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Host surveys, ixodid tick biology and transmission scenarios as related to the tick-borne pathogen, *Ehrlichia canis*

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Abstract

The ehrlichioses have been subject to increasing interest from veterinary and public health perspectives, but experimental studies of these diseases and their etiologic agents can be challenging. *Ehrlichia canis*, the primary etiologic agent of canine monocytic ehrlichiosis, is relatively well characterized and offers unique advantages and opportunities to study interactions between a monocytopathic pathogen and both its vertebrate and invertebrate hosts. Historically, advances in tick-borne disease control strategies have typically followed explication of tick-pathogen-vertebrate interactions, thus it is reasonable to expect novel, more sustainable approaches to control of these diseases as the transmission of their associated infections are investigated at the molecular through ecological levels. Better understanding of the interactions between *E. canis* and its canine and tick hosts would also elucidate similar interactions for other *Ehrlichia* species as well as the potential roles of canine sentinels, reservoirs and models of tick-borne zoonoses. This article summarizes natural exposure studies and experimental investigations of *E. canis* in the context of what is understood about biological vectors of tick-borne Anaplasmataceae.

Keywords

Ehrlichia canis; canine monocytic ehrlichiosis; host surveys; biological transmission; tick biology; Metastriata; Prostria

Introduction

Ehrlichia canis is the primary etiologic agent of canine monocytic ehrlichiosis (CME), also known as tropical canine pancytopenia, a relatively well characterized cosmopolitan tick-borne disease of dogs (Ewing, 1969; Groves et al., 1975; Kordick et al., 1999; Lewis et al.,

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1977; Mathew et al., 1996; Smith et al., 1976). *E. canis* invades and develops in canine monocytes and macrophages, which can lead to leukopenia, thrombocytopenia, fever, depression and anorexia. Some closely related pathogens, including *E. ewingii*, *E. chaffeensis*, *Anaplasma phagocytophilum* and *Neorickettsia risticii*, can cause atypical disease in dogs (Dawson and Ewing, 1992; Kakoma et al., 1994; Rikihisa, 1991). However, *E. canis* is responsible for the most common and clinically severe form of canine ehrlichiosis.

The ehrlichioses have been subject to increasing interest from veterinary and public health perspectives (Olano and Walker, 2002; Paddock and Childs, 2003; Rikihisa, 1991; Skotarczak, 2003; Walker and Dumler, 1996). Members of the genus *Ehrlichia*, which, like the closely related genus *Anaplasma*, is classified in the family Anaplasmataceae of the order Rickettsiales (Dumler et al., 2001), and all currently defined *Ehrlichia* species are biologically transmitted by ticks of the family Ixodidae. The objective of this review is to summarize current understanding of the biological transmission of *E. canis* based on natural exposure studies and experimental investigations with the canine model in the context of what is understood about biological vectors of tick-borne Anaplasmataceae.

Canine Monocytic Ehrlichiosis

CME canis can be divided into acute, subclinical and chronic phases. These phases were characterized with experimental infections of purpose-bred dogs infected by needle inoculation with *E. canis*-infected cells (Buhles et al., 1974; Buhles et al., 1975; de Castro et al., 2004; Ewing, 1969; Ewing and Buckner, 1965; Huxsoll et al., 1972; Reardon and Pierce, 1981), and this disease is reviewed in considerable detail elsewhere (Ewing, 1969; Harrus et al., 1999; Neer and Harrus, 2006; Skotarczak, 2003; Woody and Hoskins, 1991). The acute phase of experimental CME begins approximately 10 days, with a range of 8-20 days, after infection with *E. canis*, and can involve anemia, anorexia, ataxia, conjunctivitis, depression, fever, leukopenia, ocular discharge, thrombocytopenia and vomiting that end with dogs undergoing partial recovery, with clinical signs subsiding, approximately 20 to 30 days after infection. Acute CME is usually followed by a subclinical phase that can last from months to years (Baneth et al., 1996; Botros et al., 1995; Harrus et al., 1998; Waner et al., 1997). As its name suggests, there are no prominent clinical signs during this phase of the disease, but hematologic signs such as leukopenia, anemia and thrombocytopenia can still occur, with mild thrombocytopenia as the most consistent finding under experimental conditions (Waner et al., 1997).

The chronic phase of CME can be mild or severe, with recurrent clinical and hematologic signs that include pancytopenia, hemorrhage, monocytosis, lymphocytosis and weight loss (Ewing, 1969). In a retrospective study of 19 fatal cases diagnosed with chronic CME in Greece, Mylonakis et al. (2004) reported pancytopenia, bone marrow hypoplasia, anorexia, depression, severe bleeding tendencies, hypoalbuminemia and increased serum alanine aminotransferase. PCR testing of bone marrow detected *E. canis* in 13 cases and *A. phagocytophilum*, *A. platys* or *Leishmania infantum* in individual cases, but co-infections were not detected.

Ante mortem diagnosis of CME is reliant on direct or indirect detection of *E. canis* by microscopy, serology, cell culture or PCR. Microscopy can be very time consuming, and requires considerable experience and skill because both false positives and false negatives are common problems with this method. Currently available serologic methods are useful in determining if a host was exposed to an *Ehrlichia* spp., and these methods are also tractable for epidemiological, experimental and some immunological studies, but these tests are not necessarily useful for detecting active infections or if a host is clear of the organism after

treatment (Iqbal et al., 1994). Moreover, antigenic cross-reactivity between *E. chaffeensis*, *E. ewingii* and other *Ehrlichia* spp. can affect specificity when defined antigens are not used for serologic assays (Perez et al., 1996; Rikihisa et al., 1992; Wen et al., 1995). The recent availability of in-clinic ELISA testing for *E. canis* is promising. O'Connor et al. (2006), however, noted a poor sensitivity of detection with a commercially available ELISA when compared to IFA testing of canine sera with low titers (1:80 to 1:160), but good correlation between these methods was reported for samples with titers outside of this range. The authors suggested that this poor correlation at low antibody titers might be due to high IFA sensitivity or cross-reactivity with related anaplasma species. While serology is not always indicative of active infection, isolating *E. canis* by inoculating cell lines with host tissues, buffy coats, mononuclear cell fractions or peripheral blood does allow demonstration of active infection and specific identification of the pathogen (Aguirre et al., 2004a; Iqbal et al., 1994; Iqbal and Rikihisa, 1994; Keysary et al., 1996; Nyindo et al., 1971). Cultivation methods can also be sensitive, but they are technically difficult and require a relatively long, laborious and costly process.

