Invasive woody plants as foci of tick-borne pathogens: eastern redcedar in the southern Great Plains

Bruce H. Noden[1][✉], Evan P. Tanner[2,3], John A. Polo[3], and Sam D. Fuhlendorf[3]

[1]*Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, OK 74078, U.S.A.*
bruce.noden@okstate.edu
[2]*Caesar Kleberg Wildlife Research Institute, Texas A&M University-Kingsville, Kingsville, TX 78363, U.S.A.*
[3]*Department of Natural Resource and Ecology Management, Oklahoma State University, Stillwater, OK 74078, U.S.A.*

ABSTRACT: Habitat preference and usage by disease vectors are directly correlated with landscapes often undergoing anthropogenic environmental change. A predominant type of land use change occurring in the United States is the expansion of native and non-native woody plant species in grasslands, but little is known regarding the impact of this expansion on regional vector-borne disease transmission. In this study, we focused on the impact of expanding eastern redcedar (*Juniperus virginiana*; ERC) and tested two hypotheses involving relationships between habitat preferences of adult tick species in rural habitats in central Oklahoma. Using CO_2 traps, we collected ticks from two densities of ERC and grassland and screened adult ticks for the presence of pathogen DNA. We found support for our first hypothesis with significantly more *Amblyomma americanum* (Linnaeus) and *Dermacentor variabilis* (Say) collected in ERC habitats than in grassland. Our second hypothesis was also supported, as *Ehrlichia*- and *Rickettsia*-infected *A. americanum* were significantly more likely to be collected from ERC habitats than grassland. As the first evidence that links woody plant encroachment with important tick-borne pathogens in the continental United States, these results have important ramifications involving human and companion animal risk for encountering pathogen-infected ticks in the southern Great Plains. *Journal of Vector Ecology* **46 (1): 12-18. 2021.**

INTRODUCTION

Vector-borne pathogen transmission can only occur when competent arthropods (mosquitoes, ticks, fleas) interact with infectious reservoir hosts and susceptible hosts in specific environments (i.e., the 'nidus of infection' (Reisen 2010) or 'nidality' (Pavlovsky 1966)). While habitat preference and usage by disease vectors are directly correlated with landscapes, these habitats are often undergoing anthropogenic environmental changes which influence the distribution of vector species and the potential transmission of vector-borne pathogens (Foley et al. 2005, Reisen 2010, Kilpatrick 2011, Allen et al. 2017, Hassell et al. 2017, Noden and Dubie 2017). One of the predominant types of land use change occurring in the United States is the expansion of native and non-native woody plant species in grasslands and urban green spaces (i.e., woody plant encroachment; WPE) (Engle et al. 2008, Nackley et al. 2017, Stevens et al. 2017). Driven by changes in ecological processes facilitated by novel land management practices, landscape fragmentation, and climate change (Nackley et al. 2017, Scholtz et al. 2018, Wilcox et al. 2018), WPE changes abiotic conditions (e.g., temperature, moisture), large scale vegetation cover, and diversity and species composition of arthropods and wildlife (Ratajczak et al. 2012, Archer et al. 2017, Acharya et al. 2018).

One understudied consequence of WPE is its potential underlying role in influencing the nidi of transmission for vector-borne diseases. For example, in the southern Great Plains (Oklahoma and Kansas), a primary encroaching plant species is the eastern redcedar (*Juniperus virginiana*; ERC [Zou et al. 2018]). Expanding at rates of $40km^2$ per year (Wang et al. 2018), ERC is increasing throughout the Great Plains region (Zou et al. 2018). Growing in height at a steady rate of 0.5m/ year (Engle and Kulbeth 1992), invading trees form dense, closed canopy woodland areas (Engle et al. 2008, Wang et al. 2017, 2018). As ERC encroachment substantially changes abiotic conditions (e.g., temperature, humidity) and water cycling (Williams et al. 2013, Caterina et al. 2014, Acharya et al. 2017), it dramatically influences the abundance, diversity, and species composition of plants, arthropods, and wildlife at both local and regional spatial extents (Coppedge et al. 2001, Horncastle et al. 2005, Frost and Powell 2011, O'Brien and Reiskind 2013). Studies have demonstrated that ERC provides habitat for West Nile virus-infected mosquitoes (Noden and Cote, unpublished data), expanding populations of important tick species (Noden and Dubie 2017), and visual flying cues for horse flies (K. Sherrill, personal communication).

