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Original Article

Detection of *Trichinella murrelli* and *Trichinella pseudospiralis* in bobcats (*Lynx rufus*) from Oklahoma

Mason V. Reichard [a,*], Tiana L. Sanders [a], Natasha L. Prentiss [a], Stacy R. Cotey [b], Ryan W. Koch [c], W. Sue Fairbanks [d], Maria Interisano [e], Giuseppe La Rosa [e], Edoardo Pozio [e]

[a] Department of Veterinary Pathobiology, College of Veterinary Medicine, Oklahoma State University, Stillwater, OK, USA
[b] College of Forest Resources and Environmental Science, Michigan Tech University, Houghton, MI, USA
[c] Department of Integrative Biology, Oklahoma State University, Stillwater, OK, USA
[d] Department of Natural Resource Ecology and Management, Division of Agricultural Science and Natural Resources, Oklahoma State University, Stillwater, OK, USA
[e] Department of Infectious Diseases, Unit of Foodborne and Neglected Parasitic Diseases, Istituto Superiore di Sanità, Rome, Italy

A B S T R A C T

Trichinella spp. infect wild carnivores throughout the world. We determined the prevalence and mean infection intensity of *Trichinella* spp. in bobcats (*Lynx rufus*) from 41 counties in Oklahoma (USA). Tongues from 306 bobcats were examined using artificial tissue digestion. The prevalence (95% confidence interval) of *Trichinella* spp. was 5.9% (3.7%–9.2%) in which 18 of the 301 bobcats were infected. Bobcats infected with *Trichinella* spp. were detected in 10 of the 41 (24.4%; 13.7%–39.5%) counties sampled. Although variable, a statistically significant difference was not detected in the prevalence of *Trichinella* spp. among counties where bobcats were collected. The mean (standard deviation) and median (range) infection intensity of *Trichinella* sp. larvae were 30.9 (39.8) and 9.6 (0.6–119.9) larvae per gram of tissue examined. Genotyping results demonstrated that 17 bobcats were infected with *T. murrelli* and one bobcat was infected with *T. pseudospiralis*. This is the first report of *T. pseudospiralis* in bobcats and in Oklahoma. These data suggest the bobcat, as an obligate carnivore, is likely an important host in maintaining *T. murrelli* sylvatic cycles in Oklahoma.

1. Introduction

Species of *Trichinella* infect a variety of vertebrate animals throughout most regions of the world. Found predominately in carnivores and omnivores, *Trichinella* species infect mammals, birds, and reptiles (Pozio, 2005). Historically, 9 species and 3 genotypes of *Trichinella* were recognized (Pozio and Zarlenga, 2013). A new species, *Trichinella chanchalensis* (T13), was described in 2020 from wolverines (*Gulo gulo*) in Canada (Sharma et al., 2020). While all species of *Trichinella* are considered zoonotic, today the risk of infection to humans in North America is considered low and rarely occurs (Murrell and Pozio, 2011). In the United States, *Trichinella spiralis* from ingestion of undercooked infected domestic pork was historically the most likely route of infection to humans (Zimmermann, 1970). Despite its rare occurrence (Casillas and Jones, 2017), current cases of human trichinellosis in the United States arise from ingestion of wild game (e.g. bear, cougar, wild pig) containing first-stage larvae of *Trichinella* not submitted to veterinary controls (Dworkin et al., 1996; Heaton et al., 2018; Wilson et al., 2015). Wild carnivores, while unlikely to be consumed, are key hosts in maintaining sylvatic cycles of *Trichinella* spp. (Gottstein et al., 2009).

The genus *Trichinella* is a monophyletic group of morphologically identical species divided into two clades: one that encapsulate within nurse-cells in host muscle tissue, and a second that does not encapsulate (Pozio et al., 2009; Pozio and Zarlenga, 2013; Zarlenga et al., 2006). In North America, species in the encapsulated clade include *T. spiralis* (T1), *T. nativa* (T2), *T. murelli* (T5), *Trichinella* genotype T6 (T6), and *T. chanchalensis* (T13). The non-encapsulated clade is represented by only *T. pseudospiralis* (T4) in North America. *Trichinella spiralis* is the most frequently encountered species in domestic and wild pigs and is transmitted from the wild to the domestic cycle and vice versa due to poor animal production practices (Hill et al., 2010; Pozio, 2014; Pozio and Murrell, 2006; Pozio and Zarlenga, 2013). The prevalence of *T. spiralis* infection in wild animals is greatly reduced in the absence of infected pigs, although even in the absence of a domestic focus, wild carnivores have been reported to remain as reservoirs of this parasite for decades (Hill et al., 2010; Oksanen et al., 2018; Rafter et al., 2005).