PCR assays have contributed considerably to our understanding of anaplasma agents and prompted new questions regarding the infectious cycle of *E. canis* by allowing direct detection of low numbers of organisms in naturally and experimentally infected hosts. These assays usually involve amplification of genomic DNA, so expression of a specific gene is not required for pathogen detection in various hosts and tissues. *E. canis*-specific PCR assays were developed and widely used to amplify fragments of the *E. canis* 16S rRNA gene (16S rDNA) from both vertebrate and invertebrate hosts (Fenollar and Raoult, 2004; Sparagano et al., 1999). Another PCR assay was developed to amplify an outer membrane protein-1 (*omp-1*) paralog, *p30* (Stich et al., 2002), and a sensitive single-step *p30*-based assay was used to amplify *E. canis* DNA out of single tick extracts (Bremer et al., 2005; Stich et al., 2002). The increased sensitivity provided by the *p30*-based assay could be because species-specific nucleic acid sequences, like those encoding outer membrane proteins, provide greater ranges of specific targets for primer design (Stich et al., 2002; Wagner et al., 2004). Conventional PCR requires DNA isolation, amplification and gel electrophoresis, but there is work toward real time PCR assays that could be more sensitive, specific and faster without the gel electrophoresis step (Branger et al., 2004; Doyle et al., 2005; Labruna et al., 2007; Ndip et al., 2005). Although these assays can be challenging to implement, to our knowledge PCR is currently the most feasible approach to identify an active infection during all three phases of CME.

***E. canis* host surveys**

Domestic dogs

CME canis has been described around the world, but appears to be particularly prevalent in tropical regions. The first published description of canine infection with *E. canis* was from Algeria in 1935 (Donatien and Lestoquard, 1935). Studies regarding the distribution of *E. canis* and other related organisms can be complicated by difficulties associated with sensitive, specific detection of these parasites, including cross-reactivity of closely related pathogens and occasional detection of seronegative but PCR-positive, PCR-negative but seropositive or PCR-negative but culture-positive hosts.

Serologic and molecular studies detected evidence of *E. canis* among African dogs throughout the continent, including Sudan (Inokuma et al., 2006b), Zimbabwe (Kelly et al., 2004; Matthewman et al., 1993), Cameroon (Ndip et al., 2005), Ivory Coast and Gabon (Davoust et al., 2006), and Tunisia, Senegal and Chad (Brouqui et al., 1991). Positive test rates in these studies ranged from 32% (Ndip et al., 2005) to 80.8% (Inokuma et al., 2006b). Botros et al. (1995) used the *E. canis* IFA test to survey 252 dogs from five military kennels

and 122 privately owned dogs in Egypt, and reported an overall seroprevalance of 33%, with 29% among military and 41% among privately owned dogs, respectively. Dogs infested with *R. sanguineus* had a seroprevalance of 44%, while 31% of non-infested dogs were seropositive for *E. canis*. There is also serologic evidence of *E. canis* and *E. chaffeensis*, both of which are monocytotropic, among dogs in South Africa (Pretorius and Kelly, 1998), which is further complicated by the detection of an *E. ruminantium*-like pathogen in dogs with clinical signs consistent with ehrlichiosis (Allsopp and Allsopp, 2001). Several studies from southern Africa have provided important research milestones and insights into the natural history of the Anaplasmataceae, and it appears that future investigations in this region are destined to do the same.

Prior to an outbreak among military working dogs in Southeast Asia in 1967, naturally occurring canine ehrlichiosis was widely considered a mild disease characterized by fever, vomiting and naso-ocular discharge. During the Vietnam War, a highly hemorrhagic form of *E. canis* caused epizootic losses of military working dogs (Nims et al., 1971). Serological, molecular and microscopic evidence of *E. canis* infections has since been reported in dogs from many Asian countries such as China, Israel, Korea, Japan, Malaysia and Thailand (Baneth et al., 1996; Chae et al., 2003; Inokuma et al., 1999; Rajamanickam et al., 1985; Suksawat et al., 2001; Wen et al., 2003). Reports describing the seroprevalence of *E. canis* infections include 30% of stray and pet dogs in Israel, 13.6% of dogs in Japan, 0.2% of dogs in Malaysia and 22% of stray dogs in Thailand, with a 14.1% prevalence of *E. canis* detected by microscopic examination in Bangkok and surrounding areas (Jittapalapong et al., 2003). Most seropositive dogs in Thailand had higher titers to *E. canis* than to *E. chaffeensis* or *A. phagocytophylum*, thus *E. canis* appeared to be the more prevalent anaplasma species among dogs in Bangkok. In a study of 150 randomly selected dogs from Japan, seroprevalence of *E. canis* was estimated at 10% (Watanabe et al., 2006), but these authors noted considerable cross reactivity between *E. canis*, *E. chaffeensis*, *E. muris* and an *Ehrlichia* sp. isolated from *Ixodes ovatus* ticks (IOE). Notably, the latter two pathogens are indigenous to Japan (Shibata et al., 2000; Wen et al., 1995), and *E. canis* was not detected in any of 1,211 Japanese ticks examined with 16S rDNA-based nested PCR (Inokuma et al., 2003).

