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ASSAYS FOR DETECTION AND IDENTIFICATION OF THE CAUSATIVE AGENT OF MANGE IN FREE-RANGING BLACK BEARS (*URSUS AMERICANUS*)

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ABSTRACT: Three mite species (*Demodex ursi*, *Ursicoptes americanus*, and *Sarcoptes scabiei*) have been associated with mange in black bears (*Ursus americanus*). Since the early 1990s, the number and geographic distribution of mange cases in black bears in Pennsylvania, US has increased; however, the causative mites have yet to be completely defined. We evaluated several diagnostic approaches for detection and identification of mites in 72 black bears with severe lesions consistent with mange. *Sarcoptes scabiei* was morphologically identified in skin scrapes from 66 of the bears; no mites were identified in the remaining six. Histopathologic lesions consistent with sarcoptic mange were observed in 39 of 40 bear skin samples examined, and intralesional mites were observed in samples from 38 of these bears. Samples were collected from a subset of the 72 bears for PCR testing targeting both the internal transcribed spacer (ITS)-2 region and cytochrome *c* oxidase I (*cox*1) gene including 69 skin scrapes (*ITS-2* only), 56 skin biopsies (*ITS-2* and *cox*1), and 36 fecal samples (*ITS-2* and *cox*1). Skin scrapes were a more sensitive sample for PCR detection than either skin biopsies or fecal samples, and the *ITS-2* primers proved more sensitive than *cox*1. Using a commercial indirect enzyme-linked immunosorbent assay, antibodies to *S. scabiei* were detected in 45/49 (92%) black bears with confirmed mange and 0/62 (0%) cubs with no gross lesions suggestive of mange and which were born to seronegative sows. *Sarcoptes scabiei* was the predominant mite associated with mange in black bears in Pennsylvania. Diagnostically, cytologic examination of skin scrapes was the most effective approach for diagnosing active mite infestations in black bears. The evaluated serologic assay accurately detected antibodies to *S. scabiei* in most bears with confirmed *S. scabiei* infestations. Additional research is needed to determine the usefulness of this approach for larger scale surveys and for asymptomatic bears.

Key words: Black bears, diagnostic testing, mange, molecular, *Sarcoptes*, sarcoptic mange, ursids.

INTRODUCTION

Mange, a contagious skin disease of animals caused by several species of mites, is characterized by a spectrum of clinical signs including pruritus, hair loss, varying degrees of crusting or scabbing of the skin, poor body condition, secondary infections, and possible death (Bornstein et al. 2001; Pence and Ueckermann 2002). In black bears (*Ursus americanus*), mange has traditionally been a rare disease involving individual or low numbers of animals and reportedly has been associated with three different mites: *Sarcoptes scabiei* (Schmitt et al. 1987), *Ursicoptes americanus* (Yunker et al. 1980), and *Demodex ursi* (Desch 1995). However, since the early 1990s, mange has been detected in black bears in Pennsylvania, US with increasing frequency and distribution (Sommerer 2014). To date, monitoring for mange in black bears in Pennsylvania has been conducted through syndromic passive surveillance (i.e., detection of overt disease based on gross lesions); systematic surveys to identify the causative mites and the statewide prevalence of mite infestation have not been conducted. The

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latter is critical for disease monitoring and interpretation because, although annual mange cases in black bears in Pennsylvania have increased over the last 25 yr, the statewide black bear population has markedly expanded in number and range over the same period. Consequently, we do not know to what extent the perceived increase in mange is due to changes in parasite prevalence or host population.

The gold standard for diagnosing active mite infestations is cytologic examination of skin scrapes from affected skin for mite identification. The success of this technique is dependent on multiple factors including host species, quantity of mites in the sample, chronicity of lesions, and scraping the correct anatomic location. Cytology can be ineffective in animals with mild or no lesions because mite burden may be low and appropriate locations to scrape may not be apparent (Hill and Steinberg 1993; Pence and Ueckermann 2002). Similarly, animals that develop a delayed hypersensitivity reaction may have reduced mite burdens but still have traumatic skin lesions due to severe pruritus (Pence and Ueckermann 2002).