* Corresponding author at: 250 McElroy Hall, College of Veterinary Medicine, Oklahoma State University, Stillwater, OK 74078, USA.
E-mail address: mason.reichard@okstate.edu (M.V. Reichard).

Trichinella nativa, *Trichinella* genotype T6, and *T. chanchalensis* are freeze-tolerant species typically found in Arctic and sub-Arctic climates (Gajadhar and Forbes, 2010; Pozio, 2016a; Pozio and Zarlenga, 2013; Sharma et al., 2020). *Trichinella murrelli* lacks freeze resistance, has a very low infectivity for swine, and is widely distributed among wild carnivore hosts throughout temperate North America (Pozio and La Rosa, 2000; Pozio and Zarlenga, 2013). *Trichinella pseudospiralis* lacks freeze resistance and is capable of infecting both mammals and birds in a variety of climates and ecoregions (Pozio, 2016b).

The bobcat (*Lynx rufus*) is a medium-sized wild felid widely dispersed across North America. They range from central Mexico, north through the lower 48 United States, and into southern Canada (Young, 2017). In Oklahoma, bobcats occur throughout the state with population numbers increasing (Roberts and Crimmins, 2010). Like other wild and domestic felids, bobcats are obligate carnivores and must consume tissues of other animals to obtain essential amino acids (e.g., taurine) and vitamins (e.g. niacin) necessary for their survival. Bobcats use predation and scavenging strategies to obtain vertebrate prey elevating their risk of exposure to *Trichinella* spp. The purpose of the current study was to determine the prevalence and intensity of *Trichinella* spp. infection in bobcats in Oklahoma and their possible role as a *Trichinella* reservoir in the investigated region.

2. Materials and methods

2.1. Tongue collection and tissue digestion for Trichinella first-stage larvae

Bobcat tongues were collected from carcasses of legally hunted or trapped bobcats during winter 2018/2019. Tongues were removed, placed in individually labeled plastic zip-close bags, and frozen at −20 °C until processed for *Trichinella* spp. infection. Age, sex, and detailed location of collection other than county were not available for the bobcats tested. Individual bobcat tongues were tested for infection with *Trichinella* spp. by artificial digestion using similar methodology as Mayer-Scholl et al. (2017). Briefly, the superficial layer of the tongue that cannot be digested was removed and then approximately 5.0 g (to the nearest 0.1 g) of muscle tissue was weighed. The 5.0 g samples were blended with a commercial blender using a 250 mL glass jar. Blended samples were mixed with 10 mL of artificial digestive fluid (1% pepsin, 1:10,000 IU, and 1% hydrochloric acid) per 1.0 g of tissue. Digests were mixed vigorously on magnetic stir plates at 37 °C for 30 min. Then digests were immediately cooled on ice and allowed to settle for 20 min. Sediment was washed 3–5 times with tap water, by decanting supernatant, depending on the amount of cellular debris. Washing steps were performed in 50-mL centrifuge tubes. All sediment was examined for *Trichinella* larvae at 40× magnification using a stereomicroscope. The number of *Trichinella* sp. larvae were enumerated and results were recorded as the number of larvae per g (LPG) of tissue digested.

2.2. Molecular analysis

Trichinella sp. larvae recovered by artificial tissue digestion from bobcats were washed in saline, preserved in absolute ethyl alcohol, and submitted to the International *Trichinella* Reference Center (ITRC), Istituto Superiore di Sanità, Rome, Italy, for genotyping. Individual *Trichinella* sp. larvae were identified by multiplex polymerase chain reaction (PCR) analysis following the protocol described by Zarlenga et al. (1999) and modified by Pozio and La Rosa (2010). Five single larvae for each isolate were randomly selected for genotyping. If one or more of these larvae did not show any DNA amplification, additional larvae were randomly selected for genotyping. DNA was purified using a combination of the Tissue and Hair Extraction Kit (Promega) and the DNA IQ™ System Extraction Kit (Promega). The manufacturer's protocol was modified in using 20 μL as lysis buffer and 80 μL as washing volume. All DNAs from individual larva were eluted with 50 μL of elution buffer.