The European range of *E. canis* is primarily throughout areas bordering the Mediterranean Sea (Spain, southern France, Italy and Turkey), with isolated reports noted in central Europe (Trotz-Williams and Trees, 2003). Seroprevalence among dogs in northeastern Spain was found to be 16.7% (Solano-Gallego et al., 2006b), and the organism was successfully cultivated from a naturally infected dog in that region (Aguirre et al., 2004a). A study of canine sera collected in northern Sardinia noted that 50% of the samples tested were IFA-positive, with a rate of 62.5% among samples from 13-60 month old dogs (Cocco et al., 2003). In France, Jouret-Gourjault et al. (2006) intravenously inoculated four naïve Beagles with the *E. canis* Borgo 89 strain isolated from sick dog in Corsica, and observed clinical CME comparable to that reported for other strains with one case of unapparent infection. Marsilio et al. (2006) used a citrate synthase gene-based PCR assay to detect *E. canis* in three of 14 blood samples from seropositive dogs in Italy, and amplicon sequence of two of these samples revealed 99.2% identity to the Oklahoma strain. Solano-Gallego et al. (2006b) estimated the prevalence of *E. canis* with real-time PCR from blood of Italian dogs, and reported prevalence in northern, central and southern Italy of 2.9%, 8% and 9.7%, respectively. Unver et al. (2005) reported infection in 3 dogs from Ankara, Turkey, by PCR assay; analysis of a 1,388 bp 16S rRNA sequence found this strain to be identical to *E. ovina* and to a strain isolated from dogs in Venezuela, with 99.9% similarity to the Oklahoma strain. A similar study by Unver et al. (2003) demonstrated 99.9% identity between Oklahoma and Venezuelan strains to one sequenced from a naturally infected Japanese dog. A serologic survey of Swiss dogs found reactive antibodies in 2.2% of the samples tested,

the authors suggested that the dogs in question were likely exposed while traveling outside the country, because the range of *R. sanguineus* in this area is thought to be south of the Alps (Pusterla et al., 1998).

It is tempting to speculate that *E. canis* spread from the Old to the New World with its primary tick host, *R. sanguineus*. However, additional studies, perhaps involving robust molecular characterization of *E. canis* strains within both dogs and ticks on all the major continents, are needed to address this question (Pinyoowong et al., 2008). Bool and Suttmoller (1957) described the first New World infection of dogs with *E. canis* in Aruba. Since that initial report, others have characterized the occurrence of *E. canis* throughout South, Central and North America. Brazilian studies have estimated seroreactivity among privately owned dogs at 19.8-23% (Labarthe et al., 2003; Trapp et al., 2006), and PCR detected rates of 1.4-3.5% in non-thrombocytopenic dogs, 20-26.8% in mildly thrombocytopenic dogs and 63.1% in dogs with platelet counts less than 100,000/ μ l (Bulla et al., 2004; Dagnone et al., 2003; Macieira et al., 2005). Recently, Labruna et al. (2007) submitted the first report of canine infection in the Amazon region of Brazil, which involved PCR findings and sequence analysis of amplicons from 4 of 5 dogs examined (Labruna et al., 2007). In another study, PCR analysis of 55 canine blood samples from Venezuela found an infection rate of approximately 33%; a 1,408 bp 16S rDNA sequence from the Venezuelan dog *Ehrlichia* (VDE) strain cultured from one of the samples was identical to that cultured from a human blood sample reported in the same study and 99.9% identical to the Oklahoma strain of *E. canis* (Unver et al., 2001b). A study of 120 dogs admitted to vet clinics in the Yucatan, Mexico, found 44.1% of dogs seropositive with ELISA testing (Rodriguez-Vivas et al., 2005). Although there is limited information regarding the prevalence of *E. canis* in Canada, a recent article noted a relatively low seroprevalence in southern Ontario and Quebec compared to most regions of the United States (Gary et al., 2006).

The first description of *E. canis* in North America was in the U.S., from a dog in Oklahoma that was co-infected with *Babesia canis* (Ewing, 1963). Interestingly, at that time the author could not conclude whether observed intraleukocytic inclusion bodies were an undescribed schizogenous phase of *B. canis* or an infection with a second organism, but it was in the course of careful investigation of *B. canis* reproduction in experimentally inoculated dogs that leukocytic inclusions were serendipitously observed from a negative control animal, in the absence of intraerythrocytic piroplasms, and subsequent work confirmed that canine erythrocytes and leukocytes were infected with different parasites in these cases (Ewing, 1964; Ewing and Buckner, 1965). Many cases have since been recognized in Oklahoma and the southern U.S. The Oklahoma Animal Disease Diagnostic Lab found that 53% of 259 samples submitted for testing were seropositive for *E. canis* from the period of June 1986 to July 1987 (Rodgers et al., 1989). Stockham et al. (1992) found that 25 of 31 dogs presenting clinical signs of polyarthritis in Missouri had *E. canis* sera titers from 1:20 to 1:5120, with thrombocytopenia being the most common hematologic abnormality. In a survey of blood samples from 65 tick-infested dogs from northern Oklahoma, 3.1% were PCR-positive for *E. canis*, while 10.8% were seropositive (Murphy et al., 1998). A case report from the same region described concurrent infection of an Oklahoma dog displaying neurologic signs with both *E. canis* and *E. ewingii* (Meinkoth et al., 1998). Hoskins et al. (1988) performed serologic testing on both individually owned clinically ill thrombocytopenic dogs and apparently healthy dogs in south Louisiana, finding 25.9% of the ill dogs and 20.3% of the healthy dogs seroreactive. Further west, a retrospective study of sera collected in 1975 from 339 dogs in Phoenix, Arizona revealed a seroreactivity rate of 12% among all dogs, with German Shepherd dogs demonstrating the highest degree of exposure (21%) (Stephenson and Ristic, 1978). A later study of 105 canine sera from Texas and Arizona revealed a seroconversion rate of 76% with 44% of the samples testing PCR-positive with a nested 16S

rDNA-based assay; three IFA-negative samples were PCR-positive and 37 of the IFA-positive samples were PCR-negative (Wen et al., 1997).

