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RESEARCH ARTICLE

Tick-Borne Pathogens *Anaplasma phagocytophilum*, *Babesia odocoilei*, and *Borrelia burgdorferi* Sensu Lato in Blacklegged Ticks Widespread across Eastern Canada

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ABSTRACT

Blacklegged ticks, *Ixodes scapularis*, can transmit single or multiple infections during a tick bite. These tick-borne, zoonotic infections can become chronic and cause insidious diseases in patients. In the present tick-pathogen study, 138 (48.9%) of 282 ticks collected from 17 sites in 6 geographic area in eastern Canada harbored various combinations of *Borrelia burgdorferi* sensu lato (Lyme disease), *Anaplasma phagocytophilum* (human anaplasmosis), and *Babesia* spp. (human babesiosis). Overall, 167 microbial infections were detected and, of these, 25 ticks had co-infections and two ticks had polymicrobial infections. The prevalence of *Babesia* spp. was 15.2%, and the ratio of *Babesia odocoilei* to *Babesia microti* was 41 to 1 with this sole *B. microti* being detected in Nova Scotia. Notably, we provide the first documentation of *B. odocoilei* in the Maritimes. Eastern Ontario had an infection prevalence for *B. odocoilei* of 25%—the highest among the areas surveyed in this study. By far, the predominant *Babesia* sp. was *B. odocoilei*. Based on our findings, health-care practitioners need to recognize that *I. scapularis* ticks removed from patients may be carrying multiple tick-borne pathogens.

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- *Human anaplasmosis*
- *Babesia odocoilei*
- Human babesiosis
- Zoonosis

Introduction

Ticks are well known for carrying and transmitting microbial pathogens. Many of these tick-borne pathogenic microbes are of significant veterinary and medical importance, and can take a stark toll on chronic patients. East of the Rocky Mountains, the blacklegged tick, *Ixodes scapularis* (Acari: Ixodidae), harbors at least six microbial pathogens including *Borrelia burgdorferi* sensu lato (Bbsl) [1,2], *Borrelia miyamotoi* [3,4], *Babesia* spp. (Bspp) [5-7], *Anaplasma phagocytophilum* (Aph) [8-11], *Ehrlichia muris eauclairensis* [12], and Powassan Virus [13-15]. These pathogens can co-occur in ticks and be transmitted simultaneously during a tick bite [16,17].

The genus *Babesia* (Apicomplexa: Piroplasmida: Babesiidae) is a single-celled intraerythrocytic parasite that induces babesiosis in wildlife, domestic animals and humans continentwide [5,7,18-27]. Around the globe, *Babesia* spp. that are pathogenic to humans include *B. crassa*-like [28], *B. divergens* [29], *Babesia divergens*-like MO-1 [30], *B. duncani* [31], *B. microti* [32], *B. motasi* [33], *B. odocoilei*

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[7], *Babesia* sp. XXB/HangZhou [34], *Babesia* sp. TW1 [35], *Babesia* spp. CA1, CA3, and CA4 [36], and *B. venatorum* [37]. International travelers have occasion to return with any one of these *Babesia* spp.

Babesia species employ highly specific survival strategies during their intraerythrocytic development and their complex journey through the tick vector. Within the order Piroplasmida, researchers recognize at least 10 principal lineages [38]. *Babesia* sensu stricto (true *Babesia*) (clade X) embodies transovarial transmission–*B. odocoilei* (Bod) belongs to this group. On the other hand, *B. microti* belongs to *Babesia* sensu lato (clade I) which lacks transovarial transmission [38].

Since there are over 110 *Babesia* species worldwide [39], molecular identification, such as a combination of PCR, DNA sequencing, and Basic Local Alignment Search Tool (BLAST) analysis, is fundamental to confirm *Babesia* species. Using molecular identification, researchers have recently discovered *B. odocoilei* in red deer, *Cervus elaphus*, in the United Kingdom [40]. Some migratory songbirds (Order: Passeriformes) transport ticks hundreds of kilometres even to other continents and off-shore islands.

The intent of this study was to determine the prevalence and distribution of *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum*, and *Babesia odocoilei* in eastern Canada in *I. scapulars* obtained from vegetation and avian and mammalian vertebrates, including humans.