PCR amplifications were performed in 30 μL using a premixed 2× QIAGEN Multiplex PCR Master Mix (Qiagen). Two pmol/μL of each primer and 10 μL of purified DNA were used. The amplification was carried out for 35 cycles as follows: 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 30 s, plus a pre-step at 95 °C for 15 min and a post-step at 72 °C for 3 min. The PCRs were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystem, Foster City, CA, USA). Approximately 0.2 μL were used in a capillary electrophoresis analysis (Qiaxcel, Qiagen) to resolve the amplified products.

To provide controls for the DNA extraction procedure, a larva from the reference isolate of *T. spiralis* (code ISS3) was included in the analysis as an independent sample and 10 ng of DNA from a reference isolate of *T. britovi* (ISS2) were used as a control for PCR amplification.

2.3. Statistical analyses

The prevalence and intensity of *Trichinella* sp. infection was calculated according to Bush et al. (1997). Ninety-five percent confidence intervals (CI) were calculated using QuickCalcs (QuickCalcs, 2017). The prevalence of *Trichinella* spp. in bobcats with degrees of freedom (df) were compared using chi-square tests (Sokal and Rohlf, 1995) among counties in Oklahoma from which the hosts were collected. Kruskal-Wallis One Way ANOVA on Ranks (Sokal and Rohlf, 1995) was used to compare *Trichinella* sp. LPG from infected bobcats among counties. Chi-square and Mann-Whitney Rank Sum tests were performed using SigmaPlot statistical software (Systat Software Inc., San Jose, California, USA).

3. Results

### 3.1. Prevalence of Trichinella spp. in bobcats

A total of 306 bobcat tongues collected from 41 counties in Oklahoma were tested for infection with *Trichinella* spp. The overall prevalence (95% CI) of *Trichinella* spp. in bobcats was 5.9% (3.7%–9.2%). Infected bobcats occurred in a discontinuous line entailing 10 counties (24.4%; 13.7%–39.5%) extending from the southeastern corner of Oklahoma through the middle of the state (Fig. 1). While the prevalence of *Trichinella* spp. in bobcats varied among the counties, a significant difference ($X^2 = 52.714$, df = 40, $P = 0.086$) was not detected. Among *Trichinella* spp. infected bobcats (ST1), the median intensity of infection was 9.6 LPG, ranging from 0.6 LPG–119.9 LPG. There was not a significant difference (H = 9.982, df = 9, $P = 0.352$) in intensity of *Trichinella* sp. infections in bobcats among counties.

3.2. Trichinella genotyping

Banding patterns from multiplex PCR amplifications demonstrated that 17 bobcats (ST1) were infected with *T. murrelli* (ITRC codes: ISS7585, ISS7586, ISS7587, ISS7588, ISS7589, ISS7590, ISS7591, ISS7592, ISS7593, ISS7594, ISS7596, ISS7597, ISS7598, ISS7599, ISS7600, ISS7601, and ISS7602). One bobcat, from Le Flore County (ST1), was infected with *T. pseudospiralis* (ITRC code: ISS7595). No mixed infections of *Trichinella* spp. were detected in any of the bobcats sampled.