Surveys of dogs in the eastern U.S. also revealed the presence of *E. canis* in that region. Stephenson et al. (1975) recognized an early domestic case of CME in a Collie cross from Virginia, presenting epistaxis and thrombocytopenia, with a serum titer of 1:20 based on IFA. Five of 30 IFA-negative canine blood samples from Ohio were PCR-positive for *E. canis* (Wen et al., 1997). Suksawat et al. (2000) tested sera from 1,845 sick dogs admitted to the North Carolina State Veterinary Teaching Hospital, finding 44 IFA-positive dogs, 36 of which were also positive by Western immunoblotting (Suksawat et al., 2000). Among 90 adult dogs admitted to an animal shelter in eastern Tennessee, 11% were seroreactive, but all tested PCR-negative and possible exposure to another anaplasma species was not excluded (Seaman et al., 2004). The first recognized case of CME in the northeastern U.S. was in a dog in Connecticut, which led to recognition of the risk for CME in higher latitudes than previously expected (Magnarelli et al., 1990), and a 11.7% seroprevalence was reported from a retrospective study of 60 canine sera collected in 1986 in Connecticut and New York (Magnarelli and Anderson, 1993). Sampling in Rhode Island revealed a 2.9% seroprevalence for *E. canis* among 277 dogs in animal shelters (Hinrichsen et al., 2001). A large study found that 11% of military working dogs worldwide and 57% of civilian dogs in the U.S. were IFA-positive for *E. canis* (Keefe et al., 1982); the high seroprevalence among civilian dogs was skewed by practitioners submitting samples from dogs with clinical signs of CME.

Other mammalian hosts

Some investigators have observed natural or experimental *E. canis* infections of vertebrates other than domestic dogs. There is evidence that wild canids are susceptible to both *E. canis* infection and CME. An outbreak associated with a severe *R. sanguineus* infestation and significant mortality ascribed to CME was observed among wolves, dogs and wolf-dog crosses in a Florida zoo (Harvey et al., 1979). Coyotes, red and gray foxes were susceptible to infection by blood inoculation from dogs, and the gray fox was shown capable of providing an infectious blood meal for *R. sanguineus* larvae (Amyx and Huxsoll, 1973; Ewing et al., 1964). These experimental observations were supported by a report of 36% seropositivity among foxes in Israel (Fishman et al., 2004). Although a survey by Pusterla et al. (1999) failed to detect antibodies to *E. canis* in any of 1,550 sera samples from red foxes in Switzerland, this is not an area known to be enzootic for *E. canis* among the domestic canine population. Jackals sampled in Kenya and Israel displayed seroconversion at levels of 2% and 35.8%, respectively (Alexander et al., 1994; Waner et al., 1999). Inokuma et al. (2006a) identified an *E. canis* titer in 1 of 187 raccoon sera examined in Japan; these authors noted the potential exposure of this host prior to its import into Japan. Field testing and experimental trials failed to identify *E. canis* infections among capybara, deer and lemurs (Dawson et al., 1994; Labruna et al., 2007; Yabsley et al., 2004). The high similarity between 16S rDNA gene sequences of *E. canis* and *E. ovina* (Bekker et al., 2002; Unver et al., 2005), the latter of which naturally infects sheep, suggests that it would be interesting to investigate the susceptibility of their respective hosts to experimental infection.

Feline ehrlichiosis has been increasingly recognized worldwide since the first case due to natural infection was reported in France (Charpentier and Groulade, 1986). There appears to be at least two forms of feline ehrlichiosis or anaplasmosis, one attributed to *E. canis*, with inclusions in mononuclear cells, and the other to *A. phagocytophilum*, which is associated with inclusions in neutrophils (Foley et al., 1999; Lappin et al., 2004; Lewis et al., 1975). Despite an early report of failure to experimentally infect cats with *E. canis* (Ewing, 1969), the pathogenic role of *E. canis* in cats has received increasing acceptance, predominantly on the basis of the serologic evidence of natural infection and the infrequent detection of

morulae within feline leukocytes. Clinical signs of feline ehrlichiosis include anemia, anorexia, dehydration, fever, lethargy, tachypnea and thrombocytopenia (Breitschwerdt et al., 2002; Tarello, 2005), and such cases are usually identified in geographic regions that are enzootic for canine, equine or human anaplasmosis. In Thailand, *E. canis*-like infections were described in stray cats of Bangkok (Jittapalapong and Jansawan, 1993). In 2003, seropositive rates for *E. canis*, *E. chaffeensis* and *A. phagocytophilum* were 4.9, 5.2 and 2.4 %, respectively among sera taken from 287 stray cats in monasteries around Bangkok (Jittapalapong et al., 2004). Studies in Spain and the U.S. reported seroconversion among cats to *E. canis*, with the more extensive testing in Spain describing rates of 10.6 to 17.9% (Aguirre et al., 2004b; Boulay et al., 1994; Ortuno et al., 2005; Solano-Gallego et al., 2006a). Breitschwerdt et al. (2002) examined blood and sera samples from 3 cats, one from North Carolina and two from Ontario, Canada, displaying clinical evidence of ehrlichiosis (Breitschwerdt et al., 2002). Partial 16S rDNA sequences amplified from these cats were identical to one another and to a canine isolate of *E. canis*. Filoni et al. (2006) identified one seropositive Brazilian puma of 18 tested. Experimental tick transmission studies with canine and feline isolates are needed to determine whether cats are susceptible to infection with *E. canis* or a closely related species.

Human monocytic ehrlichiosis (HME) was initially thought to be caused by *E. canis* due to serological cross-reactivity and the affinity of both pathogens for monocytes and macrophages (Maeda et al., 1987). However, this hypothesis was challenged (Ewing et al., 1987) and eventually refuted through analysis of ecological, genetic and vector competence differences between *E. canis* and the ultimate etiologic agent of HME: *E. chaffeensis* (Dawson et al., 1991; Ewing et al., 1995; Rikihisa, 1991). Differences were observed between 16S rDNA sequences of these species, *E. canis* is enzootic throughout the U.S. while *E. chaffeensis* appears to be primarily restricted to southern states, and *E. chaffeensis* is transmitted by *Amblyomma americanum* while this tick species is not considered capable of transmitting *E. canis*. Although *E. canis* is not considered an agent of HME in the U.S., newer evidence indicates that a Venezuelan strain of *E. canis* might be transmitted to human beings (Perez et al., 2006; Perez et al., 1996). This recently described strain, named Venezuelan human *Ehrlichia* (VHE), is associated with human ehrlichiosis in that region and has a 16S rDNA sequence that is 99% identical to that of *E. canis*. Further work is needed to confirm whether VHE is indeed *E. canis*, and, importantly, if VHE is tick-transmissible among dogs.