The impact of tick-borne pathogens on human and animal health is becoming better understood in the U.S. (Rosenberg et al. 2018) with the southern Great Plains having a high cumulative incidence of spotted fever rickettsiosis and ehrlichiosis (Biggs et al. 2016, Springer and Johnson 2018, Gettings et al. 2020). However, it remains understudied with regard to the ecology of tick vectors and hosts and their associations with landscape change (Noden and Dubie 2017). *Amblyomma americanum*, a tick species that transmits a wide variety of pathogens (Goddard and Varela-Stokes 2009), has dramatically increased in distribution throughout the region

in recent decades (Barrett et al. 2015, Noden and Dubie 2017) while little is known regarding the ecology of other tick species (Mitcham et al. 2017). There is a significant gap in understanding how land cover influences tick vectors in the southern Great Plains and, specifically, how predominant forms of WPE contribute to the occurrence of a nidus of tick-borne pathogen transmission.

In this study, we tested two hypotheses involving relationships among habitat preferences of adult tick species in rural habitats in central Oklahoma. First, we tested whether ERC alters tick assemblages, favoring *Amblyomma americanum*, the principal transmitter of ehrlichiosis in central Oklahoma. Second, we tested whether ERC creates nidi of transmission, seen in higher likelihood of pathogen infection in ticks from ERC compared to other habitats. Based upon a previous study, we predicted that *A. americanum*, one of the principal tick vectors in Oklahoma (Heise et al. 2014, Noden et al. 2017), and *D. variabilis* would select ERC above other habitats while *Amblyomma maculatum* Koch would select grassland vegetation (Noden and Dubie 2017, Noden et al. 2020). We expanded the question to include two different densities of ERC to investigate whether levels of encroachment are important for tick distribution in a given location as well as the nidus of tick-borne pathogens.

MATERIALS AND METHODS

Field collections

Tick collections were carried out on three days between May and July, 2017 at a section of the Oklahoma State University Lake Carl Blackwell study unit in Payne County, OK, a site (0.25 km2) consisting of varying densities of ERC encroachment. We sampled ticks in three different habitats: closed canopy ERC (trees touching at least two other eastern redcedar trees), open ERC (single tree or only touching another ERC on one side), and open grassland (an area of grass at least 50 m from any tree) over a 625 m transect through the study area. Over three monthly sampling dates, three sites per habitat (total of nine sites per sampling date) were randomly chosen with each trap placed in a different site on each sampling date to ensure full recovery of ticks. Over the course of the study, 27 different sites (three sampling dates x three habitats x three sites/sampling date) (nine sites/habitat) were randomly sampled. Trapping for one h between 09:00 and 11:00 occurred using the CO_2 method which attracts ticks within a 3 m radius to a tape-outlined wooden board with dry ice (CO_2) in a centrally-located container, making it an ideal method for habitat monitoring (Koch 1987, Noden et al. 2017). After collection, we removed ticks stuck to the tape on the board and placed them into tubes containing 70% EtOH, and identified them to species, life stage, and sex using a dissecting microscope and established pictorial keys in the laboratory (Keirans and Litwak 1989, Keirans and Durden 1998, Dubie et al. 2017). Because *A. maculatum* in the United States is indistinguishable from *Amblyomma triste* (Lado et al. 2018), all *A. maculatum* references in this manuscript actually refer to the *A. maculatum* group.

