., 2006b; Read et al., 2013; Sachse et al., 2008; Van Lent et al., 2012; Wolff et al., 2015).

Single serological samples can identify exposure to the organism, but cannot determine active infections (Smith et al., 2011). The chlamydial immune response varies between species; however, it shows production of antichlamydial IgM up to two weeks post-infection, and after that time antichlamydial IgY antibody begins to be produced (Phalen, 2001). Elementary body agglutination (EBA) serological testing identifies *C. psittaci* IgM, but titers may or may not persist past the acute phase (Smith et al., 2011). Direct complement fixation (DCF) to detect IgY is considered more sensitive than agglutination methods (Smith et al., 2011), and these antibodies can persist for months to years (Phalen, 2001). However, DCF is not currently available in the United States. Another test that detects *Chlamydia* IgY to the genus level is the

Indirect Fluorescent Antibody Test (IFA); the sensitivity and specificity of this test depends on the polyclonal antibody and the bird species. False-negative results are possible from birds with acute infection when samples are taken before seroconversion (Smith et al., 2011). Further description of this test will be discussed in section 2.2.7.

b) Post mortem testing

Lesions of avian chlamydiosis can be severe and widely distributed in the tissues depending on the virulence of the strain, host susceptibility, exposure route and, concurrent bacterial infections (Andersen and Franson, 2007). Post-mortem gross lesions most commonly include hepatosplenomegaly and air sacculitis; however there are no pathognomonic pathologic lesions for this disease (Graham, 1989; Smith et al., 2011). Microscopic lesions in the spleen include multifocal necrosis and proliferation of macrophages, reticuloendothelial cells and lymphoid depletion in the white pulp (Graham, 1989; Hashimoto et al., 1985). This last lesion may lead to an inadequate immune response of the infected bird becoming susceptible to secondary infections (Hashimoto et al., 1985). The other main affected organ is the liver which can exhibit multifocal coagulative necrosis and dilation of the hepatic sinusoids (Graham, 1989; Hashimoto et al., 1985). Air sacs in acute clinical presentations often have fibrinous exudate with vacuolated macrophages and heterophils while in chronic disease there is granulomatous inflammation with histiocytosis and neovascularization (Graham, 1989). Chlamydial infection is confirmed by using IHC specific to chlamydial organisms in the damaged tissue (Smith et al., 2011). IHC has been previously described in section a) Ante mortem testing.

1.4.2.6. Treatment

Tetracyclines are the most effective drugs against chlamydial organisms; however, they are effective only against the RBs when the bacteria is replicating (Flammer, 1989). The current drug of choice to treat avian chlamydiosis is doxycycline because of its absorption and slow elimination compared to other tetracyclines (Smith et al., 2011). Generally, a 45-day regimen treatment is required because chlamydial organisms can persist within the infected cells without activity for this time period, after this period the cell turnover occurs; therefore, the organisms are able to die within the infected cells (Flammer, 1989). Doxycycline can be administered in feed, water, orally and by intramuscular injection (Smith et al., 2011). Toxicity monitoring should be performed due to the long period of treatment (Smith et al., 2011).

CHAPTER 2: *CHLAMYDIA SP.* IN FREE-RANGING POPULATIONS OF HAWKS (*BUTEO SP.*) IN CALIFORNIA

2.1. Introduction

The genus *Buteo* in North America is represented by twelve species, eight of which are found in California (California Bird Records Committee, 2015; Crossley et al., 2013). Based on habitat and size, these species are divided as either woodland or prairie-savanna buteos. Woodland buteos are smaller, resembling *Accipiter* species. These *Buteo* species include red-shouldered hawks (*B. lineatus*; RSHA) and broad-winged hawks (*B. platypterus*). In contrast, prairie-savanna species are large but not all live in the prairies. Red-tailed hawks (*B. jamaicensis*; RTHA), Swainson's hawks (*B. swainsoni*; SWHA), ferruginous hawks (*B. regalis*; FEHA), rough-legged hawks (*B. lagopus*; RLHA) and zone-tailed Hawk (*B. albonotatus*) are included in this category (Peeters and Peeters, 2005). The eighth species is the gray hawk (*Buteo plagiatus*) which was recently observed in California (California Bird Records Committee, 2015) and may be categorized as a woodland *Buteo* species because of its size.

Red-tailed hawks are considered the most common and widespread prairie-savanna hawk in North America (Cornell Lab of Ornithology, 2015a; Crossley et al., 2013). This species has an estimated breeding population of 2.3 million individuals worldwide and a conservation status of Least Concern by the International Union for Conservation of Nature (IUCN) (Cornell Lab of Ornithology, 2015a). Furthermore, this species is found throughout California especially during fall and winter months (September to February) (Peeters and Peeters, 2005). RTHA populations can be either migratory or may remain resident in a nesting territory. Western Canadian populations usually migrate to California during winter (Snyder and Snyder, 2006). Resident populations remain in their nesting territory the whole year (Snyder and Snyder, 2006). The

RTHA is considered a medium-distance migrant species. Migratory populations of RTHAs have been studied at various migratory stations in the U.S. such as the Golden Gate Raptor Observatory (GGRO) in the Marin Headlands which is considered a migratory pathway bottleneck (Binford, 1979; Hull et al., 2009). The GGRO station has reported two peak periods of migration dates of juvenile RTHAs. The first peak occurs between 15 August to 30 September and RTHAs from this peak tend to show regional dispersion through central California. The second peak is between 1 October to 30 November and includes regional dispersal as well as migratory movements with a southern direction (Hull et al., 2009). The RTHAs have a large size population with broad distribution, as well as migratory and resident populations making them an ideal bird species model to evaluate disease spread through other raptorial species that share the same habitats.