Invertebrate hosts

Less attention has been devoted to characterizing *E. canis* within its tick hosts. Both *R. sanguineus* and *Dermacentor variabilis* were demonstrated to be competent experimental vectors of the parasite (Bremer et al., 2005; Groves et al., 1975; Johnson et al., 1998; Smith et al., 1976). *E. canis* has been detected in unattached and feeding ticks in Africa (Loftis et al., 2006), Europe and the Americas. Ewing et al. (1990) found no evidence of *E. canis* infection in an *Otobius megnini* found feeding on a child with serologic evidence of ehrlichial infection, and an attempt to infect dogs with this soft tick was unsuccessful (Ewing et al., 1990). Magnarelli et al. (1991) noted organisms in hemocytes of 9.7% of nymphal and adult *Ixodes scapularis dammini* collected from Connecticut, and that these bacteria reacted with fluorescein-conjugated antiserum to *E. canis*. A similar result was reported for 6.9% of the *D. variabilis* collected. However, although they shared antigens with *E. canis*, the specific identity of these rickettsiae was not determined. In 1998, (Murphy et al.) reported use of a 16S rDNA-based PCR assay to detect *E. canis* in two pools of *R. sanguineus* males that were collected from dogs that were seropositive for *E. canis*. Similarly, Unver et al. (2001c) also found PCR evidence of *E. canis* in pools of *R. sanguineus* collected from Venezuela, and Christova et al. (2003) noted that 7% of

metastriate ticks collected from Turkey and Albania contained Anaplasmataceae, with *E. canis* in one of 28 individual *R. sanguineus* from Albania. Wielinga et al. (2006) used a 16S rDNA-based reverse line blot technique to identify amplicons consistent with *E. canis* in three of 1600 (0.19%) unattached Dutch *I. ricinus*, which, interestingly, suggests that *E. canis* or a closely related species (e.g., an *E. muris*- or IOE-like species) may infect prostriate ticks in that region.

Ticks and the transmission of *E. canis*

Biology of ixodid ticks

Ticks of the family Ixodidae biologically transmit members of the closely related rickettsial genera *Ehrlichia* and *Anaplasma*. Ixodid ticks have four development stages: egg, larva, nymph and adult (Sonenshine, 1991). Larvae and nymphs require a blood meal before molting into the next stage, and adult stage females require a blood meal prior to oviposition. Female ixodid ticks die after oviposition, but the fate of ixodid males after copulation is not as certain and varies with the biology and reproductive behaviors among groups within this family. Most authorities agree that the Ixodidae consists of five subfamilies or tribes. All but one tribe fall into a group called the Metastrata, while the more 'primitive' Prostrata contains the remaining tribe (the Ixodinae) (Barker and Murrell, 2004; Sonenshine, 1991).

There are notable differences in reproduction between prostrates and metastrates (Kiszewski et al., 2001). In contrast to prostrates, most metastrate males require a blood meal prior to sexual maturation and copulate on the vertebrate host (El Said and Swiderski, 1983; Homsher and Sonenshine, 1972, 1976; Kiszewski et al., 2001; Londt and Van Der Bijl, 1977; Londt and Spickett, 1976; Oliver and Dotson, 1993; Sonenshine, 1991; Valero et al., 1997). In addition, metastrate male ticks are attracted to feeding sites by aggregation pheromones and to feeding females by sex pheromones (Leahy and Booth, 1983; Norval et al., 1989; Norval et al., 1996; Sobhy et al., 1994; Sonenshine, 1985), suggesting means for interhost transfer and intermittent feedings on multiple vertebrate hosts. Thus, metastrate males may remain in the environment, potentially transmitting pathogens to multiple vertebrate hosts without the need for additional acquisition or molting periods.

Most ixodid species are three-host ticks with immature stages that detach from their hosts after each blood meal before molting to the next stage. One-host ticks, such as the *Rhipicephalus* subgenus *Boophilus*, seek out their hosts as larvae and remain on the same vertebrate host between molts. Although each feeding stage of *R. sanguineus*, the primary vector of *E. canis*, will readily feed on canine hosts, these are three-host ticks because engorged immature stages detach and molt away from the host.

Biological Transmission Scenarios

Each feeding tick stage or blood meal represents an opportunity to acquire and transmit pathogens (Figure 1). The majority of tick-borne pathogens are biologically transmitted, which means that these organisms also infect the invertebrate vector, where they multiply and/or develop prior to transmission to another vertebrate host. Several adverbs are used to describe pathogen passage in ticks: "transstadial" or "interstadial" describe passage of tick-borne pathogens between development stages of the same tick, while "intrastadial" describes maintenance of an infection within the same tick development stage. Thus, horizontal transmission by ticks can occur transstadially when an immature tick stage acquires the pathogen while taking a blood meal and the tick transmits the organism during a subsequent development stage, and intrastadial transmission can occur when ticks both acquire and transmit a pathogen during the same development stage. Vertical transmission by ticks is primarily thought to occur after transovarian passage from a female tick to larvae. Thus,

transovarian passage is also a form of transmission between the invertebrate hosts themselves. Although transovarian passage has been reported for tick-borne members of the rickettsial family Rickettsiaceae, this phenomenon is considered rare among the Anaplasmataceae (Groves et al., 1975; Long et al., 2003; Potgeiter, 1981; Stich et al., 1989).

The relevance of each tick transmission scenario is in large part dependent on the preferred host for each tick species and development stage. Transovarian passage is always important, especially with one-host tick species. Transstadial transmission is most important with two- or three-host ticks that have two or more stages that feed on vertebrates that are susceptible to infection with the pathogen in question. Intrastadial transmission is important with tick species that may transfer between hosts during a particular development stage. For example, intrastadial transmission could be important with adult male metastriates because they have been shown to move among and to feed on different hosts under experimental conditions, and because they are thought to persist throughout the tick season. The intrastadial transmission scenario could be even more important when interhost transfer may occur (e.g., utilization of gregarious vertebrate hosts) or when immature stages do not feed on hosts that are susceptible to infection with the pathogen. Notably, a recent report documented movement of adult *R. sanguineus*, especially male ticks, among dogs with experimentally pre-established infestations (Little et al., 2007). These results indicate that previously fed adult *R. sanguineus* will undergo interhost transfer under *ad libitum* conditions, and confirmed the potential importance of intrastadial transmission scenarios of *E. canis* with its primary vector (Bremer et al., 2005). Further investigation is needed to determine the frequency of interhost transfer of male ticks that parasitize both canine and human hosts.