Pathogen screening

We screened adult field-collected ticks for DNA for three *Ehrlichia* species and general *Rickettsia* species using established polymerase chain reaction (PCR) protocols (Trout Fryxell et al. 2017). Only adult ticks were tested due to their higher cumulative likelihood of carrying pathogens after two separate feeding events. To limit DNA contamination, all tick DNA extractions were conducted using site-specific reagents in a separate laboratory. All adult ticks were tested except *A. americanum* from three sites (two sites of closed canopy ERC [one in May and one in July] and one site of open ERC in July) from which 30% of both male and female ticks were randomly selected (due to high numbers collected) for testing to provide a valid estimate of prevalence. After washing individual adult ticks in de-ionized water and 70% ethanol, each tick was bisected and both halves were placed in a tube containing 20ul of Pro-K and 180 μl of Digestion solution provided by the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and the tubes were placed overnight at 56° C. DNA extraction occurred the following day following the manufacturer's instructions with extracted DNA stored at -20° C until testing.

Individual ticks were screened by endpoint PCR using previously published PCR protocols which targeted *Rickettsia sp.* (*gltA* [Labruna et al. 2004] and *ompA* [Eremeeva et al. 1994]) and the nested PCR assay targeting the 16S rRNA gene of *Ehrlichia* sp. (Dawson et al. 1996, Heise et al. 2010) and confirmed using the *groEL* gene of *Ehrlichia* and *Anaplasma sp.* (Tabara et al. 2007, Takano et al. 2009). Positive controls consisted of *R. rickettsii* DNA provided by Dr. William Nicholson (Ricketsial Zoonoses Branch, Centers for Disease Control and Prevention), and *E. chaffeensis* MO strain DNA provided by Dr. Susan Little (OSU School of Veterinary Science). All *Ehrlichia*-positive amplicons from the 16S rDNA assay and selected amplicons from the groEL assay and all *Rickettsia*-positive amplicons from *D. variabilis* and *A. maculatum* were bidirectionally sequenced at the Oklahoma State University Core Facility to confirm positivity and identify bacterial species. We verified each resulting sequence using BioEdit (Ibis Therapeutics, Carlsbad, CA, U.S.A.), aligned to create consensus sequences using Clustal Omega (EMBL-EBI, Cambridgeshire, UK) and divided into different groups based on sequence differences. We compared resulting consensus sequences with GenBank submissions using default conditions on NCBI BLAST where the highest % sequence identity was used to determine species similarity. However, no sequencing was performed on amplicon-positive *A. americanum* due to high numbers, limited funding, and the high probability of encountering *R. amblyommatis*, a *Rickettsia* species of unknown pathogenicity (Biggs et al. 2016), based on earlier studies in our lab (Noden et al. 2017, Mitcham et al. 2018).

Data analysis

The focus of the study was on the influence of varying densities of ERC on tick communities and tick-borne pathogens. First, we analyzed the influence of ERC habitats on the presence and mean abundance each species (*Am.*

americanum, *D. variabilis*, and *Amblyomma maculatum*). Statistical analysis was completed using SAS JMP Pro 15 (SAS Institute, Cary, NC, U.S.A.). Initially, we focused on the influence of ERC density on tick species presence using a likelihood ratio test. Next, we square-root transformed counts of each species to improve the assumptions of normality and homogeneity of variance and ran one-way ANOVA analyses, comparing the mean abundance of each species among the three habitat types. Tukey honest significant difference (HSD) (P < 0.05) was used to compare among habitat types. Because of the low numbers of replicates and infected ticks, we used a likelihood ratio test to evaluate differences among habitat types and the presence of pathogen-infected ticks among differing habitat types.

RESULTS

Trap collections

Of the 676 adult ticks collected on three trapping dates, 655 (96.9%) were *A. americanum,* followed by 10 (1.5%) and 11 (1.6%) *D. variabilis* and *A. maculatum*, respectively. ERC habitat significantly impacted the presence of all three tick species. The majority of *A. americanum* were collected in closed canopy ERC (66.7%), followed by open ERC (30.8%), with the fewest in grassland (2.4%; Likelihood ratio: χ^2=13.51, P<0.0012). All *D. variabilis* were collected in ERC (Likelihood ratio: χ^2=4.60, P<0.0319), while the majority of *A. maculatum* (81.8%) were collected in grassland habitat (Likelihood ratio: χ^2=9.0, P<0.0111). ERC habitat also impacted the abundance of all three tick species. Mean trap abundance of *A. americanum* differed significantly by canopy (F ratio=10.14, df=2, P<0.0006), with significant differences between mean tick abundances in closed canopy ERC (sqrt mean= 6.2/trap) (P<0.0004) and open ERC (sqrt mean=4.0/trap)(P<0.0367) compared with grassland (sqrt mean=0.8/trap). Mean trap abundance of *D. variabilis* did not differ

