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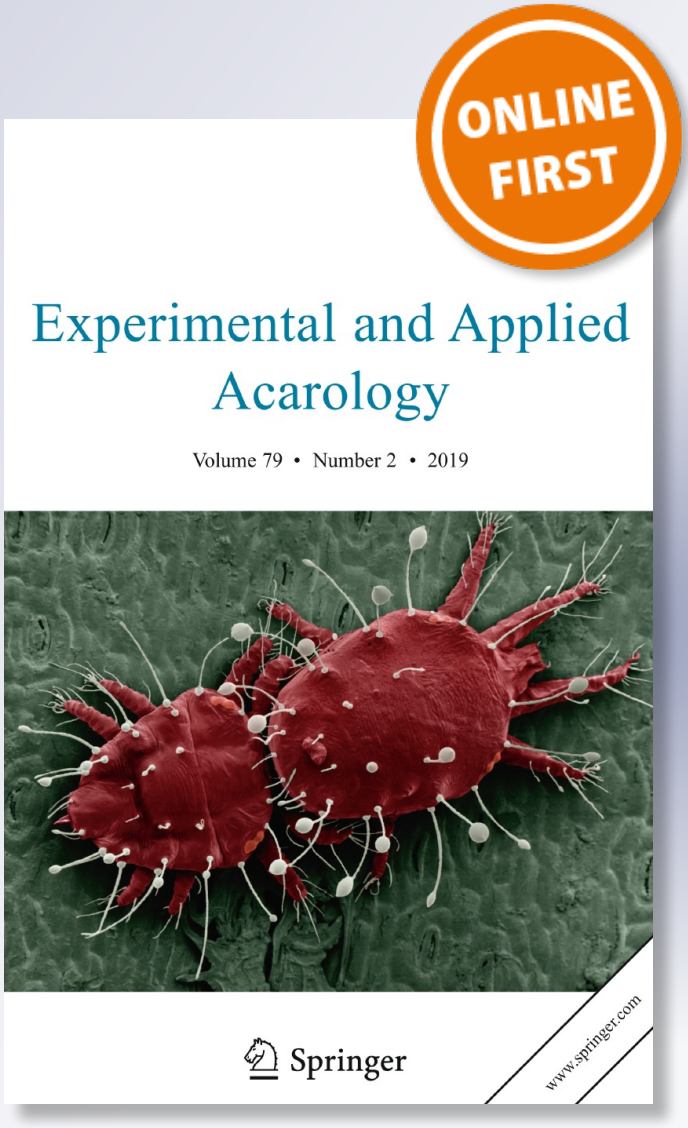

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A standardized method for the construction of a tick drag/flag sampling approach and evaluation of sampling efficacy

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Abstract
Drag sampling and flagging are two of the most effective and widely applied techniques to monitor tick populations. Despite the importance of this sampling strategy, there is a lack of standardized protocols for the construction of an inexpensive tick drag/flag. To this end, we provide a step-by-step protocol that details the construction of a tick drag/flag. We provide evidence of efficacy by comparing results obtained over 3-months at 108 locations within the William B. Bankhead National Forest, Alabama, USA. Overall, our drag/flag sampling approach yielded 1127 larvae, 460 nymphs, and 53 adults for a total of 1640 ticks representing three species. We detected significant patterns in *Amblyomma americanum* abundance for nymphs and adults with greater counts in June ($\beta = 0.91 \pm 0.36$, 95% CI 0.55–1.27; $\beta = 2.44 \pm 0.63$, 95% CI 1.81–3.07, respectively) and July ($\beta = 0.73 \pm 0.36$, 95% CI 0.37–1.09; $\beta = 1.65 \pm 0.66$, 95% CI 0.99–2.31, respectively) as compared to August. We also detected a significant difference in tick captures by tick drag/flag fabric type with greater captures when muslin was used as compared to flannel ($\beta = 1.07 \pm 0.06$, 95% CI 1.01–1.13). Our goal is to provide instructions to assemble a highly effective tick drag/flag using minimal supplies. Evaluation and improvements of sampling techniques is essential to understand impacts of landscape management and larger stressors, such as climate change on tick populations but also for enhancing detection of invasive non-native species.