The Swainson's hawk (*Buteo swainsoni*) is a long-distance migrant species. It migrates regularly from North America to Mexico and South America and back thorough the year (Clark and Wheeler, 2001; Snyder and Snyder, 2006). These birds begin to arrive in North America in March as their breeding season is during April and May. When the breeding season is complete, contrary to other raptors, this species usually has very social behavior such as foraging, communal overnight roosting and migrating (Snyder and Snyder, 2006). This species is considered an early migrant because they begin to migrate south at the beginning of October (Binford, 1979). Although Swainson's hawks have a stable population worldwide with a breeding population at 580,000 individuals and a global conservation status of Least Concern by the IUCN, their numbers have been drastically declining particularly in Argentina and California where this population has its non-breeding and breeding range, respectively (Cornell Lab of Ornithology, 2015b). In Argentina, the main concern is the decline due to organophosphate

toxicities. In the 1990s, these compounds were used in agricultural lands to control grasshoppers causing thousands of SWHAs deaths (Cornell Lab of Ornithology, 2015b; Snyder and Snyder, 2006) while in California this species is considered a state-threatened species (California Department of Fish and Wildlife, 2015) due to loss of prey and the decreased number of nesting sites associated with loss of riparian and wetland habitat (Bloom, 1980; Cornell Lab of Ornithology, 2015b). However, little research has been done to determine whether diseases are also affecting this species in this range.

Avian chlamydiosis is a zoonotic disease known to most commonly be caused by *Chlamydia psittaci* in birds. However, recent studies suggest that there are other *Chlamydia* species involved that may cause this disease in birds such as *Chlamydia avium* and *Chlamydia gallinacea* the latter of which has been suspected to cause disease in humans but currently there is no evidence to support that it causes disease in birds (Laroucau et al., 2009b; Sachse et al., 2014). Few studies have investigated the role of raptors as natural reservoirs of *C. psittaci* (Blomqvist et al., 2012; Fowler et al., 1990; Gerbermann and Korbel, 1993; Mirande et al., 1992; Ortega et al., 2012; Schettler et al., 2003; Schettler et al., 2001), and fewer reports describe this disease in ill raptors in rehabilitation centers (Hawkins, 2010; Johns et al., 2009; Kalmar et al., 2014; Zabka et al., 2004). In California, avian chlamydiosis has been recognized in *Buteo* species (red-tailed hawks, Swainson's hawks, and ferruginous hawks) presented to wildlife rehabilitation centers and identified by qPCR in combination swabs, whole blood cytology and/or tissue immunohistochemistry (IHC) (Hawkins, 2010; Johns et al., 2009; Zabka et al., 2004). Clinical signs include lethargy, weight loss, moderate-to-severe leukocytosis and radiographic evidence of splenomegaly (Hawkins, 2010). However, to our knowledge, there are no studies evaluating the prevalence of this organism in free-ranging populations of raptors in any area of the United

States. Therefore, the objective of this study was to estimate the prevalence of *Chlamydia* sp. in free-ranging populations of hawks (*Buteo* sp.) from Northern California. We hypothesized that we would identify < 8.4% prevalence of this bacterium in free-ranging populations of *Buteo* species. This 8.4% prevalence of chlamydial organisms was found by positive IHC of primarily hepatic and splenic tissues of sick hawks (*Buteo* sp.) from the total *Buteo* sp. cases presented to the Veterinary Medical Teaching Hospital, School of Veterinary Medicine at the University of California, Davis between 2000-2013 (Hawkins, 2015). In addition, we compared *Chlamydia* sp. status with species (RTHAs and SWHAs), age (fledgling, juvenile and adult), sex (male and female), season (summer, fall, winter, spring), bird geographic range (resident, medium-distance migrant, long-distance migrant) of hawks (*Buteo* sp.) populations sampled in Northern California and we hypothesized that there would not be any significant difference between species, age or sex and the *Chlamydia* sp. status.

2.2. Methodology

2.2.1. Location

Birds were opportunistically sampled throughout nine counties in California (Colusa, Marin, Orange, Plumas, San Diego, Sierra, Siskiyou, Sutter and Yolo) during routine banding programs from July 2013 to February 2015. The main sources of samples were from RTHAs from the Golden Raptor Observatory in the Marin Headlands, Sausalito California (37°50'N, 122°30'W) which were sampled during the fall and winter seasons, and from SWHAs from a breeding colony in Macdoel, Siskiyou California (41°50'N, 122°W) sampled during summer season.

2.2.2. Animal population of the study

Buteo species were the primary focus of the study as to date avian chlamydiosis has been most commonly identified in these species (Fowler et al., 1990; Hawkins, 2010; Johns et al., 2009; Mirande et al., 1992; Zabka et al., 2004). Red-tailed hawk and SWHA populations are common species that are captured for annual leg banding programs; therefore, a great number of samples came from these two species; however, when available other *Buteo* species were included.