Material and Methods

Tick collections

Ixodes scapularis larvae, nymphs and adults were collected from avian and mammalian hosts using superfine-pointed, stainless steel forceps. These ticks were removed by bird banders, wildlife rehabilitators, veterinarians, biologists, and the public. Ticks from humans were removed and submitted directly by the individuals themselves. In addition, part of the *I. scapularis* adults were collected by flagging dry, low-lying vegetation during bimodal questing periods in spring and fall. For this study, we divided the eastern part of Canada into six geographic areas that consisted of three forest regions (i.e., Deciduous/Carolinian, Acadian, Great Lakes-St. Lawrence) totalling 17 collection sites. Wherever possible, fully engorged ticks were kept alive until they molted to the next life stage. All ticks were preserved in microcentrifuge tubes with 94% ethyl alcohol for further processing. All ticks were identified taxonomically using a previously established key [41].

Molecular detection of microbial pathogens

Ethanol storage medium was allowed to evaporate off whole ticks before cuticle disruption using a bead mill homogenizer (Fisher Scientific, Waltham, MA, USA) followed by genomic DNA extraction using the PureLink Genomic DNA Mini Kit (Invitrogen, Waltham, MA, USA) according to manufacturer's instructions with elution in 80 µL Genomic Elution Buffer.

Real-time PCR amplification was performed using 2 µL of extracted DNA in a 20 µL reaction of Taqman Fast Advanced Master Mix (Applied Biosystems) using published primers and probes for the 16S gene of *B. burgdorferi* sensu lato [42], and *msp2* gene of *A. phagocytophilum* [43]. The molecular *B. burgdorferi* s.l. probe specifically eliminates *B. miyamotoi* [42]. A pathogen was considered positively detected when the Cycle Threshold (CT) was less than 40 with a characteristic curve, and all positives were run in duplicate to reduce the possibility of false positives. Conventional PCR amplification for the 18S gene of *Babesia* spp. was performed and visualized on a 1% gel using methods and reagents as previously described [24]. All PCR reactions included molecular-grade water and synthetic gBlock gene fragments (Integrated DNA Technologies) of *B. burgdorferi* (MH781147.1), *A. phagocytophilum* (AY151054.1), and *B. microti* (MT974173.1) as controls.

PCR products were prepped for sequencing using either ExoSTAR-IT (Applied Biosystems) or PureLink Quick Gel Extraction Kit (Invitrogen). All sequencing was performed at the Genomics Shared Resource laboratory at the Comprehensive Cancer Center within the Ohio State University using forward and reverse primers [44,45]. Manual edits and alignments were performed in the program CLC Main Work bench v.21.0.3 (Qiagen) followed by Basic Local Alignment Search Tool (BLAST) in GenBank (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Tick collection

In total, 282 *I. scapularis* ticks (larvae, nymphs, adults) were selected from 286 ticks because four belonged to other species of ticks. The *I. scapularis* ticks (150 questing, 132 blood fed) were collected from six geographic areas consisting of 17 sites in eastern Canada, namely Nova Scotia, southwestern Ontario, eastern Ontario, northwestern Ontario, southeastern Manitoba, and southern Quebec. Tick collections began 01 April 2021 and continued until 28 November 2021.

Pathogen detection

Single and mixed infections were encountered in *I. scapularis* collected in eastern Canada. Overall, 138 (48.9%) of 282 ticks harbored one or more microorganisms (Table 1). Various combinations of *Borrelia burgdorferi* s.l., *A. phagocytophilum*, and *Babesia* spp. were either double or triple (Table 2). Overall, 167 microbial infections by geographic area were detected in 282 ticks (Table 3). Our data conveyed 111 single infections, 25 doubles (50 infections), and 2 triples (6 infections) (Table 2). The distribution of

Table 1: Detection of microorganisms in *Ixodes scapularis* ticks collected in eastern Canada, 2021.

Number of ticks with a microorganism	138 (48.9%)
Ticks with a single microorganism	111 (39.4%)
Ticks with multiple microorganisms	27 (9.6%)
Total surveyed ticks	282 ticks

Table 2: Polymicrobial infections in *Ixodes scapularis* ticks collected in eastern Canada, 2021.