Keywords Acari · Fabric · Population assessment · Surveillance · Tick-borne disease

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Introduction

Globally, ticks pose a significant threat to human and animal well-being as they parasitize every class of terrestrial vertebrates (Sonenshine and Roe 2014). Ticks also transmit the greatest variety of disease-causing agents as compared to all other hematophagous arthropods (Jongejan and Uilenberg 2004). Although advances in molecular biology have increased the probability of tick-borne pathogen detection, as well as identification of new pathogen species and strains of public health concern (de la Fuente et al. 2008; Dantas-Torres et al. 2012), the ability to assess pathogen risk and prevalence in a geographic location depends on in situ surveillance of competent tick vector species distribution and abundance (Ogden et al. 2010; Medlock and Jameson 2010; Braks et al. 2011; Jameson and Medlock 2011). Further, in situ surveillance activities provide baseline data for native and exotic tick vector species distribution and abundance, thus modification, evaluation, and improvements of existing tick sampling techniques is essential to understand the impacts of landscape management and larger stressors, such as climate change on tick populations and communities, spatial patterns of current and future tick-borne pathogen risk (Eisen 2008; Leighton et al. 2012; Rochlin 2018; Selmi et al. 2018), and detection of introduced and exotic tick species (Beard et al. 2018).

Common techniques for sampling tick populations include, (1) flagging-dragging, (2) carbon dioxide traps, (3) collection from captured hosts, (4) collection from ambulatory human host(s), (5) extraction from soil and litter samples (Ginsberg and Ewing 1989; Barton 1995), (6) using live, caged animals as sentinel hosts (Mather and Mather 1990), (7) aspiration of host burrows and nests (Logan et al. 1993), and (8) artificial nest-box traps (Wilson 1994). It is beyond the scope of this manuscript to discuss each sampling technique, however, systematic comparison and evaluation of biases associated with common techniques have been conducted by Ginsberg and Ewing (1989) as well as Schulze et al. (1997). Herein, we focus on flagging-dragging which is the most widely used technique to sample questing ticks (Carroll and Schmidtmann 1992; Tack et al. 2011; Rulison et al. 2013).

Flagging-dragging, also referred to as sweeping, is an effective technique in which fabric (e.g., muslin, cotton flannel, corduroy, etc.) is lined on a single side with rod or dowel and is passed over the ground and/or vegetation layer level to collect questing ticks. Flagging differs from dragging in that the apparatus is held by the user and passed over vegetation in a sweeping or waving motion (Rulison et al. 2013). Although each method differs in application, both rely on the fabric to contact questing ticks, which attach to the fabric via claws terminal to the pretarsus. Dependent on habitat type, phenology, and questing strategy of particular tick species, flagging as compared to dragging (or vice versa) may be more useful for determining presence and abundance of ticks in an area (Falco and Fish 1992; Fourie et al. 1995; Ginsberg et al. 2004; Tack et al. 2011). In this manuscript, we demonstrate effectiveness of our design by comparing results obtained from sampling at multiple sites that vary in vegetation structure within mixed pine hardwood forests in the southeastern United States. In addition, we provide step-by-step instructions to assemble a highly effective tick drag/flag that requires minimal supplies for construction as well as scanning electron microscopy (SEM) images of fabrics used to discuss effects of fabric material properties on tick sampling performance.

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Materials and methods

Study area

Our study occurred in the William B. Bankhead National Forest (BNF; 34.2270° N, 87.3461° W), which is a 72,000 ha multi-use forest managed by the United States Forest Service that spans Lawrence, Winston, and Franklin counties in the southern Cumberland Plateau of northwestern Alabama, USA. Mean annual temperature of the study area is 13 °C and rainfall is 147 cm (Nobles et al. 2009). The BNF represents one of the largest tracts of contiguous forests in the southeastern United States with the forest community composed primarily of upland pine (~51%) and hardwood species (~49%) with an udic soil moisture regime (Gaines and Creed 2003; Nobles et al. 2009). Predominant tree species in the BNF include Virginia Pine (*Pinus virginiana* Mill.), Loblolly Pine (*Pinus taeda* L.), Scarlet Oak (*Quercus coccinea* Muenchh.), Chestnut Oak (*Quercus prinus* L.) and Southern Red Oak (*Quercus falcata* Michx.; Schweitzer et al. 2015). For additional ecological characteristics of the study site, please see Gaines and Creed (2003), Nobles et al. (2009), and Sutton et al. (2013).

Sampling location determination

We determined random sampling locations primarily within Lawrence county in the upland pine forest stands of the BNF via the "create random points" tool in ArcMap 10.4 (ESRI 1999–2016) of which we sampled 36 randomly generated locations per month from 1 June to 30 August 2016 (N = 108) between 10:30 and 14:00 h. We randomly determined sampling location order via the random number generator function in Microsoft Excel®. At sampling locations, we measured 30 × 30 m quadrats via a 50 m open reel fiberglass tape measure. Within each quadrat, we delineated 30 m sampling transects at intervals of 0, 10, 20, and 30 m, for a total of 4 transects per quadrat (N = 432 transects; Fig. 1).