Sample size was calculated according to previous methodology in the following formula $n = p(1-p)z^2/d^2$ (Zhang et al., 2015). Based on the true 8.4% prevalence of *Chlamydia* positive *Buteo* sp. case submissions to the Veterinary Medical Teaching Hospital, University of California, Davis between 2000 to 2013 (Hawkins, 2015), we calculated the sample size for an expected prevalence of 1%, 2.5%, 5% and 7.5% with deviations of the true prevalence of 7.4%, 5.9%, 3.4% and 0.9%, respectively, and a 95% confidence ($z=1.96$). The obtained sample sizes were 7, 27, 158 and 3290 for the expected prevalence of 1%, 2.5%, 5% and 7.5%, respectively. Therefore, we chose to use the expected prevalence of 5% and a minimum sample size of 158 for this study.

2.2.3. Bird trapping, sample collection and transportation

Birds were trapped during routine annual banding and monitoring programs. RTHAs and adult SWHAs were trapped by using previously described methods including dho-ghaza traps, bal-chatris and bow nets (Bloom et al., 2007). In order to have access to SWHA chicks, the tree where the nest site had been identified was climbed, and the chick was brought to the ground and banding and sampling was then performed. All state and federal permits for trapping/banding

were obtained for all of the sampling sites. Collection of samples for this study was approved by the University of California, Davis Institutional Animal Care and Use Committee (IACUC#17291).

No bird was maintained in hand for more than 30 minutes, and all birds were released back to the site where they were captured immediately after sampling. Species, sex, age, season, bird geographic range, weight, and tarsus depth were recorded when possible. Two types of samples were collected: a multi-mucosal site swab and whole blood. The conjunctiva, choana, and the cloaca were swabbed with a single sterile rayon tipped swab with semi-flexible polystyrene handle (25-806 1PR, Puritan®, Guilford, Maine, USA), placed in a sterile cryovial (Figure 1). After collection, swabs were stored on ice packs during transportation and conserved at room temperature or at -20°C until laboratory submission. One to 2 mL whole blood was collected from the medial metatarsal vein and placed into a lithium heparin tube which was placed on ice packs until arrival to the field lab. Whole blood was centrifuged ≤ 8 hours after collection in the field sites and plasma was separated. Swabs and plasma were shipped from field sites to the Hawkins laboratory at University of California, Davis on either icepacks for the swabs or dry ice for plasma samples. In the Hawkins laboratory, plasma was thawed and divided into 2 aliquots of a minimum of quantity of 75 μ l, placed into separate sterile cryovials and stored at -80°C until serological evaluation. Then, all samples were shipped from the UC Davis laboratory to the Infectious Diseases Laboratory, University of Georgia, Athens, Georgia USA for the qPCR and *C. psittaci* EBA (IgM) antibody evaluation, and to the Avian and Wildlife Laboratory, University of Miami, Miami, Florida for *Chlamydia* spp. IgY antibody testing.



Figure 1. Multi-mucosal swab of a red-tailed hawk, collected on a single swab in this order: (a) conjunctival swab, (b) choanal swab, and (c) cloacal swab

2.2.4. DNA extraction

QiaAmp DNA minikit (catalog no. 51304; Qiagen, Inc) was used to extract DNA from the swabs according to the manufacturer's instructions. A total of 200 μ l of Qiagen elution buffer was used to dilute the extracted DNA (Mitchell et al., 2009).

2.2.5. Real Time quantitative PCR (qPCR)

Amplification reactions were performed based on the *ompA* gene specific for microorganisms of the genus *Chlamydia* in a LightCycler® 480 System. A three marker panel was used (Ppac, GTpc, and GT-F). The first one was the Ppac marker, composed by 109 bp (5'-gaacccTATTGTTTGCCGCTACGGGT“t”C-3' forward, labeled; 5'TCCTGAAGCACCTTCCCACA-3' reverse, unlabeled), the GTpc marker was the second one with 274 bp (5'-gaactcaTGTGCAACTTTAGGAGCTGAG“t”TC-3' forward, labeled; 5'-GCTCTTGACCAGTTTACGCCAATA-3' reverse, unlabeled), and the third one was the GT-F marker with 98bp (5'-gacgcCATTCGTGAACCACTCAGCG“t”C-3' forward, labeled; 5'-CTCCTACAGGAAGCGCAGCA-3' reverse, unlabeled) (Mitchell et al., 2009). The reaction mixture contained: 12.5 μ l of 2X master mix (100 nM for each forward and reverse primers), 0.15 μ l of platinum *Taq* polymerase, 5 ng of a template, and nuclease-free water to get a final

volume of 25 µl. The conditions of cycling were: 1 cycle at 95°C for 2 minutes, then 45 cycles at 95°C for 5 s and 62°C for 15 s, with data acquired at the 62°C step in the green channel (Mitchell et al., 2009). *Chlamydia* spp. positive samples were tested in triplicate.

2.2.6. Sequencing and HRM analysis

Chlamydia positive samples were amplified within the target region of the *ompA* gene based on a previously published method (Mitchell et al., 2009). The sequence product was compared to all known *C. psittaci* genotypes (A, B, C, D, E, F, G, E/B, M56, and WC), and *C. pneumoniae* (Mitchell et al., 2009) by using high-resolution melt (HRM) analysis using light upon-extension chemistry. This test was performed in a temperature range of 75°C to 85°C increasing constantly by 0.05°C. Before separation fluorescence normalization regions were between 75.5°C to 76°C and after separation were between 84°C to 84.5°C (Mitchell et al., 2009).