Double	Bbsl/Aph 12
Double	Bbsl/Bspp 8
Double	Aph/Bspp 5
Triple	Bbsl/Aph/Bspp 2

Bbsl: *Borrelia burgdorferi* sensu lato; Aph; *Anaplasma phagocytophilum*; Bspp: *Babesia* spp.

Table 3: Detections of microbial pathogens in *Ixodes scapularis* collected within eastern Canada, 2021.

		Microbial infections (%)		
No. of Ticks	Region	Bbsl	Aph	*Babesia* spp.
137	Nova Scotia	46 (33.6)	37 (27.0)	20 (14.6)
30	Southwestern Ontario	4 (13.3)	1 (3.3)	3 (10.0)
56	Eastern Ontario	7 (12.5)	13 (23.2)	14 (25)
12	Northwestern Ontario	4 (33.3)	1 (8.3)	2 (16.7)
5	Southeastern Manitoba	2 (40)	0 (0)	0 (0)
42	Southern Quebec	2 (4.8)	7 (16.7)	4 (9.5)
282		65 (23)	57 (20.9)	43 (15.2)

Total microbial infections: 167

microbial infections by geographical regions is available in table 3 and figure 1.

The cursory itemization of *A. phagocytophilum* in *I. scapularis* ticks parasitizing avian or mammalian hosts was 24 dogs (71%), six songbirds (25%), and one human (4%).

In total, 43 babesial detections (41 *Babesia odocoilei*, 1 *Babesia microti*, 1 *Babesia* spp. 20-5A74) were detected in *I. scapularis* ticks.

An *I. scapularis* larva was collected from a Swainson's Thrush, a ground-frequenting songbird, on 22 Aug 2021 at Ste-Anne-de-Bellevue, Quebec; the fully engorged larva molted to a nymph in 32 d. This nymph, which followed the larva-nymph molt, was infected with *A. phagocytophilum*. To our knowledge, this phenomenon represents the first documentation of transstadial passage (larva to nymph) of *A. phagocytophilum* harbored by *I. scapularis* collected from a passerine bird.

Borrelia burgdorferi s.l. and *B. odocoilei* were detected in *I. scapularis* at Rondeau Provincial Park within the Deciduous Forest region. Questing adults of *I. scapularis* were collected on 01 November 2021 during fall, bimodal questing activity.

With the exception of southeastern Manitoba, which had a small collection of five *I. scapularis* adults, each of the 3 pathogens (i.e., Bbsl, Aph, Bspp) was detected in all areas (Table 3, Figure 1).

Discussion

This study contributes to the growing scientific evidence that *I. scapularis* harboring *B. burgdorferi* sensu lato, *Anaplasma phagocytophilum*, and *Babesia odocoilei* are widespread in eastern Canada. Migratory songbirds play a role in the widespread dispersal of juvenile *I. scapularis* harboring several tick-borne, zoonotic pathogens, including Bbsl, Aph, and Bod. When left untreated or inadequately treated, these zoonotic pathogens cause insidious and malevolent diseases, and are an ongoing public health risk, especially for those engaged in occupations or recreational activities with high tick exposure, such as outdoor enthusiasts and workers (i.e., mushroom collectors, hikers, hunters, loggers, and First Nations people) [46,47]. Epidemiologically, several ticks harbored mixed infections. In addition, we found that the *B. burgdorferi* s.l. was the most prevalent tick-borne zoonotic pathogen followed closely by *A. phagocytophilum* and *Babesia* spp. Since *Babesia* spp. are piroplasmids, and are treated with antiparasitics, we wanted to learn more about their