Tick drag/flag sampler construction

For tick sampling, we constructed two tick drag/flag samplers (described below), using either cotton muslin or cotton flannel fabric. Our choice of fabric for the tick drag/flag were based on recommendations in Armed Forces Pest Management Board (2012) and Lindquist et al. (2016). We constructed our tick drag/flag using the following items: (1) 100% cotton white flannel fabric (2 in quantity), (2) 100% cotton bleached white muslin fabric (2 in quantity), (3) diamond braided rope bright color (4.7 mm × 22.8 m), (4) wood round dowel (pine, 2.2225 cm × 1.2192 m), (5) white industrial strength Velcro (4.57 m × 5.08 cm; Fig. 2a), and (6) all-purpose stainless steel blade scissors (20.3 cm). Prior to construction of the tick drag/flag, we cut sampling fabric into four 1 × 1 m square panels (two cotton flannel and two cotton muslin). We stitched together square panels of the same fabric along all four edges using a double fold hem technique. Of note, sewing was completed by a professional seamstress as it was more cost-effective than purchasing necessary equipment (e.g., sewing machine) to ensure fabric panels were sewn to maximize durability. We aligned the fabric panels (one cotton and one flannel) with a wood dowel such that ~4 cm and 18 cm of space was left between ends of dowel and fabric (Fig. 2b). To ensure precision, we encourage marking the wood dowel at 4 cm and 18 cm from each end. We aligned Velcro "strips" with the fabric and markings on dowel (Fig. 2c, d) and removed the plastic

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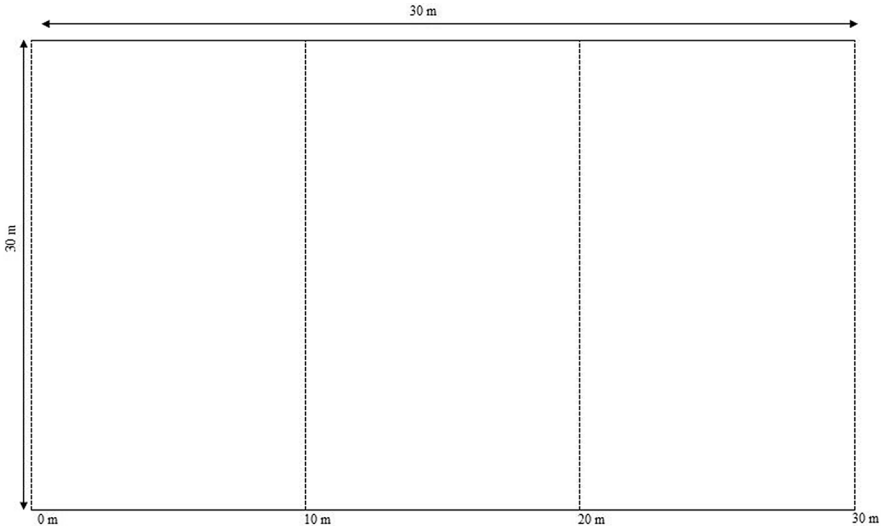

Fig. 1 Illustration of 30 m × 30 m sampling quadrat with drag transects at intervals of 0, 10, 20, and 30 m

film that lined the loop side of the Velcro and carefully attached it to the dowel to make sure no wrinkles or pockets formed (Fig. 2e). We combined the dowel and fabric panel to ensure that both adhered to each other via the Velcro strips to complete construction of the 1 × 1 m tick flag (Fig. 2f). We left an ~ 18 cm space left on the dowel to serve as a handle to perform tick sampling via a flagging technique. We cut diamond braided rope bright color to ~ 2 m and tied each loose rope end to the wood dowel (Fig. 2g). Lastly, the tick drag/flag can be rolled up and fastened down with the diamond braid rope to enable easy storage and carrying (Fig. 2h).

We do not provide trade names, manufacturers, and/or distributors because this publication is solely for the purpose of providing scientific information. However, based on our review all materials can be purchased online from a variety of vendors and shipped internationally dependent on shipping laws and regulations of destination country or region.