2.2.7. Serology

Each plasma sample was separated into two aliquots and sent to two different commercial laboratories in the U.S. for serological testing. One batch of plasma aliquots was submitted to the Infectious Diseases Laboratory, University of Georgia, Athens, Georgia USA for evaluation of *C. psittaci*-specific IgM antibody titers by the elementary body agglutination test (EBA) while the other set of plasma aliquots was sent to the Avian and Wildlife Laboratory, University of Miami, Miami, Florida to perform the indirect immunofluorescent assay (IFA) to evaluate the IgY antibody titers to the genus *Chlamydia*. The difference between these two serological assays is that the EBA relies on the detection of IgM antibodies to *C. psittaci* infective particles (elementary bodies) that are produced in the early stage of disease (acute immune response)

whereas for the IFA test, the reagent is based on detecting IgY antibodies to the reticulate bodies at the genus-level, which appear after the intracellular stage of the infection (Cray and Bonda, 2005).

Elementary body agglutination test has almost 100% specificity to *C. psittaci* antibodies, specifically more for *C. psittaci* strain A and less to strain E (Ritchie, 2015). A prepared suspension of *C. psittaci* elementary bodies from the isolation of chicken embryos infected with an original turkey isolate was mixed directly with the plasma of question on a slide by using a mechanical rotator for 3 minutes at 150 rpm. Results were considered positive if there was agglutination, and negative in the absence of agglutination (Grimes et al., 1994). Compared to other serological tests such as the direct complement fixation (DCF) or latex agglutination (LA) assays, the EBA generally produces low titers (Grimes, 1993). The interpretation of the results is a titer over 1:20 indicates an active *C. psittaci* infection and a titer of 1:10 is considered to be suspicious for infection (Grimes et al., 1996).

Indirect immunofluorescent assay was performed by using fixed slides stored at -70°C with the inclusion stage of infection of *C. trachomatis* which previously have been grown in McCoy cells on IFA slides under 5% CO₂ and 37°C in an Eagle media with supplements (Bossart et al., 2011). Slides were thawed out at room temperature and diluted plasma samples were added. Dilution of the samples was made with phosphate-buffered saline (PBS). Then slides were incubated at 37°C for 30 minutes and a PBS wash was performed (Bossart et al., 2011). FITC anti-chicken IgY antibody was overlayed as secondary antibody for an additional incubation at 37°C for 30 minutes more; there was a subsequent wash and counterstain with Evan's Blue stain. A fluorescence microscope was used to evaluate specific reactivity comparing samples with positive and negative controls (Bossart et al., 2011). The cut off titer of the test is

1:5 based on a significant number of cases predominated by psittacines with confirmed or suspect infection (Cray, 2015; Cray and Bonda, 2005). Seropositive samples are considered to reflect subclinical infection, previous exposure or infection (Cray and Bonda, 2005).

2.2.8. Statistical analysis

The results were first evaluated in an EXCEL spreadsheet database and then analyzed with the statistics software STATA 13®. The estimated prevalence was calculated with its binomial confidence interval. The Fisher's exact test was used to analyze the association of the variables species, sex, age, and bird geographic range with positive/negative birds. The categories of the variables are defined as (1) species: identification of *Buteo* species was performed by experienced field researchers; (2) sex: defined as *male*, *female* or *unknown* in juvenile and adult birds was defined by experienced biologists. The differentiation was based on the size compared to mate nest, weight, and tarsus depth of the bird (Pitzer et al., 2008; Vennum, 2015). Weight was done by using a gram scale while tarsus depth was measured in millimeters at the narrowest point of the thickness of the tarsometatarsus (Golden Gate Raptor Observatory, 2012), (3) age: defined as *fledgling* when the bird was found in the nest; *juvenile*, a bird with first year coloration plumage, and *adult* bird with a complete mature plumage, (4) season: defined as the date of sample collection: *summer* (June 1 - August 31), *fall* (September 1 - November 30), *winter* (December 1 - February 28/ 29 in a Leap Year), *spring* (March 1 to May 31), and (5) geographic range: based on the species migratory pathway and known territorial activity of the bird that was previously banded. The subcategories include: *medium-distance migrant birds* are RTHAs and other *Buteo* birds that usually fly throughout western North American with migratory pathways in Northern and Southern California; *long-distance migrant birds* are SWHAs which have a geographic range in North America and South America and RLHA which

migrate to the Arctic tundra to breed; and *resident birds* are defined as birds that are not known to migrate such as RSHA, and RTHA known to range within a given territory because they were banded in that territory before.

2.3. Results

A total of 297 free-ranging *Buteo* birds were sampled obtaining 291 multi-mucosal swabs and 78 plasma samples. The collection was performed from July 2013 through February 2015 from nine counties in California: Colusa (n=1), Marin (n=85), Plumas (n=2), Sierra (n=1), Siskiyou (n=195), Sutter (n=3), Yolo (n=1), Orange (n=1), and San Diego (n=8). The number of each species is listed in Table 1. The majority of samples were collected in Northern California and only nine birds were sampled from Southern California; however, these nine birds may come from the Northern migratory pathway where Northern California may be part of their route. Therefore, we considered all samples from Northern California. Red-tailed hawks were sampled during the fall and winter seasons, and SWHAs from a breeding colony during summer season. None of the birds were captured in the spring.

Four positive samples were identified from the 291 qPCR samples, two of them were from red-tailed hawks of Marin County and two from Swainson's hawks of Siskiyou County. The overall percentage prevalence (95% confidence interval) of Chlamydiaceae in free-ranging *Buteo* species sampled in these counties of California was 1.37% (0.38- 3.48) by using qPCR analysis of multi-mucosal site swabs; the prevalence was 2.11 (0.26-7.40) in red-tailed hawks and 1.08 (0.13-3.85) in Swainson's hawks (Table 1).