Molecular tests have been utilized in animals with mange for both mite detection and genetic characterization. As a screening tool, molecular approaches have yielded variable results (Amer et al. 2014; Makouloutou et al. 2015; Zhao et al. 2015). Similar to cytology, the success of this diagnostic method is dependent on the quantity of mites in the sample. The most frequently used gene target is the internal transcribed spacer (ITS)-2 region in nuclear ribosomal DNA. Although commonly used, most studies have shown that *S. scabiei ITS-2* sequences from different hosts and geographic regions exhibit low diversity and thus have limited value in epidemiologic studies (Berrilli et al. 2002; Makouloutou et al. 2015). Other gene targets, including mitochondrial genes coding for cytochrome *c* oxidase subunit 1 (*cox*1) and 16S ribosomal RNA, have identified distinct, geographically separate, and host-adapted *S. scabiei* populations and may be preferable for epidemiologic studies (Amer et al. 2014; Makouloutou et al. 2015; Zhao et al. 2015).

Histologic examination of skin is commonly used to characterize mange lesions and to detect the presence of intralesional mites (Schmitt et al. 1987; Deem et al. 2002; Nimmervoll et al. 2013). In addition, histology can identify coinfections with other pathogens (e.g., parasites, bacteria, and fungi) that may contribute to the disease process and have impacts on treatment efficacy. One important limitation of histology is that the species of mite cannot be identified in most cases. As described earlier, there is extensive variation in pathology, coinfections, and mite burdens (Pence and Ueckermann 2002; Nimmervoll et al. 2013). Such variation may complicate the interpretation of some diagnostic tests. Consequently, histology is an important tool to distinguish between mange and other skin disease that may be grossly indistinguishable including dermatophilosis, trauma, allergic dermatitis, and hypothyroidism (Newman et al. 1975; Harper et al. 1988; Storms et al. 2002).

Cytology, PCR, and histology are successfully used for diagnosis of active mite infestations. Serologic assays may identify individuals that have been previously exposed, including animals with chronic infestations lacking overt lesions and individuals with resolved infestations. Antibodies to *S. scabiei* have been successfully detected in free-ranging species, including red fox (*Vulpes vulpes*) and Iberian ibex (*Capra pyrenaica*), by using commercial enzyme-linked immunosorbent assays (ELISA) developed for use in domestic dogs (*Canis lupus familiaris*) rather than for each wildlife species (Bornstein et al. 2006; Nimmervoll et al. 2013; Ráez-Bravo et al. 2016). Serologic assays for *S. scabiei* have not been evaluated in bears.

Because mange is a concern for black bears, we need to identify the causative mites of mange in bears to understand the epidemiology and risk factors of the disease. However, a lack of information on diagnostic options currently limits this research. Our objectives were to identify the causative agents of mange in black bears from Pennsylvania and evaluate

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the utility of several assays (cytology, PCR, histology, and serology) for diagnosing mange in black bears.

MATERIALS AND METHODS

Sample collection

During 2014–17, we collected diagnostic samples from 72 black bears from Pennsylvania with gross lesions consistent with severe mange, as defined by alopecia over 50% or more of the body and markedly crusted skin (Fig. 1A). We collected samples from bears with mange that were found dead, euthanized due to severity of clinical presentation, or that were harvested by hunters. Multiple diagnostic samples, including skin scrapes and full-thickness skin samples collected from affected areas, blood, and feces, were collected from each bear. However, not all sample types were available for each individual animal. Skin scrapes were preserved in 70% ethanol, and full-thickness skin samples were preserved in 70% ethanol for PCR analysis and in 10% neutral-buffered formalin for histologic examination. Blood was collected in serum separator tubes, centrifuged, and the resulting serum stored at –20 C until testing. Feces were collected following protocols used previously for notoedric mange in bobcats (*Lynx rufus*) and frozen at –20 C until PCR testing (Stephenson et al. 2013). If necessary, euthanasia of black bears with severe mange was conducted by personnel of the Pennsylvania Game Commission following agency standard operating procedures. All animal sampling protocols were reviewed by and approved by University of Georgia's Institutional Animal Care and Use Committee (A2013-10-016 and A2015-05-13).

We also collected serum samples from 6- to 11-wk-old bear cubs from Pennsylvania to serve as serologic negative-control samples. The Pennsylvania Game Commission personnel sampled bear cubs during annual den visits to radiocollared sows in 2016 and 2017. All cubs were sampled while they were still in the den; none had gross lesions consistent with mange and all were from sows that were seronegative for *S. scabiei*. Small volumes of blood (0.5–1.0 mL) were collected via the jugular or femoral vein into serum separator tubes and then processed and stored as described above.

Cytology

Ethanol-fixed skin scrapes were examined under dissecting light microscopes and mites were identified to species using published keys and confirmed by PCR and sequence analysis (Peltier et al. 2017). Voucher specimens were deposited in the Collection of Arthropods, Georgia Museum of Natural History, University of Georgia, Athens, Georgia.