Sampling description

At each sampling quadrat, we assigned randomly the order that transects were sampled using either cotton muslin or flannel fabric. We used one sampler at intervals of 0 and 20 m while the other was used at intervals of 10 and 30 m ($n = 216$ transects sampled using muslin flag-drag and $n = 216$ transects sampled using cotton flannel flag-drag). To increase surface area and capture efficiency for tick sampling, we laid the sampling cloth flat on the ground, and maintained full contact of the tick drag/flag with the ground for the duration of the transect. Tick sampling was completed by two different researchers, however, differences in gait between researchers was not a factor as both had similar height and mass (researcher 1: male, height = 1.82 m, weight = 87 kg; researcher 2: male, height = 1.8 m, weight = 82 kg). We removed all sampled adult and nymph ticks with forceps and stored them in 40 mL amber glass vials that contained 90% molecular biology grade ethanol. We removed all sampled larvae with a lint roller (Scotch-Brite™) and stored each lint roller

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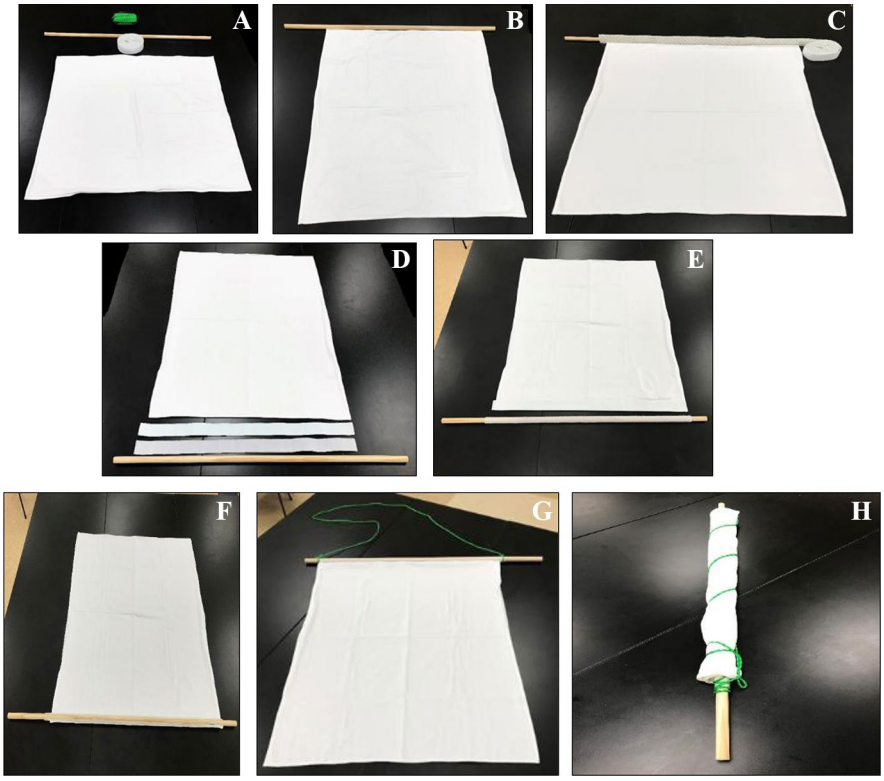

Fig. 2 **a** Cotton white flannel fabric (1 × 1 m), diamond braided rope bright color, wood round dowel (2.2225 cm × 1.2192 m), and white industrial strength Velcro (4.57 m × 5.08 cm). **b** Fabric panel aligned to wood dowel with ~ 4 cm and 18 cm of space left between ends of dowel and fabric. **c** Velcro aligned with fabric and markings on dowel. **d** Velcro cut to length of fabric panel. **e** Loop side of Velcro attached to dowel and hook side of Velcro attached to fabric panel. **f** Tick flag (1 × 1 m) with ~ 18 cm handle. **g** Diamond braided rope bright color cut at 2.5 m attached to wood dowel to complete assembly of 1 × 1 m tick drag. **h** Tick flag-drag tightly rolled up with rope spiraled around fabric to secure apparatus and enable ease of transport

sheet in plastic resealable bags (7.62 × 10.16 cm). We labeled sample vials and bags with sample date, geographic coordinates (longitude and latitude), and drag/flag fabric. At the end of each transect, researchers were checked from boots to waist height for ticks. If present, we collected and stored ticks via the methods described above. We identified ticks to species via morphology-based keys (Clifford et al. 1961; Keirans and Durden 1998) in a laboratory environment.