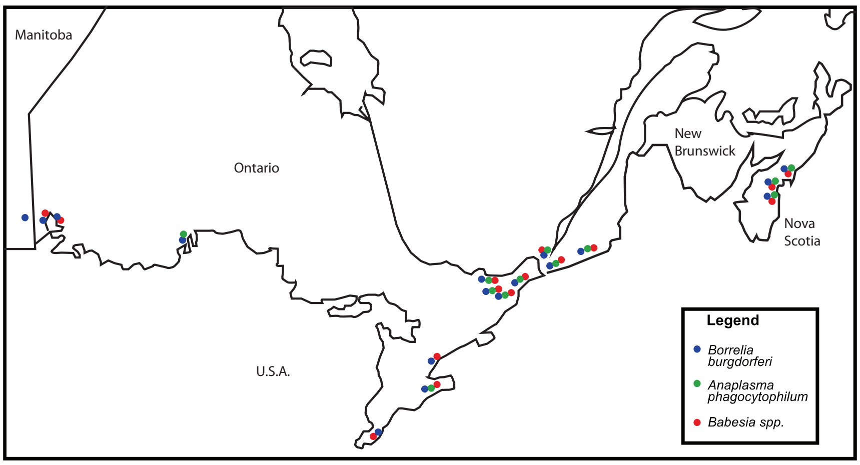

Figure 1 Map of eastern Canada showing locations where *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum, and Babesia* spp. were found.

prevalence and pathogenicity in eastern Canada. Because of the vast topography in eastern Canada, we spearheaded sampling where *I. scapularis* ticks are known to be present. Our sampling only encompassed a fraction of the total land area of eastern Canada. Markedly, polymicrobial infections were prominent in this ectoparasite study.

Enzootic transmission of *Babesia odocoilei*

When an *I. scapularis* feeds on a *B. odocoilei*-infected hosts (i.e., cervids, passerines), it has the potential to acquire *B. odocoilei*. During the blood meal, *B. odocoilei* is stored in the midgut lumen. Gametocytes change to kinetes (infective spores) in the midgut epithelium of the female, and then migrate to various organs (i.e., ovaries, salivary glands). When the *I. scapularis* female is mated, the kinetes-infected eggs are laid on the forest floor. When the gravid female lays a clutch of eggs in mid-summer, the kinete-infected eggs hatch to larvae. These newly laid eggs, which number approximately 1,000, start host-seeking within a day of hatching. This ecological situation poses a pronounced epidemiological risk to the public venturing into the outdoors during temperate weather.

Transovarial transmission occurs exclusively with *Babesia* sensu stricto (true *Babesia*) (Clade X), namely *B. odocoilei* [19,38,48]. Mated, replete *I. scapularis* females, which are infected with *B. odocoilei*, can perpetuate infection for generations–α unique, dynamic feature of the enzootic transmission cycle. In order to maintain the infective kinetes, transstadial passage (larva to nymph or nymph to adult) occurs during the molt. When an infective *I. scapularis* tick feeds on a host, kinetes change to sporozoites, and they directly infect red blood cells. In contrast, *B. microti*, a member of *Babesia* sensu lato (clade I), lacks the ability of transovarial transmission [38]. Epidemiological, the high prevalence of *B. odocoilei* presents a much greater threat in the environment than *B. microti*.

Adaptive strategies of *Anaplasma phagocytophilum*

In the present study, 59 (20.9%) of 167 infections were recorded as *A. phagocytophilum*, an obligate intracellular bacterial pathogen. This bacterium was the second-most prevalent pathogen. Whenever an *A. phagocytophilum*-infected *I. scapularis* parasitized a migratory songbird, expansion of this bacterium will vary depending range of local and migratory flight [49]. The fact that *A. phagocytophilm*-infected *I. scapularis* were removed from various avian and mammalian hosts (i.e., dogs, songbirds, human), shows that movement can be short- and long-distance [26]. In essence, *A. phagocytophilum* is a multi-host pathogen with highly adaptive strategies exibits a wide range of movement [50].

We provided the first documentation of transstadial passage (larva to nymph) of *A. phagocytophilum* in *I. scapularis* collected from a songbird. In addition, this bird parasitism suggests that the songbird (Swainson's Thrush, *Catharus ustulatus*) was infected with *A. phagocytophilum*, and this bird species is a competent host (Figure 2). This event also suggests transovarial transmission of *A. phagocytophilum* in *I. scapularis*. Our findings reiterate the fact that songbirds play an important role in the wide dispersal of *A. phagocytophilum* [26].

Figure 2 Swainson's Thrush, juvenile, parasitized by *Ixodes scapularis* larvae. One larva molted to a nymph *via* transstadial passage, and was infected with *A. phagocytophilum*. Photo: Ana Morales.