Table 1. Prevalence of Chlamydiaceae in free-ranging hawks (*Buteo* sp.) in California by species

Species	n	qPCR (+)	Prevalence (%) (95% CI)
Red-tailed hawk <i>Buteo jamaicensis</i>	95	2	2.11 (0.26-7.40)
Swainson's hawk <i>Buteo swainsoni</i>	185	2	1.08 (0.13-3.85)
Ferruginous hawk <i>Buteo regalis</i>	5	0	-
Red-shouldered hawk <i>Buteo lineatus</i>	3	0	-
Rough-legged hawk <i>Buteo lagopus</i>	3	0	-
TOTAL <i>Buteo</i> species	291	4	1.37 (0.38-3.48)

n: Number of birds, **qPCR:** Real time PCR, **CI:** 95% Confidence Interval

Although we performed statistical testing and there was no statistical difference identified between the positive/negative qPCR animals and species, age, sex, and geographic range (Table 2), the power of the analysis was not high enough to allow for conclusions to be drawn from our data. For example, Fisher's exact test was done for the variable species including RTHAs and SWHAs which had the highest numbers of individuals and the positive/negative qPCR animals; however, the power was only 3.82%. The variable sex includes the categories female, male and unknown. Females and males were only recorded in juvenile and adult birds which had adequate morphometric characteristics and body weight or birds that had a nest mate and size comparison

was able to be made. None of the birds were sampled during spring season. Birds with unknown geographic range were also excluded from the analysis (Table 2).

Table 2. Analysis of the association of *Chlamydia* sp. prevalence and species, age, sex, season, and geographic range in free-ranging hawks (*Buteo* sp.) in Northern California

Variable	Categories	n	qPCR (+)	Prevalence (%) (95% CI)	p value*
Species	Red-tailed Hawk	95	2	2.11 (0.26-7.40)	0.61
	Swainson's Hawk	185	2	1.08 (0.13-3.85)	
Age	Fledgling	183	2	1.09 (0.13-3.89)	0.72
	Juvenile	82	2	2.44 (0.30-8.53)	
	Adult	26	0	-	
Sex	Female	32	1	3.13 (0.08-16.22)	0.54
	Male	65	1	1.54 (0.04-8.28)	
	Unknown	194	2	1.03 (0.13-3.67)	
Season	Summer	187	2	1.07 (0.13-3.81)	0.34
	Fall	81	1	1.23 (0.03-6.69)	
	Winter	23	1	4.35 (0.11-21-95)	
	Spring	0	0	-	
Geographic range	Resident	11	0	-	0.65
	Medium-distance migrant	87	2	2.3 (0.28-8.06)	
	Long-distance migrant	188	2	1.06 (0.13-3.79)	

n: Number of birds, **qPCR:** Real time PCR, **CI:** 95% Confidence Interval

*p-value based on Fisher's Exact test

There were three atypical positive samples and one atypical weak positive sample based on the signal produced by the melt curve and amplitude. The *C. psittaci* assay used runs 46 cycles where the positive control starts to amplify at cycle 18. The three atypical positives started to amplify between cycle 20 and 30 while the atypical weak positive started to amplify at cycle 39; therefore, this last sample had low amplitude even though it had similar melt curve in relation to the other samples. Genotyping via sequencing of the ompA gene was performed on

the cDNA from the three positive qPCR samples but genotyping was not performed on the weak positive sample due to insufficient target cDNA for analysis. High-resolution melt analysis with light upon-extension was used to compare if any *C. psittaci* known genotype sequence or *C. pneumoniae* sequence was encountered. Chlamydial DNA sequence was identified as related but not identical to the target sequences of *C. psittaci* controls; therefore it was called atypical. The sequence did not align completely with any of the known *C. psittaci* genotypes (A-F, E/B, M56, and WC) including the G strain identified previously in RTHAs. The most likely related strain was M56 but due to the small number of base pairs compared the positive samples could not be directly determined to be *C. psittaci* M56. Furthermore, none of the three samples had sequences related to *C. pneumoniae*. Therefore, the DNA detected on the initial qPCR test was considered an atypical Chlamydiaceae organism.

A total of 78 plasma samples were collected; however, not all the samples could be submitted for both antibody tests to both commercial laboratories because the collected plasma volumes were not the amount required for the laboratories to analyze. Seventy-eight plasma samples were evaluated for EBA testing and only 51 samples for IFA. All samples were negative, regardless of antibody testing methodology. Unfortunately, only one positive qPCR sample had a corresponding plasma sample. The EBA and IFA serology tests results obtained from this sample were also negative (Table 3).