Scanning electron microscopy

Samples of each fabric type were mounted on 26 mm diameter aluminum stubs using double-stick tape, and then coated with a gold/palladium mixture using a Hummer 6.2 (Anatech USA, Hayward, CA, USA) sputtering apparatus. The preparations were examined and photographed with a Hitachi S-3400N (Hitachi High Technologies America, Schaumburg,

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IL, USA) scanning electron microscope in the Middle Tennessee State University (MTSU) Interdisciplinary Microanalysis and Imaging Center (MIMIC).

Data analysis

To assess the effectiveness of our tick drag/flag method and fabric type, we evaluated monthly differences of collected ticks using a general linear mixed model (GLMM) via the lme4 package (v.1.1-18-1; linear mixed effects models using 'Eigen' and S4; Bates et al. 2015). We assumed a Poisson distribution due to presence of numerical count data, and considered tick count (e.g., larvae, nymphs, and adults) as the response variable, sampling month and fabric type as fixed effects, and site and plot number as nested random effects ($\alpha = 0.05$).

Results

We collected 1,640 ticks via drag sampling at 108 randomly generated locations from 1 June to 30 August 2016 within the William B. Bankhead National Forest (Table 1). Our tick drag/flag constructed from muslin fabric accounted for ~ 74% of total ticks collected whereas flannel fabric accounted for ~ 26% of captures. Of the 108 drag sampling surveys, larvae were the most abundant life stage collected ($n = 1127$; ~ 69% of total ticks) followed by nymphs ($n = 460$; 28%) and adults ($n = 53$; 3%). Lone star ticks (*Amblyomma americanum*) accounted for 98.12% ($n = 38$ adults, $n = 452$ nymphs, and $n = 1119$ larvae) of total captures followed by blacklegged ticks 1.10% (*Ixodes scapularis*; $n = 3$ adults, $n = 1$ nymph, and $n = 15$ larvae) and American dog ticks 0.73% (*Dermacentor variabilis*; $n = 12$ adults, $n = 0$ nymphs, and $n = 0$ larvae). We collected voucher specimens for *A. americanum* nymphs and larvae (accession numbers USNMENT00862274 and USNMENT00862295, respectively) as well as *I. scapularis* nymph and larvae (accession numbers USNMENT00862273 and USNMENT00862270, respectively), which were deposited in the U. S. National Tick Collection (Statesboro, GA). Based on recent research by Lehane et al. (2019) and distributional reporting criteria of Dennis et al. (1998), we also provide new Alabama county records for *D. variabilis* populations as established in Lawrence County ($n = 11$) and reported in Winston County ($n = 1$).

We captured 9 (± 67.0 SD) larval, 2 (± 5.0 SD) nymph, and 1 (± 0.6 SD) adult ticks per sampling quadrat using muslin fabric and approximately 2 (± 8.0) larval, 2 (± 5.0) nymph, and 1 (± 0.5) adult ticks per sampling quadrat using flannel fabric.

Table 1 Total number of ticks collected ($N = 1640$) at the William B. Bankhead National Forest by month, life-stage and tick drag/flag fabric type

Fabric	June			July			August			Σ
	Larvae	Nymphs	Adults	Larvae	Nymphs	Adults	Larvae	Nymphs	Adults	
Muslin	0	104	17	839	66	10	124	59	1	1220
Flannel	0	65	17	82	114	6	82	52	2	420
Total	0	168	34	921	180	16	206	111	3	1640

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Based on total adults collected during drag sampling, we detected a significant effect of sampling month, with greater captures in June ($\beta = 2.44 \pm 0.63$; 95% CI 1.21–3.67) and July ($\beta = 1.65 \pm 0.66$, 95% CI 0.36–2.94) as compared to August (Fig. 3a), with average counts as follows: June (0.47 ± 0.10), July (0.22 ± 0.07), and August (0.04 ± 0.02). Similarly, we detected a significant effect of sampling month for nymphs collected during drag sampling, with greater tick counts in June ($\beta = 0.91 \pm 0.36$; 95% CI 0.20–1.62) and July ($\beta = 0.73 \pm 0.36$; 95% CI 0.02–1.44), as compared to August (Fig. 3b), with mean counts as follows: June (2.93 ± 0.56), July (2.5 ± 0.71), and August (1.54 ± 0.64). We did not report statistical analysis related to larval ticks due to lack of model convergence, which was likely due to large count variation among sampling events (Fig. 3c). Averaged total tick counts across all life stages (i.e., adult, nymph, and larvae) did not differ by sampling month (June: $\beta = 0.02 \pm 0.38$; 95% CI −0.72–0.76; July: $\beta = 0.59 \pm 0.38$; 95% CI −0.15–1.33; Fig. 3d). Nested random effects varied among sampling month and life stage for collected ticks such that site and plot number accounted for $0.47\% \pm 0.69$ of the variance for adults, $1.45\% \pm 1.21$ for nymphs and $2.13\% \pm 1.46$ for total tick counts across all life stages.