significantly overall by canopy (F ratio=3.20, df=2, P<0.0587), but there were significant differences between mean tick abundances in grassland (sqrt mean=0.0/trap) and closed canopy ERC (sqrt mean=0.6/trap) (P<0.0489). Mean trap abundance of *A. maculatum* differed significantly by canopy (F ratio=5.22, df=2, P<0.0131), with significant differences between mean tick abundances in grassland (sqrt mean=0.7/trap) (P<0.0116) compared with closed canopy ERC (sqrt mean=0.0/trap).

Pathogen prevalence

Prevalence rates for *E. ewingii* (3.8%), *E. chaffeensis* (1.2%), and Panola Mountain Ehrlichia (0.5%) were determined from 423 individual *A. americanum* collected from different habitats (Table 2). All sequences for 16 *E. ewingii*, five *E. chaffeensis*, and two Panola Mountain Ehrlichia-infected ticks had 100% identity with published sequences (*E. ewingii* accession numbers: MN336353, KJ907744); (*E. chaffeensis* accession number: NR_074500); (Panola Mountain Ehrlichia accession number: HQ658904.1). Prevalence rates for pathogen presence differed by habitat type among tick species. The five *E. chaffeensis*-infected *A. americanum* were collected from two closed canopy ERC sites while the two Panola Mountain Ehrlichia-infected *A. americanum* were collected in ERC (one closed and one open); all were collected in July. In agreement with these trends, there was a significantly greater likelihood of collecting *E. ewingii*-infected *A. americanum* ticks in closed canopy ERC (7/9 sites – 77.8%) compared with open ERC (0/9 sites) or grassland (1/9 – 11.1%) (Likelihood ratio: ^{2}=17.002, P<0.0002).

Rickettsia prevalence rates differed among *A. americanum* (63/423 [14.9%]), *D. variabilis* (3/9 [33.3%]), and *A. maculatum* (7/11 [63.6%]). All sequences for three *R. rhipicephali*-infected *D. variabilis* and seven *Candidatus* R. andeanae-infected *A. maculatum* had 100% identity with published sequences (*R. rhipicephali* accession number

Table 1. Total numbers of adult ticks collected by species in three habitats (closed and open eastern redcedar [ERC] and grassland) in central Oklahoma, 2017.

		A. americanum				*D. variabilis*				*A. maculatum*			
		May	June	July	Total	May	June	July	Total	May	June	July	Total
Closed ERC	Site 1	69	12	49	130	2	1	0	3	0	0	0	0
	Site 2	34	10	54	98	0	0	0	0	0	0	0	0
	Site 3	20	9	180	209	1	0	3	4	0	0	0	0
	Total	123	31	283	437	3	1	3	7	0	0	0	0
Open ERC	Site 1	5	19	7	31	0	0	0	0	0	0	0	0
	Site 2	38	37	3	78	0	1	0	1	1	1	0	2
	Site 3	2	5	86	93	0	1	1	2	0	0	0	0
	Total	45	61	96	202	0	2	1	3	1	1	0	2
Grass	Site 1	5	0	0	5	0	0	0	0	0	1	1	2
	Site 2	6	0	0	6	0	0	0	0	0	3	2	5
	Site 3	4	1	0	5	0	0	0	0	0	2	0	2
	Total	15	1	0	16	0	0	0	0	0	6	3	9

CP003342 and *Candidatus* R. andeanae accession number MG887826E). There was a significantly greater likelihood of collecting *Rickettsia*-infected *A. americanum* from closed canopy ERC (9/9 sites – 100%) and open ERC (8/9 sites – 88.9%) compared with grassland (3/9 sites – 33.3%) (Likelihood ratio: χ^2=13.167, P<0.0014). Conversely, *A. maculatum* collected from grassland (5/9 sites – 55.6%) were significantly more likely to be *Candidatus* R. andeanae-infected than those collected from closed canopy ERC (0/9 sites) and open ERC (1/9 sites – 11.1%) (Likelihood ratio: χ^2=9.96, P<0.0069).