Tick surveys

Reports that distinguish tick stages and sexes collected in surveys often underscore the importance of differences in the biology and behavioral ecology among tick tribes. For example, surveys of unattached questing ticks provide evidence that equivalent numbers of female and male ixodid offspring reach maturity. Among the surveys summarized in Table 1, the average yields of unattached adult prostriate female and male ticks were 39 and 61%, respectively, while unattached metastriate female and male yields averaged 53 and 47%, respectively. However, there was no statistical difference, according to a one-tailed Student's T-test, between the mean percentages of unattached female and male ticks of either the prostriates ($P>0.2$) or metastriates ($P>0.1$) among these reports. None of the surveys of unattached ticks reported any test to determine whether these ticks had previously fed during their adult stage.

There is evidence that metastriate males persist on hosts longer than females under natural conditions. Table 2 provides a summary of studies where burdens of adult female and male ticks were reported for various mammalian hosts. Relative percentages of male and female ticks collected from different hosts in separate studies are also presented due to substantial differences in numbers of ticks collected, host species, environmental factors, seasons of collection and experimental designs. Despite the production of equal proportions of female and male offspring, adult male metastriate ticks outnumber females in the majority of these reports where ticks were collected from vertebrate hosts. An interesting observation is made when the numbers of metastriate ticks reported in Table 2 are broken down into the three tribes represented in these studies (Rhipicephalinae, Amblyomminae and Haemaphysalinae): on average male ticks make up 65% of the adult ticks collected for each tribe. In addition, the differences between percent female and male ticks collected from hosts for each metastriate tribe were statistically different ($P<0.001$ for rhipicephalines and amblyommines, and $P<0.01$ for haemaphysalines).

Interestingly, there is not a difference between the percentage of Haemaphysalinae males and females reported in one investigation summarized in Table 2 (Horak et al., 1987). At first glance this observation may suggest that these male and female ticks persisted on dogs at the same rate in this study. However, this is not necessarily the case when one considers that these results represented total tick numbers removed from four dogs every 2 to 4 weeks over the course of three years, thus these values are closer to the total number of ticks that attached to these dogs rather than the number of ticks on the hosts at a single time point. Therefore, although this observation supports the idea that equivalent numbers of metastriate female and male ticks feed in nature, this particular study design was not conducive with estimating the persistence of ticks over time because the ticks were periodically removed from the same hosts.

Conversely, the percentage of prostriate (Ixodinae) female ticks (80%) collected from mammalian hosts was greater ($P < 0.001$) than the percent males found on these hosts, which supports the paradigm that male *Ixodes* spp. may not be important pathogen vectors in nature. This observation meshes well with prostriate biology, because these ticks are often nidicolous and mate on or off of the host (Kiszewski et al., 2001; Sonenshine, 1991), so there is a conceivable advantage for males of many prostriate species to remain off the host.

These summaries support the paradigm that equivalent numbers of female and male ixodids are produced under natural conditions (Table 1) and that the ratio of the sexes found on hosts is reflective of where the males of the different subfamilies mate and that metastriate males can persist on hosts after mating (Table 2). These hypotheses require additional testing, in part through more surveys that distinguish tick development stages in addition to their species and infection status.

Intrastadial Transmission

There is considerable evidence of anaplasma pathogen transmission through interhost transfer of ticks. Experimental studies have provided evidence for an epizootiological role of three-host male ticks as intrastadial vectors of *A. marginale* after interhost transfer (Andrew and Norval, 1989; Coan et al., 1987; Kocan et al., 1992; Stiller and Coan, 1995; Stiller et al., 1987; Stiller et al., 1989; Zaugg et al., 1986). For example, one study involved the release of differentially marked ticks on to an *A. marginale*-infected calf that was penned with a naive calf. Of the ticks recovered from these calves, 30.8% were found to have transferred between hosts (Coan et al., 1987; Stiller and Coan, 1995). In addition, the naive calf developed clinical anaplasmosis 25 days after being penned with the infected calf; 21 days after the first two males from the infected calf were found to have transferred. It was also demonstrated in another study that intrastadially infected *D. andersoni* male ticks could continually transmit *A. marginale* to multiple hosts without any apparent loss of virulence (Kocan et al., 1992). *A. hebraeum* males placed on a host infected with *E. ruminantium* were found to transfer between hosts sharing the same stall in sufficient numbers to transmit a lethal dose of this pathogen (Andrew and Norval, 1989). Furthermore, epidemiological investigations have suggested that one-host tick species such as *R.* (subgenus *Boophilus*) *microplus* and *D. albipictus* were associated with natural transmission of *A. marginale* (Bock et al., 1999; Ewing et al., 1997), which corroborates at least one report of experimental transmission (Aguirre et al., 1994). Intrastadial or transstadial transmission after interhost transfer are the most likely scenarios in such cases because transovarial transmission is considered unlikely for Anaplasmataceae (Bram and Roby, 1970; Long et al., 2003; Potgeiter, 1981; Stich et al., 1989) and because *R. Boophilus microplus* has been reported to transfer between bovine hosts (Mason and Norval, 1981). A unique finding of a questing *R. Boophilus decoloratus* adult further supports the idea that interhost transfer of one-host ixodid ticks may occur under natural conditions (Spickett et al., 1991). Further investigations are needed to evaluate the importance of (1) interhost transfer of one-host

ticks and (2) intrastadial transmission scenarios for zoonotic agents and their tick vectors that parasitize both canine and human hosts.