Fabric type: sampling performance

In regard to fabric type, we detected a significant effect such that total tick counts (total larvae, nymphs, and adults) were greater ($\beta = 1.07 \pm 0.06$, 95% CI 0.95–1.22) during surveys conducted with muslin fabric (Fig. 4a, b; 11.29 ± 6.46) as compared to surveys conducted with flannel fabric (Fig. 5a, b; 3.89 ± 0.89). We did not detect a significant effect

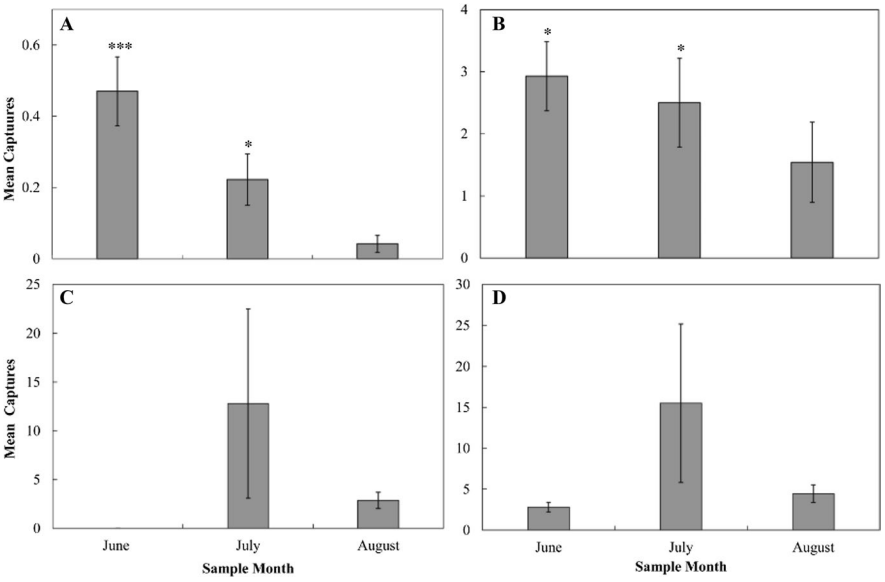

Fig. 3 Average number of **a** adults, **b** nymphs, and **c** larvae, collected by month during drag sampling event. **d** Total tick counts across all life stages (i.e., adult, nymph, and larvae). Asterisks indicate significant differences (***$P \leq 0.001$; *$0.01 \leq P \leq 0.05$)

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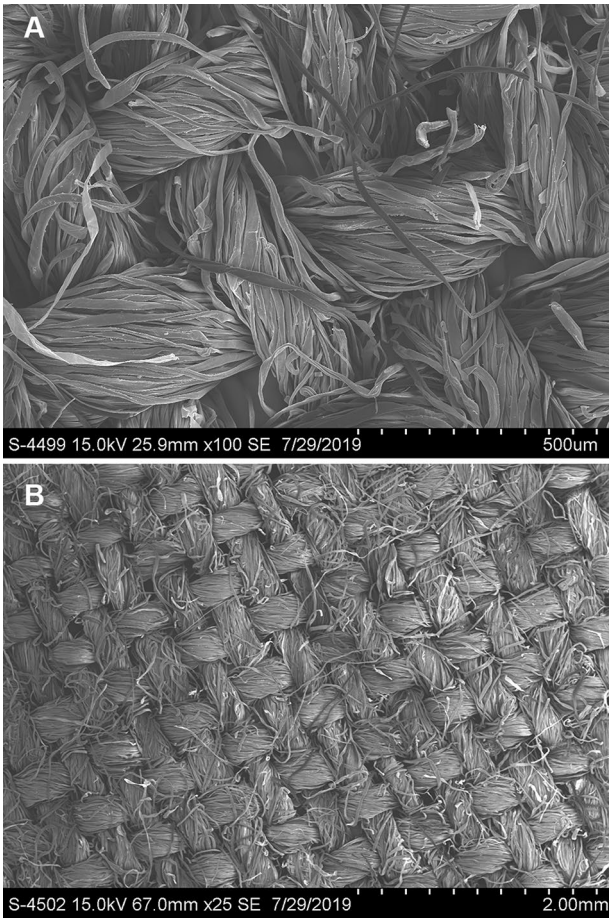

Fig. 4 **a** Scanning electron microscope image of cotton muslin fabric at 100× magnification. **b** Scanning electron microscope image of cotton muslin fabric at 25×magnification

of fabric type on total counts for adults ($\beta = 0.11 \pm 0.27$, 95% CI −0.53–0.22) and nymphs ($\beta = -0.01 \pm 0.09$, 95% CI −0.19–0.18).