DISCUSSION

We found that invasive ERC altered tick assemblages, particularly of *A. americanum* in central Oklahoma, with significantly more adult *A. americanum* collected per trapping event in closed canopy ERC compared with grassland or open ERC habitat. This same relationship was observed for *D. variabilis* but not for *A. maculatum*, which was associated with grassland habitat. The importance of this preference was augmented by the higher likelihood of encountering *Ehlrlichia*-infected and *Rickettsia*-infected *A. americanum* in closed canopy ERC habitats. This is the first evidence that links WPE with important tick-borne pathogens in the

Table 2. Prevalence of three *Ehrlichia* species in ticks collected from different habitats (eastern redcedar [ERC] and grassland) in central Oklahoma, 2017.

Species	Habitat	Sites	May # positives	May # tested	May % prevalence	June # positives	June # tested	June % prevalence	July # positives	July # tested	July % prevalence	Total prevalence
Ehrlichia ewingii	Closed ERC	Site 1	2	25	8.0	2	13	15.4	0	49	0.0	
		Site 2	3	34	8.8	0	10	0.0	2	54	3.7	
		Site 3	2	20	10.0	2	9	22.2	2	53	3.8	
		Total	7	79	**8.9**	4	32	**12.5**	4	156	**2.6**	**5.6**
	Open ERC	Site 1	0	5	0.0	0	19	0.0	0	7	0.0	
		Site 2	0	38	0.0	0	37	0.0	0	3	0.0	
		Site 3	0	2	0.0	0	5	0.0	0	24	0.0	
		Total	0	45	**0.0**	0	61	**0.0**	0	34	**0.0**	**0.0**
	Grass	Site 1	0	5	0.0	0	0	0.0	0	0	0.0	
		Site 2	1	6	16.7	0	0	0.0	0	0	0.0	
		Site 3	0	4	0.0	0	1	0.0	0	0	0.0	
		Total	1	15	**6.7**	0	1	**0.0**	0	0	**0.0**	**6.3**
Ehrlichia chaffeensis	Closed ERC	Site 1	0	25	0.0	0	13	0.0	3	49	6.1	
		Site 2	0	34	0.0	0	10	0.0	2	54	3.7	
		Site 3	0	20	0.0	0	9	0.0	0	53	0.0	
		Total	0	79	**0.0**	0	32	**0.0**	5	156	**3.2**	**1.9**
	Open ERC	Site 1	0	5	0.0	0	19	0.0	0	7	0.0	
		Site 2	0	38	0.0	0	37	0.0	0	3	0.0	
		Site 3	0	2	0.0	0	5	0.0	0	24	0.0	
		Total	0	45	**0.0**	0	61	**0.0**	0	34	**0.0**	**0.0**
	Grass	Site 1	0	5	0.0	0	0	0.0	0	0	0.0	
		Site 2	0	6	0.0	0	0	0.0	0	0	0.0	
		Site 3	0	4	0.0	0	1	0.0	0	0	0.0	
		Total	0	15	**0.0**	0	1	**0.0**	0	0	**0.0**	**0.0**
Panola Mountain *Ehrlichia*	Closed ERC	Site 1	0	25	0.0	0	13	0.0	0	49	0.0	
		Site 2	0	34	0.0	0	10	0.0	0	54	0.0	
		Site 3	0	20	0.0	0	9	0.0	1	53	1.9	
		Total	0	79	**0.0**	0	32	**0.0**	1	156	**0.6**	**0.4**
	Open ERC	Site 1	0	5	0.0	0	19	0.0	0	7	0.0	
		Site 2	0	38	0.0	0	37	0.0	0	3	0.0	
		Site 3	0	2	0.0	0	5	0.0	1	24	4.2	
		Total	0	45	**0.0**	0	61	**0.0**	1	34	**2.9**	**0.7**
	Grass	Site 1	0	5	0.0	0	0	0.0	0	0	0.0	
		Site 2	0	6	0.0	0	0	0.0	0	0	0.0	
		Site 3	0	0	0.0	0	0	0.0	0	0	0.0	
		Total	0	11	**0.0**	0	0	**0.0**	0	0	**0.0**	**0.0**