4. Discussion

Previous reports of *Trichinella* spp. occurrence in bobcats include British Columbia, Colorado, Georgia, Idaho, Montana, Nova Scotia, South Dakota, and Wyoming (Table 1). Conversely, *Trichinella* spp. were not detected in 1 bobcat from Iowa (Zimmermann et al., 1962), 126 from Nova Scotia (Smith and Snowdon, 1988), and 69 from Texas (Pozio et al., 2001; Stone and Pence, 1978). Reports on the prevalence of *Trichinella* spp. in bobcats are highly variable and range from 0.0% to
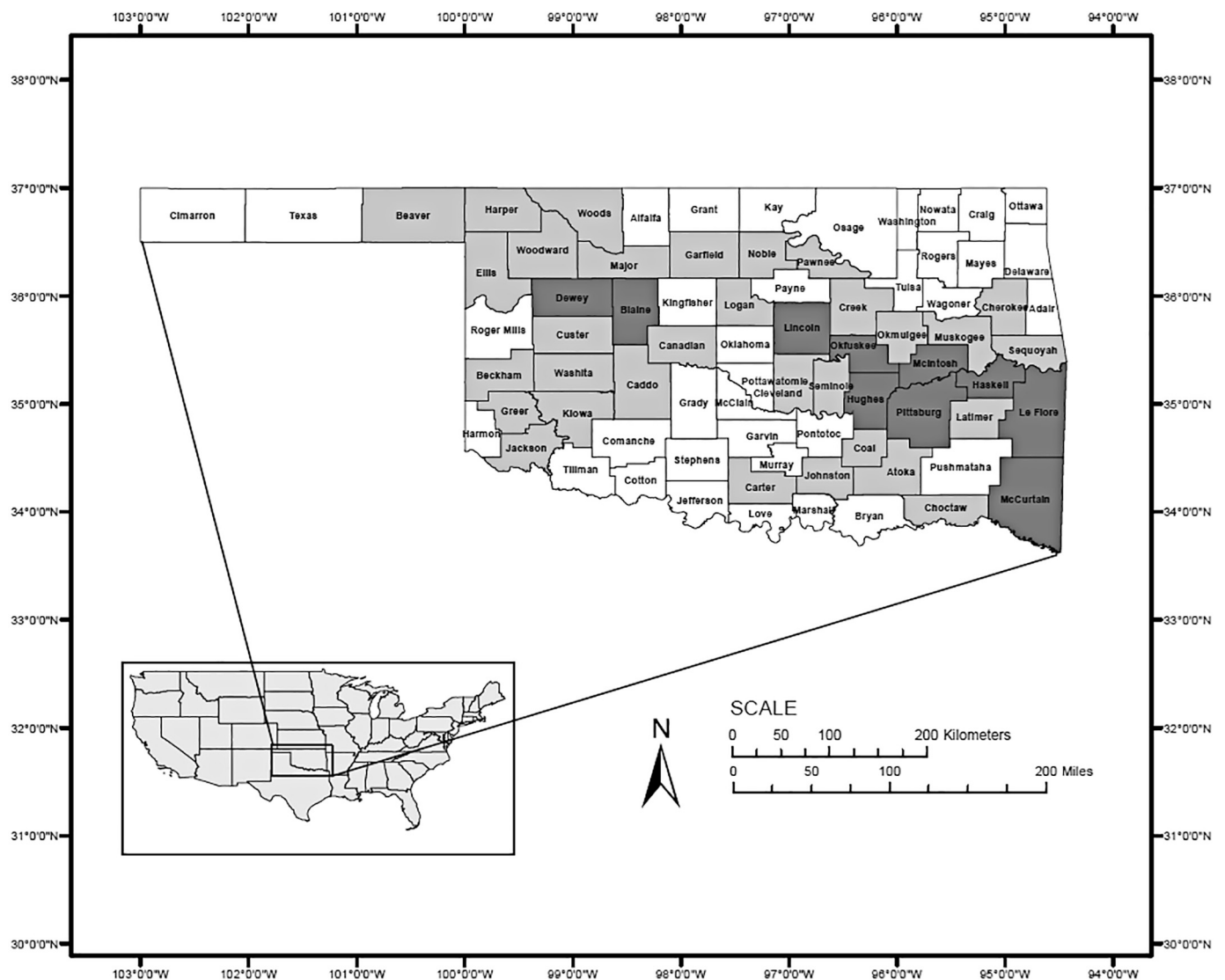
Fig. 1. Counties in Oklahoma where bobcats (*Lynx rufus*) were sampled during winter 2018/2019. Counties from which *Trichinella* spp. were detected are shaded in dark gray. Counties from which bobcats were sampled but infection not detected are shaded in light gray.

~17.0% (Table 1). Prevalence is a descriptive statistic often used as a point estimate (Bush et al., 1997) to express the general occurrence or frequency of a parasite within a population. Prevalence should be interpreted with caution as it is highly influenced by sampling strategy and the number of samples obtained from a population. Our data is biased in that we collected tongues from legally hunted or trapped animals. Nevertheless, an overall prevalence of 5.9% is comparable to what others have reported (Table 1). The intensity of *Trichinella* sp. infection in wild animals generally range from 0.1–10.0 LPG with intensities >50.0–100.0 LPG exceptional (Dick and Pozio, 2001). We report an overall mean of 30.9 LPG and median of 9.6 LPG that ranged from 0.6–119.9 LPG. Our data, along with those of reported previously on the mean intensity of *Trichinella* spp. in bobcats (Table 1), suggest a similar worm burden across the range of this host.