Discussion

Our results demonstrate that our tick drag/flag is effective for collection of three different tick species and larval, nymph, and adult tick life stages. Additional advantages of our tick drag/flag are: (1) it can be constructed in 4 (tick flag) and/or 5 (tick drag) straightforward steps; (2) it is durable and therefore can be used repeatedly over a collecting season; (3) all supplies can be purchased locally or online in most regions of the world; (4) it is flexible such that it can be used as either a drag and/or flag based on habitat composition and life stage of tick species of interest; (5) it enables statistically valid comparison of sampling technique due to size of sampling material (e.g., 1 × 1 m) which permits investigators to quantify sampling efforts and capture rates for flagging versus dragging in an area/habitat type of interest; (6) ease of disassembly of the drag/flag from the Velcro lining strips enabling collection of ticks that have migrated to areas of the tick drag that would normally

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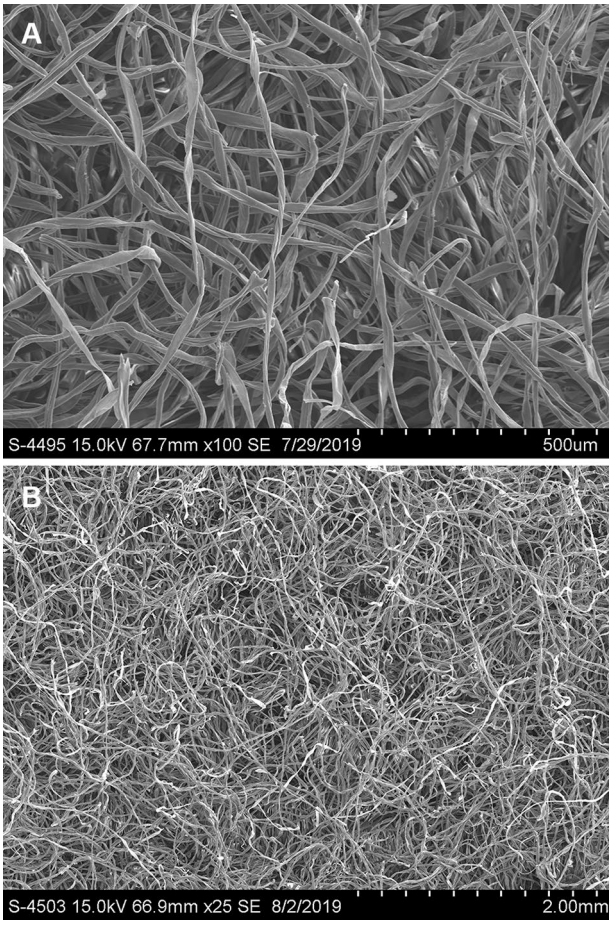

Fig. 5 **a** Scanning electron microscope image of cotton flannel fabric at 100 × magnification. **b** Scanning electron microscope image of cotton flannel fabric at 25 × magnification

not be accessible (e.g., a loop sewn into fabric to connect it at the dowel) as well as general cleaning; and (7) it is lightweight and can be conveniently rolled up and fastened down via the drag rope which enables ease of transport.

Amblyomma americanum represented the most commonly sampled species followed by *I. scapluaris*, and *D. variabilis*. Larval *A. americanum* accounted for ~ 70% of total ticks collected during our surveys as they are known to quest in large clusters ranging from hundreds to thousands of individuals (Schulze et al. 1986, 1997). Based on phenology and geographic location, our findings are consistent with previous research where this species is most active such that adults are most active from April to June, nymphs from May to July and August to September, and larvae from July to September (Semtner and Hair 1973; Newhouse 1983; Kollars et al. 1999, 2000; Goddard 2007; Mays et al. 2016). We attribute the observed sampling trends to ambient weather conditions associated with sampling time (late morning to early afternoon) and season (summer) as meteorological conditions play a significant role in tick activity patterns (Atwood and Sonenshine 1967; Semtner and Hair 1973; Duffy and Campbell 1994; Schulze et al. 2001; Schulze and Jordan 2003). If sampling efforts were expanded to include spring (March, April, and May) and fall months (September, October, and November) our collection data would likely yield comparatively

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different tick species richness. For example, Durden et al. (1991) and Heine et al. (2017) found that *I. scapularis* and *D. albopictus* were more active during November to January in Alabama as compared to *A. americanum* and *D. variabilis*.