continental United States in a region impacted by ehrlichiosis and spotted fever group rickettsiosis (Drexler et al. 2016, Heitman et al. 2016, Rosenberg et al. 2018, Springer and Johnson 2018, Gettings et al. 2020). These results have important ramifications involving human and companion animal risk for encountering pathogen-infected ticks in the southern Great Plains. The widespread scale that WPE is occurring in the United States (e.g., ≥330 million ha affected in the U.S.) and its complete transformation of ecosystems (e.g., from grassland to forest) sets it apart from small-scale woody cover changes (e.g., shrub invasions in forests) or land use change in former agricultural areas which also impact tick-borne pathogen transmission (Allan et al. 2010, Mierzejewska et al. 2015, Bajer et al. 2017, Adalsteinsson et al. 2018).

Tick species are highly influenced by the habitats in which they encounter hosts and survive moisture deficits (Goddard and Varela-Stokes 2009). This study demonstrated that significantly more *A. americanum*, a tick normally associated with humid litter habitat, are found in closed canopy ERC compared with open ERC or grassland habitat. Grasslands can functionally change and transition to woodlands at low thresholds of woody cover (~18.5% [Loehle et al. 1996]), illustrating the highly dynamic systems in which these tick species occur. As ERC encroachment occurs in pasture ecosystems, micro-abiotic changes in temperature and humidity initiate changes in water cycling, including water infiltration, runoff, and storage in the soil (Williams et al. 2013, Caterina et al. 2014, Acharya et al. 2017). The high water content of eastern redcedar litter enables moisture-dependent ticks to survive in places where they have not been previously collected (Noden and Dubie 2017). These changes in micro-climate directly impact the plants, wildlife, and livestock who use it for shelter from wind and extreme heat, thus providing hosts for blood meals to continue tick life cycles.

This study also demonstrated that pathogen-infected ticks are also more likely encountered in particular habitats with a higher likelihood of encountering *E. ewingii*-infected and *Rickettsia*-infected *A. americanum* in closed canopy ERC compared with open ERC and grassland habitats. Often, tick-borne pathogens are distributed in clusters in a given landscape as the specific tick species associates with a habitat that provides refuge for the vertebrate pathogen-reservoir host (Eisen and Eisen 2008, Estrada-Pena et al. 2013). White-tailed deer (*Odocoileus virginianus*), a mammalian host for *E. ewingii* (Yabsley et al. 2002), uses ERC throughout central and western Oklahoma (Gee et al. 1991), thereby linking the WPE with the presence of a specific tick-borne pathogen. While recognizing the specific nature of the habitat question posed in this study, future work is needed to compare tick abundance and pathogen presence in differing habitats in the central cross-timbers region as well as habitat distribution in arid, western Oklahoma where ERC is expanding.

This habitat-focused study identified that closed canopy ERC habitat plays a role in the nidus of infection for specific tick species in central Oklahoma. While limited in time and scope as well as using a collection method that mainly attracts *A. americanum* (Koch et al. 1987), this study

initiated a process of inquiry into specific environmental and ecological aspects of ERC that may play a critical role in the harborage of *A. americanum* and *D. variabilis* ticks in the southern Great Plains. The connection of tick-borne disease with invasive plants suggests the importance of land management working with public health programs to benefit human and animal health. The results indicate a need for targeted extension and outreach activities to educate specific segments of the general population, as well as livestock producers, regarding the benefits of ERC removal in rural and urban areas. Future studies are needed to expand the scope of study to include other plant types as well as varying geographical areas in the Great Plains where ERC invasion is occurring (Zou et al. 2018), in addition to whether ERC provides a refugia that enables activity in the winter months.

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