Experimental transmission of *E. canis*

Several interesting studies were focused on tick transmission of *E. canis*. Seminal work demonstrated that *E. canis* could be transstadially transmitted between dogs with *R. sanguineus* nymphs or adults (Groves et al., 1975; Lewis et al., 1977; Smith et al., 1976). These investigations leave no doubt that *R. sanguineus* is an important vector of *E. canis*, because all three feeding stages of this tick species readily attach to canine hosts, thus *E. canis* is likely to be transstadially transmitted by *R. sanguineus* in nature. This tick species is particularly problematic due to its somewhat unique ability to persistently infest buildings and kennels (Dantas-Torres, 2008; Harvey et al., 1979). *E. canis* probably multiplies within the midgut cells, hemocytes and salivary glands of its tick host (Smith et al., 1976); however, more extensive investigation of the parasite's development in ticks is needed. Another finding from early studies was that transstadial transmission of *E. canis* could not be demonstrated when ticks were allowed to acquisition feed on dogs in the subclinical or chronic phase of CME (Lewis et al., 1977), thus it is often assumed that dogs cannot serve as infection reservoirs once past the acute phase of CME. However, although difficult to detect, *E. canis* infections can persist in dogs long after the acute phase of CME, possibly with cyclic rickettsemia and recrudescent disease, so the potential of such hosts as sources of tick infection should not be disregarded until the aforementioned report is confirmed by additional, exhaustive, experimental and epidemiological investigations.

One *E. canis* strain, the Oklahoma isolate, was reportedly non-transmissible by *R. sanguineus*, presumably due to extensive passage in a mammalian cell line (Mathew et al., 1996). Interestingly, a subsequent study demonstrated infection of adult stage *R. sanguineus* with the same Oklahoma *E. canis* strain, but transmission by these ticks was not reported (Unver et al., 2001a). Taken together these two studies suggest that the Oklahoma strain might be infective for but not transmissible by *R. sanguineus*, which could provide a novel opportunity to explore events between acquisition and transmission of this pathogen by *R. sanguineus*.

At least one investigation demonstrated that *D. variabilis* is also capable of transstadially transmitting *E. canis* under experimental conditions (Johnson et al., 1998). Epidemiological implications of this finding are difficult to estimate because, while *D. variabilis* adult stages readily feed on dogs, the immature stages have a reported predilection for natural infestation of smaller mammals (Apperson et al., 1993; Clark et al., 2001; Gage et al., 1992; Jackson et al., 1975; Kollars, 1996; Sonenshine et al., 1966). Although there are a few reports of *D. variabilis* nymphs attached to canids in nature (Bishopp and Trembley, 1945; Bloemer and Zimmerman, 1988; Brillhart et al., 1994; Kollars, 1993; Kollars et al., 2000), intrastadial transmission of *E. canis* would probably be more important when this tick species is involved. Further work is warranted to determine if male *D. variabilis* are capable of intrastadial transmission of *E. canis* and *ad libitum* interhost transfer.

As previously mentioned, male *R. sanguineus* were reported capable of both intrastadial and transstadial transmission of *E. canis* under experimental conditions (Bremer et al., 2005). These transmission scenarios took place in the absence of female ticks, indicating that copulation was not required for multiple feedings or pathogen acquisition and transmission by the male ticks. The absence of an intermittent molt in the intrastadial transmission cycle would mean a shorter requisite time period between pathogen acquisition and transmission. Cross sectional surveys of natural canine tick burdens indicated that the majority of adult *R. sanguineus* collected were male (Horak et al., 1995a; L'Hostis et al., 1998), supporting the paradigm that male *R. sanguineus*, like other metatritates, may persist after their female

counterparts have left the host. Moreover, a recent report indicated that adult male *R. sanguineus* will transfer among canine hosts under experimental conditions (Little et al., 2007), confirming the potential role of this tick stage in the transmission of pathogens.

Another observation was that milder acute phase CME was associated with transmission of *E. canis* by male ticks compared to intravenous inoculation with the same strain in carrier blood (Bremer et al., 2005). Several factors may contribute to the severity of CME, which include strain of the agent, infective dose, and age, sex, immunocompetence, concomitant disease and breed susceptibility of the canine host (Rikihisa, 1991). Other reports associated with experimental tick transmission of *E. canis* indicated more severe clinical signs when feeding large numbers of transstadially infected male and female ticks (Johnson et al., 1998; Mathew et al., 1996). Therefore more severe acute CME might result with large tick numbers or the presence of immature or female ticks. At least one study demonstrated severity of disease associated with subcutaneous inoculation with different amounts of *E. canis* in frozen stabilate (Gaunt et al., 1996). However, it is difficult to estimate infectious organism numbers in peripheral monocytes in blood from an *E. canis* carrier (with leukopenia) compared to ticks allowed to acquisition feed on experimental hosts during acute CME and presumably peak rickettsemia. Further work is needed to determine whether clinical differences after tick versus needle inoculation could be due to infective dose. Another consideration is that female ticks, which were included in other investigations, might affect the severity of disease. Female ixodid ticks process considerably more host blood than males, which in turn requires elimination of greater volumes of water via saliva, a known medium of pathogen transmission and vertebrate immunomodulation (Sonenshine, 1991). Therefore it is possible that female tick feeding could result in transmission of larger pathogen numbers or a more pronounced pharmacological effect on the vertebrate host (Bergman et al., 1995; Stich et al., 1993; Wikel et al., 1994). Regardless of the mechanism(s) underlying the differences in severity of clinical signs, these results underscore the relevance of experimental tick transmission in characterization and development of control measures for *E. canis* and other tick-borne pathogens, especially those characterized mainly through needle-inoculation of experimental hosts.

Concluding remarks

Although the complex triad of canid-*E. canis*-*R. sanguineus* interactions are relatively well characterized compared to other *Ehrlichia* spp., there is still a great deal to learn about the infectious cycle of this pathogen and the etiology of CME. The role of different *R. sanguineus* development stages in natural transmission of *E. canis* is difficult to estimate, because all stages of this tick species have a strong preference for canine hosts. However, in the absence of an intermittent molt, the intrastadial transmission cycle would require considerably less time from pathogen acquisition to transmission. Further investigations are needed to determine if *E. canis*-infected male ticks can transmit the pathogen to multiple hosts. If so, *R. sanguineus* males could remain in the environment, transmitting *E. canis* to multiple canine hosts without any acquisition or molting period, and subsequent vertebrate hosts would be more rapidly exposed to the pathogen. Control of the adult male tick populations would then be important for limiting outbreaks of CME, and the logistics of those treatments, including timing and areas treated, would have to be evaluated. More questions remain regarding the effects of tick burdens, stages and copulation on acquisition and transmission of *E. canis* and the effect of immature and female ticks on the severity of subsequent disease. Although *D. variabilis* transmits *E. canis* transstadially, intrastadial transmission might be more important because it appears to be the most likely natural transmission scenario between dogs by this tick species. Attempts to answer these and future questions regarding this enigmatic transmission and disease model is expected to advance our understanding of the infectious cycle of *E. canis*, to augment understanding of the

ehrlichioses and to stimulate interest in the potential roles of dogs as models, sentinels and sources of tick-borne pathogens.