Reports of *Trichinella* spp. from the 77 counties of Oklahoma are limited. In humans, infection with *Trichinella* spp. has been rare with only two cases reported since 1989 (Graves et al., 1996; McAuley et al., 1991). In wild animals, 6 of 425 (1.4%) feral pigs (*Sus scrofa*) sampled from 2006 to 2010 had antibodies to *Trichinella* spp. (Hill et al., 2014). *Trichinella* sp. larvae were recovered in coyotes (*Canis latrans*) collected from Creek county (4 of 6), and Okmulgee county (1 of 33; Reichard et al., 2011). However, bobcat samples also collected and tested from

these two counties yielded no *Trichinella* spp. larvae. The current study is limited in that only a small portion of frozen tongues from bobcats were available for *Trichinella* spp. testing. Additional samples from other striated muscles (e.g., diaphragm, masseter, intercostal muscles) could have revealed additional positive samples. Similarly, all tongues were frozen to prevent decomposition prior to being tested for *Trichinella* spp. infection. As such, the freezing/thawing process kill the freezing susceptible species, such as *T. murrelli* and *T. pseudospiralis*, causing possible damage to the cuticle of larvae and consequently, leakage of cellular content. It is also possible that *Trichinella* spp. killed by freezing may sediment more slowly, further reducing the sensitivity of their detection. The problem is even more pronounced in the larvae of non-encapsulated species, in the present case *T. pseudospiralis*, as they are not protected by the collagen capsule, so that the artificial digestion has a longer time of action against the cuticle.

Regardless of genotype, the persistence of *Trichinella* larvae in putrefying flesh is also influenced by the environment: high humidity and low temperatures favor survival even when the muscle tissue is completely liquefied. This adaptive mechanism of survival is a biological character displayed by all taxa in the genus *Trichinella*; the survival in host carcasses is longer for the encapsulated than for the non-encapsulated species (Pozio, 2016a; Rossi et al., 2019). The climate of

Table 1

Prevalence and mean larvae per gram (LPG) intensity of *Trichinella* spp. in bobcats from North America.

Location	*Trichinella* spp.	No. Positive/tested (%)	Reference
British Columbia, Canada	*Trichinella* spp.	1/1 (100%)	Smith and Snowdon (1988)
Colorado, USA	*Trichinella* spp.	4/394 (1.0%)	Olsen (1960)
Georgia, USA	*T. murrelli*	Reported, no numbers listed[a]	Dick and Pozio (2001), Pozio and La Rosa (2000)
Idaho, Montana, Wyoming, USA	*T. spiralis*[b]	5/29 (17.2%)[c]	Worley et al. (1974)
Montana, USA	*T. spiralis*[b]	Reported, no numbers listed[a]	Dick and Pozio (2001)
Nova Scotia, Canada	*Trichinella* sp.	1/24 (4.2%)[d]	Gajadhar and Forbes (2010)
Oklahoma, USA	*T. murrelli*	17/306 (5.6%)[e]	Current study
Oklahoma, USA	*T. pseudospiralis*	1/306 (0.3%)[f]	Current study
South Dakota, USA	*T. spiralis*[b]	1/153 (0.7%)	Schitosky and Linder (1981)
Southern Canada	*T. nativa* or *Trichinella* T6	Reported, no numbers listed	Dick and Pozio (2001)

[a] Reported as isolate obtained but number of positive/tested bobcats not reported.

[b] Larvae were identified as *Trichinella spiralis* before the multispecies concept of the genus *Trichinella*.

[c] Mean LPG 36.4, range 0–351.

[d] 0.04 LPG.

[e] Mean LPG 32.6, range 0.6–119.9.

[f] LPG 2.7.

Oklahoma ranges from humid subtropical (Köppen-Geiger Climate Classification = Cfa) in the east to semi-arid (Köppen-Geiger Climate Classification = BSk) in the west (Weather Atlas, 2020; Kottek et al., 2006). All infected bobcats in the current study originated from the Cfa climate zone, where high humidity favors survival of *Trichinella* sp. larvae in host carrions compared to the semi-arid climate. However, the number of bobcats sampled from the Cfa and BSk climates were not equal as only 17 of the 306 bobcats sampled were from the arid climate of Beaver, Ellis, and Harper counties (Fig. 1). This difference in respective samplings from Cfa and BSk climates could also reflect the distribution of bobcats in these two ecoregions. The topography of Oklahoma is relatively flat with an average elevation of 366 m that ranges from 87 to 1516 m (Johnson and Luza, 2008). Bobcats infected with *Trichinella* spp. were recovered from 8 of 10 counties in ecoregions at elevations of 305 m or lower. Elevation of the other two counties, Dewey and Blaine, where *Trichinella* spp. were detected was 610 m. Oklahoma landscape is comprised of vast plains, elevated karst plateaus, and folded, low mountains that are divided into 12, level III ecoregions (Woods et al., 2005). In the current study, bobcats with *Trichinella* spp. were found in 6 of the 12 ecoregions that comprise the state (Fig. 1).