Comparison of sampling performance based on fabric type showed that our tick drag/flag constructed of cotton muslin fabric collected significantly more total ticks as compared to our tick drag/flag constructed of cotton flannel fabric. This may be due to how each fabric type is manufactured. For example, the muslin fabric used in our study is sewn via plain weave construction with low thread count thereby producing small interstices within the fabric structure. This may provide increased opportunity for questing ticks to hook the interstitial space(s) of the fabric with outstretched claws and remain attached. In contrast, the flannel fabric used in our study is sewn via a loose, twill weave construction. As is characteristic of flannel fabric, it was brushed mechanically to raise fine fibers from the loosely spun cotton and form a nap on both sides for increased softness, water-repellent properties, and other changes in texture (Gioello 1982; Redmore 2012). The manufactured nap of flannel often runs in a single direction; therefore, it has a soft/smooth or rough feel only in one direction. This may have implications for sampling effectiveness of tick drag/flags constructed of flannel fabric because if the apparatus is passed along the vegetation layer in the direction of the nap, the raised cotton fibers will lie flat creating a smooth, sheer surface, which may reduce the ability of ticks to attach via outstretched claws during questing activities. Raised fine fibers of flannel also may enable collected larvae to go undetected by samplers as they can crawl beneath raised fibers thereby decreasing visibility and/or hindering collection with forceps which has been observed in previous research (Gil de Mendonça 2018). If left undetected or embedded in the flannel, this may lead to sampling bias as well as provide a pathway for range expansion and/or introduction of ticks and their associated pathogens as the tick drag is transported to new sampling locations (Gil de Mendonça 2018).

Assessment of sampling effectiveness is important for any monitoring program. White cotton flannel fabric (Zimmerman and Garris 1985; Falco and Fish 1992; Tsunoda et al. 2004; Cohen et al. 2010; Tack et al. 2011; Allerdice et al. 2017) as well as rubberized cotton flannel (Savage et al. 2013; Centers for Disease Control and Prevention 2018), corduroy (Schulze et al. 1997; Ostfeld and Lewis 1999; Daniels et al. 2000; Ginsberg et al. 2004; Mays et al. 2016) and muslin (Hoch et al. 1972; Ginsberg and Ewing 1989; Li and Dunley 1998; Stein et al. 2008; Lane et al. 2013) are all commonly used fabric types for collection of ticks via drag/flag sampling. However, to our knowledge, only two systematic studies comparing capture success rate based on tick drag/flag fabric types exist. Research by Vassallo et al. (2000) found toweling fabric as compared to cotton, woolen flannel, and "molleton" (soft thick cotton) optimized collection of nymphal *Ixodes ricinus* during drag sampling events conducted within a forest ecosystem (Rambouillet forest, France). Gil de Mendonça (2018) determined that tick drags constructed of white flannel fabric outperformed a "heavier and thicker fabric with a complex structure" for collection of *Ixodes ricinus* larvae within mixed woodlands (Bavaria, Germany), however, the author also recognized localized, large concentrations of larvae at hatching sites led to large count variation among sampling events. We observed the same trend in our study and therefore, could not determine preference for either one of the materials used for the drags. However, our results as well as research by Vassallo et al. (2000) and Gil de Mendonça (2018) suggests fabric type may be an important variable to consider when constructing tick drag/flags. We encourage replication of our tick drag/flag sampler design over different areas and time as this would greatly improve our understanding of the effects of fabric type when surveying tick communities.

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Development of a standardized, affordable, and replicable approach to sample ticks is important due to epidemiological significance of this organismal group. As ticks are known to cause spotted fever rickettsioses, human granulocytic anaplasmosis, tick-borne encephalitis, Heartland virus, Babesiosis, and many other zoonotic diseases in humans and wildlife (Goodman et al. 2005; Savage et al. 2013; Shock et al. 2014; Vaughn et al. 2014), monitoring techniques that can be replicated with little cost are essential to understand how tick populations and communities change in response to landscape management and larger ecological change (e.g., landuse and climate change). Such methodologies will equip public health agencies with monitoring techniques to detect changes in tick populations and better monitoring of tick-borne pathogen prevalence.

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