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Figure 1. Pathogen acquisition and transmission scenarios

Transstadial (*a.k.a.* Interstadial) transmission occurs when a pathogen is acquired by an immature tick stage (*i.e.*, a larva or a nymph) and is transmitted by a subsequent developmental stage. Transovarian transmission occurs when a pathogen is transmitted by a generation subsequent to the one that acquires the pathogen. Intrastadial transmission occurs when the same tick stage both acquires and transmits a pathogen.

Table 1
Summary of surveys reporting questing three-host tick sexes

Tick species	Collection Method	Adult Ticks Collected		Source
		Males (%)	Females (%)	
<i>Ixodes ricinus</i>	CO ₂ Trap	381 (41)	553 (59)	(Gray, 1985)
	Drag	156 (54)	135 (46)	
<i>I. scapularis dammini</i>	CO ₂ Trap	1.1 (88)	1.6 (13)	(Solberg et al., 1992) ^a
	Drag	0.7 (83)	0.1 (17)	
	Human host	0.5 (36)	0.1 (64)	
<i>Amblyomma americanum</i>	CO ₂ Trap	89.9 (48)	160.6 (52)	
	Drag	6.1 (38)	6.6 (62)	
	Human host	3.2 (44)	5.2 (56)	
<i>A. cajennense</i>	Drag	253 (42)	212 (58)	(Oliveira et al., 2000) ^b
<i>Rhipicephalus spp.</i>	Drag	36 (61)	49 (39)	(Spickett et al., 1992)
	Drag	116 (54)	98 (46)	(Zieger et al., 1998a)
<i>Dermacentor variabilis</i>	Drag	549 (41)	705 (59)	(Harlan and Foster, 1990)
<i>Haemaphysalis leachi</i>	Drag	31 (54)	20 (46)	(Spickett et al., 1992)

^aData are reported as means of ticks collected at four survey times

^bData presented are estimates (by this author) of the total values reported for a 2 yr period

Table 2
Summary of surveys reporting three-host tick sexes attached to mammals^a

Tick Species	Hosts	Adult Ticks Collected		Source
		Males (%)	Females (%)	
Haemaphysalinae				
<i>Haemaphysalis leachi</i>	Dogs	1184 (58)	843 (42)	(Horak, 1995) ^b
		2426 (39)	3720 (61)	(Horak et al., 1987) ^c
	Caracals	57 (63)	34 (37)	
<i>H. spinulosa</i>		60 (75)	20 (25)	
Rhipicephalinae				
<i>Rhipicephalus</i> spp.	Zebra	816 (66)	420 (34)	(Zieger et al., 1998b) ^d
	Bushpig	12 (45)	14.5 (55)	
	Impala	28.9 (73)	10.6 (27)	
	Tsessebe	66.7 (65)	36.6 (35)	
	Hartebeest	685 (79)	186 (21)	
	Eland	3389 (69)	1488 (31)	
	Greater kudu	866 (49)	892 (51)	
	Sable	62 (81)	15 (19)	
	Water-buck	1161 (63)	688.1 (37)	
	Kafue lechwe	280 (76)	87 (24)	
	<i>R. sanguineus</i>	Dogs	525 (56)	408 (44)
585 (71)			244 (29)	(L'Hostis et al., 1998)
<i>R. simus</i>		131 (67)	64 (33)	
		612 (45)	739 (55)	(Horak et al., 1987) ^c
	Leopard	375 (72)	146 (28)	
<i>R. appendiculatus</i>		2481 (57)	1854 (43)	
<i>R. pravus</i>	Scrub Hares	191 (78)	55 (22)	(Horak et al., 1993)
		101 (81)	24 (19)	(Horak et al., 1995b)
<i>R. kochi</i>		93 (76)	29 (24)	
<i>R. turanicus</i>		72 (73)	26 (27)	
<i>Dermacentor variabilis</i>	White-tailed deer and Feral Swine	94 (51)	89 (49)	(Allan et al., 2001)
	Raccoons	51 (58)	37 (42)	(Pung et al., 1994)
		96 (47)	110 (53)	(Ouellette et al., 1997)
Amblyomminae				
<i>Amblyomma americanum</i>		51 (59)	36 (41)	
	White-tailed deer	73 (53)	65 (47)	(Allan et al., 2001)
<i>A. maculatum</i>	and Feral Swine	516 (64)	293 (36)	
<i>A. variegatum</i>	Hartebeest	24 (80)	6 (20)	(Zieger et al., 1998b) ^d
	Eland	30 (70)	13 (30)	
Ixodinae				
<i>Ixodes scapularis</i>	White-tailed deer and Feral Swine	314 (29)	780 (71)	(Allan et al., 2001)

Tick Species	Hosts	Adult Ticks Collected		Source
		Males (%)	Females (%)	
	Raccoons	3 (11)	24 (89)	(Pung et al., 1994)
<i>I. texanus</i>		0 (0)	135 (100)	(Ouellette et al., 1997)
<i>I. pilosus</i>	Dogs	38 (23)	130 (77)	(Horak et al., 1987) ^c
<i>I. rubicundis</i>	Caracals	132 (36)	232 (64)	

^a Data represents ≥80 total adult ticks (rows 1-25) or ≥15 adult ticks per host (rows 26-37).

^b Domestic dogs diagnosed with canine babesiosis (a tick-borne disease) in South Africa.

^c Collected every 2 to 4 weeks from 4 dogs over 3 years; single collection from 50 caracals and 2 leopards.

^d Data is reported as mean number of ticks per host.