Bobcats can be found in any Oklahoma county. The home range of bobcats in Oklahoma is variable and has been reported to be from 7.3–28.5 km^2 for females and 17.1–72.1 km^2 for males (Rolley, 1983). Estimates of the sex and age structure based on carcasses collected from hunted and trapped bobcats in Oklahoma suggested a sex ratio of 50:50 with a mean age of 2.3 yr (Rolley, 1983; Rolley, 1985). In Oklahoma, 5.5% of 549 bobcats were ≥ 6.5 yr (Rolley, 1983). Maximum age of wild bobcats is thought to be 16 yrs (Anderson and Lovallo, 2003; Knick et al., 1985). Unfortunately, age and sex data of bobcats tested for *Trichinella* spp. infection in the current study were not available. The diet of bobcats in Oklahoma consists mostly of rodents (e.g., *Sigmodon hispidus*, *Neotoma floridana*, *Peromyscus* spp., *Sciurus niger*), cotton-tail rabbits (*Sylvilagus floridanus*), and other small, wild vertebrates including birds and reptiles (Litvaitis, 1981; Rolley and Warde, 1985; Whittle, 1979). Since *T. murrelli* shows a high infectivity for *Peromyscus leucopus* and *Peromyscus maniculatus* (Yao et al., 1997), it can be hypothesized that the bobcat acquires infection by preying on these small rodents which in turn can

become infected through their scavenger activity even though their main diet is based on insects and plants.

Among sylvatic cycles, mammals with cannibalistic and scavenging behaviors (e.g., members of the families Canidae, Procyonidae, and Ursidae) host the majority of the *Trichinella* spp. biomass; however, others belonging to members of the families Felidae and Mustelidae can also be infected (Pozio, 2000). In addition to bobcats, other wild carnivores, scavengers, and omnivores known to occur in Oklahoma (Caire et al., 2019; American Society of Mammalogists, 2020; Shaughnessy Jr. and Cifelli, 2017) include: badger (*Taxidea taxus*), long-tailed weasel (*Mustela frenata*), mink (*M. vison*), river otter (*Lutra canadensis*), striped skunk (*Mephitis mephitis*), eastern spotted skunk (*Spilogale putorius*), western spotted skunk (*S. gracilis*), hog-nosed skunk (*Conepatus mesoleucus*), swift fox (*Vulpes velox*), red fox (*V. vulpes*), gray fox (*Urocyon cinereoargentus*), coyote, raccoon (*Procyon lotor*), ringtail (*Bassariscus astutus*), black bear (*Ursus americanus*), Virginia opossum (*Didelphis didelphis*), and feral pigs. Feral pigs can be infected with *T. pseudospiralis* (Gamble et al., 2005; Hill et al., 2014), but are not considered good hosts for *T. murrelli*. In Oklahoma, *T. murrelli* has been detected only in coyotes (Reichard et al., 2011), and bobcats (current study).

Apex mammalian predators that historically inhabited Oklahoma but have since been extirpated include the red wolf (*C. rufus*), gray wolf (*C. lupus*), and grizzly bear (*U. arctos*) (Caire et al., 2019). These large carnivores were likely host to *Trichinella* spp. in Oklahoma. Cougars (*Puma concolor cougar*) are rare in Oklahoma. The Oklahoma Department of Wildlife Conservation reports that there is no substantial evidence to support the existence of a viable population of cougars in Oklahoma (Oklahoma Department of Wildlife Conservation, 2020). The few cougars that have been found in Oklahoma were thought to be transient animals dispersing from other locations (Thompson and Jenks, 2005).