Babesia odocoilei discoveries

Babesia odocoilei is reported for the first time in each of these different geographic regions/areas, including Nova Scotia, south-central Quebec, Ottawa area, far-eastern Ontario, northwestern Ontario, and Rondeau Provincial Park. In particular, 3 (25%) of 12 *I. scapularis* adults collected in south-central Quebec were infected with *B. odocoilei*.

In the present tick-pathogen study, we found that the predominant *Babesia* sp. was *B. odocoilei*, and 15.2% of the *I. scapularis* ticks were infected with this piroplasmid. Our findings are congruent with prevalence results within the USA, such as Indiana (10.3%) [20], Wisconsin (11%) [51], and Pennsylvania (16.1%) [14]. In all of these apicomplexan studies, the predominant *Babesia* sp. was *B. odocoilei* (Figure 3). Although the majority of medical professionals have steered away from diagnosing and treating human babesiosis caused by *B. odocoilei*, a scant few have accepted the task [5,7]. Importantly, our study shows that *B. odocoilei* is present in the environment and infect humans. Explicitly, white-tailed deer, *Virginianus odocoileus*, are of reservoirs of *B. odocoilei* [19], and *I. scapularis* are vectors [5,7,18-26].

In southwestern Ontario, *B. odocoilei* was documented for the first time at Rondeau Provincial Park. We also detected Bbsl in *I. scapularis* adults questing in the park. Bbsl was previously reported in this established population of blacklegged ticks [52], and now, again, we document a significant prevalence of Bbsl in 4 (33%) of 12 *I. scapularis* adults which indicates that this breeding colony continues to be an endemic area for Lyme disease. Ecologically, we also provide substantive evidence that *Babesia* pathogens are present in this Deciduous/Carolinian region (Figure 3).

Figure 3 White-throated Sparrow, adult, parasitized by an *Ixodes scapularis* nymph that was infected with a *Babesia* pathogen. Photo: Nancy Furber.

In northwestern Ontario, *B. burgdorferi* s.l. infection prevalence (33.3%) was synonymous to Nova Scotia (33.6%). Researchers previously found an established population of *I. scapularis* on Corkscrew Island, located in Lake of the Woods, and it had a Bbsl prevalence of 73% [53].

Even though 43 *Babesia* detections were discovered, only a single *B. microti* was detected. The species ratio of *B. odocoilei* to *B. microti* was 41:1. Unequivocally, *B. odocoilei* is the predominant *Babesia* sp. among our samples. The sole *B. microti* was detected in a questing *I. scapularis* female at Lunenburg, Nova Scotia; this location is on the Atlantic flyway, and is situated within the Acadian region. Of note, researchers isolated *B. burgdorferi* s.l. from an *I. scapularis* nymph collected from a Common Yellowthroat, a ground-foraging songbird, at Bon Portage Island, Nova Scotia at a migratory stopover [54].

This transatlantic flight path would be one avenue for *B. odocoilei* to cross the Atlantic Ocean. The Northern Wheatear, *Oenanthe Oenanthe*, a small passerine bird has the flight potential to transport *B. odocoilei*-infected *I. scapularis* from northeastern Canada to Greenland to the United Kingdom and onward to Sub-Sahara Africa, and provide a naturally occurring, interconnecting link between the Eastern Hemisphere and the Western Hemisphere.

Consistent with our *Babesia* findings, only two *B. microti* piroplasmids were detected, stateside, in 299 *I. scapularis* adults tested in Pennsylvania [14]. Moreover, a Maryland study revealed that none of the 348 *I. scapularis* nymphs was positive for *B. microti* [55], and shows a clear-cut paucity of *B. microti*.