Table 3. Description of *Chlamydia* positive birds by species, band number, age group, approximate age, sex, season and test results (qPCR, EBA, and IFA)

Species, Band #	Age group, Approx age	Sex	Season	Tests		
				qPCR	EBA	IFA
RTHA, 1907-04280	Juvenile, <1y	Male	Winter	ATYPICAL POSITIVE	N/A	N/A
RTHA, 1957-05288	Juvenile, <1y	Female	Fall	ATYPICAL POSITIVE	N/A	N/A
SWHA, 1687-15796	Fledgling, 36d	Unknown	Summer	ATYPICAL WEAK POSITIVE	NEGATIVE	NEGATIVE
SWHA, 1947-20400	Fledgling, 36d	Unknown	Summer	ATYPICAL POSITIVE	N/A	N/A

qPCR: Real time PCR, **EBA:** Elementary Body Agglutination, **IFA:** Indirect Immunofluorescent Assay, **RTHA:** Red-tailed hawk, **SWHA:** Swainson's hawk, **y:** year, **d:** days, **N/A:** No Available

2.4. Discussion

Buteo species within sampled counties in Northern California show an overall prevalence of 1.37% (4/291) of *Chlamydia* sp. organisms by using qPCR analysis of multi-mucosal site swabs. Our results were similar to those obtained in Sweden where there was an overall prevalence of 1.3% (4/319) of *C. psittaci* in peregrine falcons (*Falco peregrinus*) and white-tailed sea eagles (*Haliaeetus albicilla*) determined by qPCR and sequencing of the ompA gene of cloacal swabs (Blomqvist et al., 2012) but differs greatly from the prevalence in Spain where Chlamydiaceae organisms were found in 85.7% (n=64) of samples from 12 species of raptors from choanal and conjunctival swabs by conventional PCR (Ortega et al., 2012). However, the number of birds sampled in Spain was less than 100 while the number of samples of the Swedish and our study were approximately 300 birds.

This study indicates that the identified atypical chlamydial organisms from the four samples in this study do not align completely to any known *C. psittaci* strain (A-G, E/B, M56, WC) or *C. pneumoniae* when the sequence was compared via the ompA gene; however, this sequence was similar but not identical to the known *C. psittaci* M56 strain. Therefore, the chlamydial organism may be (1) another previously reported *Chlamydia* species not identified with these testing modalities, (2) a novel *Chlamydia* species not previously reported, or (3) a novel *C. psittaci* strain.

If the chlamydial DNA sequence identified in this study is one of the previously reported *Chlamydia* species, it could be any of the other nine recognized *Chlamydia* species that were not evaluated using these testing modalities. *Chlamydia abortus*, *C. muridarum* or *C. suis* have been previously identified in other raptor species such as common (*Falco tinnunculus*) and lesser (*Falco naumanni*) kestrels (Lemus et al., 2010). *C. muridarum* could occur due to rodent consumption and thus would be identified only while passing through the digestive tract of the raptor. This is plausible because even though we only had serology for one of the positive birds, there were no antibody titers in any of the birds from this study suggesting an immune response to infection with any chlamydial organism. The sampled SWHA breeding population has a varied diet composed mainly by rodents (Belding's ground squirrels, pocket gophers, voles), reptiles (snakes and lizards), small birds (magpies, flickers, kestrels, starlings), insects (grasshoppers) and other mammals (Vennum, 2015). Therefore, wild rodents may be a source of *C. muridarum*; however, to our knowledge there are no reports confirming that this *Chlamydia* species is present in these particular rodent species. In contrast, reptiles which are commonly affected by *C. pneumoniae* (Bodetti et al., 2002; Hotzel et al., 2001; Jacobson et al., 2004; Reed et al., 2000) may not be a source of chlamydia for the *Buteo* sp. in this study due to the three

DNA chlamydial sequences found were not comparable with *C. pneumoniae*. No definitive correlations between the found small atypical chlamydial DNA sequence and *C. pneumoniae* could be done because of the small amount of material to sequence. Although there is not yet any report of *C. avium* or *C. gallinacea* in raptors, these bacteria cannot be excluded as a source of *Chlamydia* from small birds to the hawks via ingestion. Whether the chlamydial DNA sequences found in the four positive hawks (*Buteo* sp.) came from a prey item also could not be determined because we used multi-site mucosal swabs, including swabbing the gastrointestinal tract via the cloaca with swab from the choana.

The identification of chlamydial-like DNA sequences in these hawks may also suggest a new *Chlamydia* species. One of the chlamydial sequences identified from a white-tailed sea eagle in Sweden was closely related to a new *Chlamydia* species from poultry in France (Blomqvist et al., 2012; Laroucau et al., 2009b). It only differed by 12 base pair positions to this new species while when compared to *C. psittaci* it had only 71% homology (Blomqvist et al., 2012). This new species was later identified as *C. gallinacea* (Laroucau et al., 2009b; Sachse et al., 2014). Recent reports demonstrate that there is a diversity of non-classified Chlamydiaceae in asymptomatic free-ranging seabirds such as northern gannets (*Morus bassanus*), European herring gulls (*Larus argentatus*), common murrelets (*Uria aalge*), chinstrap penguins (*Pygoscelis antarcticus*), brown skuas (*Catharacta antarctica*), southern giant petrels (*Macronectes giganteus*), and snowy sheathbills (*Chionis albus*) (Aaziz et al., 2015; Isaksson et al., 2015); therefore, it is also possible that new chlamydial species are present in terrestrial birds such as raptors. It would be important to isolate and sequence the new chlamydial species in these birds in order to understand the ecology of the avian chlamydial organisms in free-ranging raptors.