In the absence of large apex predators, extant mesocarnivores assume the role of *Trichinella* reservoirs and key species for epidemiological investigations on the circulation of these zoonotic pathogens (Gottstein et al., 2009; Roemer et al., 2009). Bobcats and coyotes are both considered generalists and can survive in a variety of habitats (Buskirk and Zielinski, 2003). However, bobcats are obligate carnivores whereas coyotes have a diverse diet that can include insects and fruits as well as vertebrate animals (Bekoff and Gese, 2003). In areas of sympatry, the two mesocarnivores rarely physically confront one another (Litvaitis and Harrison, 1989) and use similar resources independently (Neale and Sacks, 2001). Analysis of interference competition between the two indicate that predation by coyotes can be a source of bobcat mortality, which may (Henke and Bryant, 1999; Litvaitis, 1981; Nunley, 1978; Robinson, 1961) or may not (Fedriani et al., 2000) be negatively correlated with populations of the wild felid across different habitats. Regardless, coyotes are considered predators of bobcats (Anderson and Lovallo, 2003) with multiple reports of the wild canid killing the wild felid (Anderson, 1987; Gipson and Kamler, 2002; Jackson, 1986; Knick, 1990; Toweill, 1986); whereas the corollary is rare. In most cases of coyote attacks, the bobcats were relatively small specimens, such as adult females and juveniles. Cannibalism among bobcats is rare with only a few reported instances (Gashwiler et al., 1960; Litvaitis et al., 1982; Zezulak and Minta, 1987).

The majority of the *Trichinella* spp. recovered from bobcats in the current study were identified as *T. murrelli*. This finding was expected as *T. murrelli* is the predominant species found in wild carnivores in temperate North America (Pozio and La Rosa, 2000). Described in 2000, *T. murrellli* is an encapsulated, freeze-susceptible species that rarely infects domestic animals (Pozio and La Rosa, 2000). Previous reports of *T. murrelli* in animals from North America include black bear (Dubey et al., 2013; Hall et al., 2012; Pozio and La Rosa, 2000; Pozio et al., 2001), raccoon (Hill et al., 2008; Pozio and La Rosa, 2000; Scandrett et al., 2018), red fox (Pozio and La Rosa, 2000; Scandrett et al., 2018), mink (Pozio, 2000), coyote (Hill et al., 2008; Pozio and La Rosa, 2000; Pozio et al., 2001; Reichard et al., 2011), cougar (Gajadhar and Forbes,

2010; Reichard et al., 2017), domestic horse (*Equus caballus*; Pozio and La Rosa, 2000; Scandrett et al., 2018), and domestic dogs (*Canis familiaris*; Dubey et al., 2006).

The finding of one bobcat infected with *T. pseudospiralis* was unexpected, yet, not surprising. *Trichinella pseudospiralis* is an unencapsulated species that is found throughout the world and infects both mammals and birds (Pozio, 2016b). Before the advent of the multispecies concept and the use of molecular tools for the identification of *Trichinella* sp. muscle larvae at the species level (Zarlenga et al., 2020), nematode larvae resembling those of the genus *Trichinella* were detected in a pomarine jaeger (*Stercorarius pomarinus*; (Rausch et al., 1956), Cooper's hawk (*Accipiter cooperi*; Wheeldon et al., 1983), and great horned owl (*Bubo virginianus*; Zimmermann and Hubard, 1969) from North America, but their identification as *T. pseuospiralis* can be only suspected but not confirmed (Pozio, 2005). More recently, *T. pseudospiralis* has been identified by molecular tools in a black vulture (*Coragyps atratus*; Lindsay et al., 1995), wild boar (Gamble et al., 2005), cougars (Gajadhar and Forbes, 2010; Reichard et al., 2017), Florida panthers (*Puma concolor coryi*) (Reichard et al., 2015), and a wolverine (Sharma et al., 2019) from North America. To the best of our knowledge, this is the first report of *T. pseudospiralis* from Oklahoma and in bobcats.

Transmission of sylvatic *Trichinella* spp. occurs through predation and scavenging activities of vertebrate hosts. It is unclear what infected prey species bobcats are ingesting to become infected with *Trichinella* spp. Future studies should be conducted to investigate rodent prey species to elucidate the transmission cycle of *T. murrelli* in the region. Considering that bobcats are infected over several different counties in a diverse array of ecoregions and show a similar prevalence of infection as coyotes from the same region (Reichard et al., 2011), the wild felid likely plays an important role in maintaining sylvatic cycles of *T. murrelli* in Oklahoma.

Ethical statement

This research did not include any experimentation on animals.

Declaration of Competing Interest

All authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vprsr.2021.100609.

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