Pathogenic mechanism of *Babesia species*

Certain *Babesia* spp., such as *B. odocoilei*, have stealth-like survival mechanisms, including cytoadherence and sequestration [56]. During cytoadherence, *Babesia*-infected red blood cells adhere to endothelial cells. Whereas, during sequestration, self-perpetuating entanglements build up in capillaries, and occlude and restrict blow flow. These entanglements, which include uninfected and infected erythrocytes, hold themselves together configured by fibrin bonding. The smaller capillaries in the white matter of the brain promote cytoadherence and sequestration. Consequently, the occlusion of these tiny capillaries deprives the brain tissues of oxygen and nutrients. Tiny capillaries in the brain are especially affected. Such occlusions cause ischemia and inflammation and, thus, proliferate fatigue, impaired cognition and organ dysfunction. In addition, sequestration has been demonstrated in cats by *Babesia lengau* infection [57], *Babesia bovis* infection in cattle [58], and *Babesis canis* infection in dogs [59]. Sequestering *Babesia* species can complete their life cycle within capillary/venule entanglements, and dodge the spleen and be inert to the circulating immune system [56]. In essence, these microscopic morasses become trapped in the capillaries and, with time, capillaries turn out to be loaded with infected erythrocytes.

One tick-pathogen study in Ontario revealed that 20% of the adult *I. scapularis* ticks were infected with *B. odocoilei* [26]. In another study, researchers have found that *I. scapularis* adults collected from cats and dogs in the Huronia area of central Ontario had an infection prevalence of 71% for *B. odocoilei* [25]. In stark contrast, several researchers did not detect any *B. microti*, which is a non-sequestering *Babesia*, in Ontario and Quebec [5,21-26]. Human patients are being diagnosed and treated clinically for *B. odocoilei* infection in Ontario and Quebec [5,7]. Importantly, *B. microti* lacks transovarial transmission, and only maintains itself in *I. scapularis* for a maximum of one generation. On the other hand, *B. odocoilei*-infected *I. scapularis* can remain infective for generations without feeding on *B. odocoilei*-infected hosts. Not only is the *I. scapularis* larva (0.75 mm) hard to see, hordes of these infective larvae on the forest floor create a sobering epidemiological hazard to outdoor venturers and forest workers. In reality, *I. scapularis* adults will quest in the winter months when ambient temperatures are above freezing and there is no snow cover.

Pathologically, clinical symptoms of human babesiosis will be asymptomatic to severe, and may be fatal. Common symptoms of human babesiosis may include profound fatigue, increased thirst, digital numbness, sleep disturbance, cold intolerance, cognitive impairment, muscle aches (especially legs), loss of balance, inflammation, and intolerance to physical exertion [5,7]. In advanced cases, patients can have coma-like symptoms. Left untreated or inadequately treated, *B. odocoilei* infections are normally recalcitrant and persistent.

Conclusion

We provide the first authentic documentation of *B. odocoilei* in the Maritimes. Epidemiologically, *B. burgdorferi* s.l. was the most prevalence microbial pathogen followed closely by *A. phagocytophilum*, and ensued concomitantly by

B. odocoilei. Co-infections were most common, and clinically should be systematically investigated. We documented *Babesia spp.* in new regions, namely Nova Scotia, far-eastern Ontario, northwestern Ontario, and south-central Quebec. In eastern Canada, *B. burgdorferi* s.l., *A. phagocytophilum*, and *B. odocoilei* is prevalent, and nearly half of the *I. scapularis* ticks harbored zoonotic pathogens. Pointedly, ticks are nature's dirty syringes. The wide dispersal of *I. scapularis* infected with three microbial pathogens indicates that songbirds play an integral role in the dispersal of these zoonoses. With a ratio of *B. odocoilei* to *B. microti* of 41 to 1, *B. odocoilei* has been the predominant *Babesia* sp. in *I. scapularis* ticks. Grounded on our molecular findings, healthcare practitioners must be aware that any *I. scapularis* tick can have *A. phagocytophilum*, *B. odocoilei* and *B. burgdorferi s.l.* infections. Since *B. odocoilei* infections are new on the medical horizon, testing laboratories and clinicians must address this pathogen and its associated zoonosis with empirical and scholastic vigour.

Acknowledgments

Ethical consideration

Ethical approval to collect ticks from songbirds was granted directly to wildlife rehabilitators by Birds Canada, and veterinarians and wildlife rehabilitators are licensed to remove ticks from domestic and wildlife animals.

Authors' contributions

Conceptualization and design: JDS and RRP. Collection and methodology: JDS, EM and RRP. Formal analysis: JDS, EM and RRP. Drafting of manuscript: JDS and RRP. Accuracy of data analysis: JDS and RRP. All authors have read and agreed to the final manuscript.

Competing interests

The authors declare no conflict of interest.

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