It is also possible that an unknown *C. psittaci* strain was in the raptors of our study. SWHAs and RTHAs have been previously identified having clinical disease associated with chlamydial organisms in California by IHC, PCR of tissues and swabs, and blood smear (Hawkins, 2010; Johns et al., 2009; Zabka et al., 2004). In an outbreak of avian chlamydiosis in RTHAs in Northern California the sequence of the organism was closely related to the mammalian strain M56, and it was proposed to be named the *C. psittaci* G strain (Zabka et al., 2004). However, the birds reported in this study were considered to be clinically healthy as they were sampled at banding stations. Therefore, it is possible that infected birds showing evidence of clinical disease may have a more virulent *Chlamydia* species or *C. psittaci* strain than the asymptomatic hawks of our study, or it may be the same strain that causes disease in weakened birds. Studies on the complete chlamydial DNA sequence of this organism are needed. A recent study has shown that there is a number of genetic variations among certain genes involved in host preferences and virulence between *C. psittaci* genotypes; therefore, host-switching may occur (Wolff et al., 2015). If a mammalian *C. psittaci* strain was infecting and also causing clinical disease in birds, it may show that *C. psittaci* is able to rearrange its genes in order to switch hosts, or it may be that the strain was originally from birds.

From the 78 samples submitted to evaluate chlamydial IgM titers by EBA, and the 51 samples to evaluate chlamydial IgY titers by IFA not a single sample was even weakly positive. Previous studies described a high seroprevalence of *Chlamydia* sp. in wild raptors such as 63% in Germany, and 41 to 44% in Northern California in the 1980's by using ELISA testing methods (Fowler et al., 1990; Schettler et al., 2001). These findings may differ due to (1) antibody test, (2) time of infection, (3) species immune response, and/or (4) handling artifacts.

Elementary body agglutination test is a highly sensitive and specific test for detecting *C. psittaci* antibodies and is not known to cross react with other chlamydial species (Ritchie, 2015). If our qPCR products are a non *C. psittaci* organism, it is unlikely that the concomitant antibody plasma sample would cross-react with that of the EBA antibody test for *C. psittaci*. In the case of IFA, which is a test based on *C. trachomatis* antigen, it may not recognize the *Chlamydia* species because it targets the omp2 gene shared between the chlamydial species (Bas et al., 2001; Bossart et al., 2011). Although it is known that the omp2 gene is in all Chlamydiaceae, this gene is not present in some *C. pecorum* strains (Hartley et al., 2001a). In addition, to our knowledge, there is no published literature that mentions the existence of the omp2 gene in *C. gallinacea* and *C. avium*. Therefore, it is possible that chlamydial DNA identified in this study either does not have the omp2 gene, or the birds were not actually infected with the organism, thus an antibody response was not mounted.

Some ELISA tests have high sensitivity and low specificity (Phalen, 2001). These tests used to detect chlamydial lipopolysaccharides (LPS) and glycolipids common to all *Chlamydia* species (Ruppanner et al., 1984). The high prevalence identified from published studies from Northern California in the 1980s and more recently from Germany may be due to the lack of specificity of the ELISA tests that were used. In the case of the early Northern California study the ELISA test was based on antigen from an ovine *C. psittaci* (Ruppanner et al., 1984) while the German study used a commercial ELISA (R-Biopharm GmbH, 64293 Darmstadt, Germany) developed to detect *C. psittaci* antibodies independently of the bird species (Schettler et al., 2001). Unfortunately, this test is not available commercially. Ketodeoxyoctanoic acid (KDO) is a carbohydrate constituent of LPS in most Gram negative bacteria which is also found in chlamydial glycolipid antigens (Nurminen et al., 1983). Thus, cross-reactions with these bacteria

may have artificially increased the number of positive samples in these previous studies by ELISA, and thus the serology results of these previous studies may not be comparable to the results of our study.

The time of infection may play a role in the overall lack of antibody titers seen in this study as well, because generally pathogens have a prepatent period in the host which cannot be detected with common diagnostic methods (Phalen, 2001). It is possible that the qPCR positive birds may have been exposed to Chlamydiaceae bacteria and sampled for this study before they could develop an immune response that could be identified by serologic tests. It is common to find the antigen by PCR within the first days of infection, whereas the humoral immune response may require up to two weeks (Phalen, 2001). Therefore, if the single qPCR positive bird with plasma for serological testing in this study was sampled within this period, EBA and IFA results would be negative. Moreover, EBA and IFA results could have been negative because of chlamydial organisms just passing through the gastrointestinal tract because of the consumption of a prey with chlamydial organisms.

Immune response to chlamydial organisms can also differ between birds, avian species and time to antibody production. Generally, the chlamydial immune response begins approximately two weeks post-infection producing antichlamydial IgM, and after that time antichlamydial IgY antibody begins to be produced (Phalen, 2001) but this time can vary by species. For example, cockatiels seem to be poor producers of immunoglobulins, especially IgM; therefore EBA test is not a valuable diagnostic tool in this species (Grimes et al., 1994). In addition, IFA in cockatiels has been shown to produce a lot of false negatives; therefore this test is also not useful in this species (Hofle, 1999; Phalen et al., 1999; Phalen, 2001). EBA testing has been useful in a wide range of species including crowned plovers (*Vanellus coronatus*), macaws

(*Ara* sp.), Amazon parrots (*Amazona* sp.) and rheas (*Rhea* sp.) (Grimes et al., 1994); however is suggested to be not to be useful for some small psittacines (budgerigars and lovebirds) (Phalen, 2001) and columbid species because EBA is less sensitive in these species (Phalen et al., 1999). Indirect immunofluorescent assay was also found to be useful in a variety of birds including toucans (*Ramphastidae*), blue-fronted parrots (*Amazona aestiva*) and macaws (*Ara* sp.). To our knowledge, in the case of raptor species there is not previous published literature identifying the usefulness or disadvantages of either of these two serology tests. However, IFA has been considered to obtain accurate results from raptors (Cray, 2015).

Serologic test results may also have been altered by sampling technique and transportation conditions to the laboratory (Phalen, 2001). In this study, due to field conditions sometimes an inadequate blood volume was collected because there were not experienced people to collect blood samples from the birds. For future studies, there is a necessity to ensure appropriate training for the collection of samples for all the field collaborators and also have a person of the project involved in the collection of the samples in order to maximize the number of samples collected appropriately. Whole blood samples were commonly centrifuged at the end of the day, or unfortunately frozen without separating the plasma, allowing for hemolysis of some samples. Grossly hemolyzed samples have been observed to produce a high background on IFA methods and the possibility of false positive reactions (Cray, 2015). For future studies, training of personnel responsible for processing the sample will be helpful to reduce this potential artifact. Although we used dry ice to transport the samples within and outside CA, samples were exposed to multiple temperature changes such as transportation between field sites to UC Davis laboratory, thawed to separate plasma into two aliquots at the UC Davis laboratory and, during out-of-state overnight transportation to the other laboratories. Therefore, results may

be artifactual due to undesirable temperature variation conditions giving false negatives, as was reported in patients tested serologically for syphilis that became negative due to temperature changes of the sample (El-Zaatari and Martens, 1994).

We did not find any statistical difference between the presence of chlamydial organisms and species, age, sex, season or bird geographic range. However, the small sample size and small overall number of positive samples make it difficult to compare these variables. Further studies with larger sample numbers are needed to elucidate if there is any relationship between these variables and chlamydial-like organism in free-ranging birds.

Swainson's hawks are a state-threatened species (California Department of Fish and Wildlife, 2015). Even though environmental threats (loss of prey and the decreased number of nesting sites) have been recognized (Cornell Lab of Ornithology, 2015b), the association of infectious diseases and reduced populations have been poorly studied to date. The identification of chlamydial organisms particularly in this species may have an impact in the health of the free-ranging populations if it is an avian chlamydial species which may mean another potential threat for this species. In the case of RTHAs which have a widespread and well-established population, the main impact could be that avian chlamydial organisms will not only affect individuals, but these bacteria could be spread geographically to other avian species because of the medium-distance migratory characteristic of this species and may affect sympatric avian populations. RTHAs and SWHAs are sympatric species, and interspecific and intraspecific territoriality has been reported (Rothfels and Lein, 1983); therefore, cross infections may occur.

It is common to recognize that juvenile RTHAs are susceptible to avian chlamydiosis (Johns et al., 2009; Mirande et al., 1992; Zabka et al., 2004) or that nestling raptors are positive to Chlamydiaceae organisms (Blomqvist et al., 2012; Lemus et al., 2010). In our study, juvenile migratory RTHAs were positive to *Chlamydia* sp.; however it is important to mention that we only could test 17/26 adult RTHAs while there were 78/82 RTHA juveniles. The great number of sampled juvenile RTHAs was because they are commonly found in the migratory path of the Golden Gate Raptor Observatory (Hull et al., 2009), where the majority of migratory RTHA samples were obtained. The positive juvenile birds were asymptomatic which in contrast to previous clinical reports finding juvenile RTHAs positive on qPCR for this organism also developed the clinical syndrome (Hawkins, 2010; Johns et al., 2009; Mirande et al., 1992; Zabka et al., 2004). This may be due in part to several factors such as the *Chlamydia* strain involved, environmental factors, concomitant diseases and immune response of the bird. In breeding season *Chlamydia* sp. can spread from parents to chicks effectively (Blomqvist et al., 2012). In this study, two SWHAs of approximately 36 days of age were positive on qPCR. The positive SWHA was the only chick on the nest while the weak positive SWHA had a sibling that tested negative. Therefore, the weak positive SWHA could be suggested to be related to a prey item rather than to transmission from the parent since the sibling was negative. However, this hypothesis could not be evaluated in the other SWHA chick where there could still be the potential for chlamydial organisms to be transmitted by the parent.

2.5. Conclusions

This study identified a prevalence of 1.37% of *Chlamydia* sp. in the sampled free-ranging hawks (*Buteo* sp.) from 9 counties in Northern California via qPCR, confirming our hypothesis that the prevalence in free-ranging populations of these birds is less than 5%. The prevalence detected here is less than the prevalence of chlamydial organisms by IHC of primarily hepatic and splenic tissues of sick hawks (*Buteo* sp.) from the total *Buteo* sp. cases presented to the Veterinary Medical Teaching Hospital, School of Veterinary Medicine at the University of California, Davis between 2000-2013. Our results included two positive fledgling Swainson's hawks and two positive juvenile red-tailed hawks over the total of 291 *Buteo* sp. sampled. The identified atypical chlamydial ompA DNA sequence was not identical to any known strain of *C. psittaci* (A-G, E/B, M56, WC) or to *C. pneumoniae*. However, it was most similar to the *C. psittaci* mammalian strain M56. In addition, all serological testing for *Chlamydia* spp. was negative for all samples. The most likely hypothesis is that this identified atypical *Chlamydia* sp. by qPCR from multi-mucosal site swabs was from a mammalian prey item passing through the gastrointestinal tract of these *Buteo* sp. Additional studies which can identify and sequence these bacteria in raptors and potentially prey items may establish if these bacteria can affect the health status of *Buteo* sp. with further population implications. It is also possible that a new *Chlamydia* sp. or a new low pathogenic *C. psittaci* strain is present in free-ranging *Buteo* sp. populations in Northern California, but the lack of either EBA or IFA antibody tests reduce this possibility.

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