Molecular detection

Skin scrape material was centrifuged at 2,500 × G for 10 s, the ethanol was removed, and the pellet washed with 500 µL phosphate-buffered saline during another centrifugation. The remaining pellet was divided into two samples and dried under a fume hood. To determine if grinding of the sample material would improve DNA extraction from mites and improve sensitivity of PCR, one sample was ground with a micropestle (Kimble Chase, Grainger, Houston, Texas, USA) while the other sample was extracted without grinding. We extracted DNA with a commercial extraction kit (DNeasy Blood and Tissue Kit, Qiagen, Valencia, California, USA) following manufacturer's instructions. For skin biopsy samples, we extracted DNA from three separate sections of skin (25 mg each) to maximize detection of mites. Sections of skin were independently washed and dried as described above and, prior to DNA extraction, each skin sample was ground with a micropestle (Kimble Chase). We extracted DNA from two separate approximately 800-µg fecal samples which were ground with a micropestle prior to extraction (Qiagen).

We amplified mite DNA by PCR using primers RIB-18 and RIB-3 to amplify the *ITS-2* gene (Zahler et al. 1999) and primers CytoF and CytoR to amplify a partial region of the *cox*1 gene (Walton et al. 2004). Both ground and non-ground sets of DNA from skin scrapes were tested using the *ITS-2* primers while only the ground set was tested with the *cox*1 primers. Of the three DNA extractions from full-thickness skin samples, one was tested using the *ITS-2* primers and the remaining two were tested using the *cox*1 primers. One of each of the two DNA extractions of feces sample was tested with *ITS-2* and *cox*1 primers.

We took precautions to prevent and detect contamination including performance of DNA extraction, PCR reaction setup, and product analysis in distinct, designated areas. Negative water controls were included in each set of DNA extractions. For each batch of PCR reactions, the extraction negative control, a new water negative control, and a positive control (DNA sample from *S. scabiei*) were included. To confirm mite identity, representative PCR amplicons were purified from a GelRed stained 1.5% agarose gel using a gel purification kit (Qiagen) and bidirectionally sequenced at the University of Georgia Genomics Facility (Athens, Georgia).

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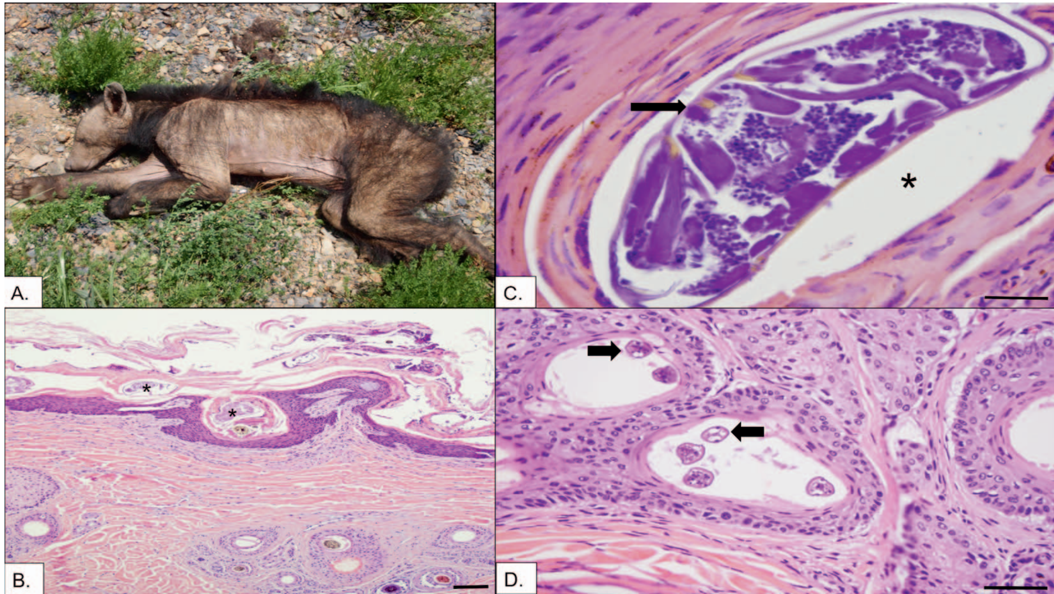

FIGURE 1. A. Gross image of a black bear (*Ursus americanus*) in Pennsylvania, USA that was severely affected by mange. Notice the extensive regions of crusting and alopecia affecting approximately 90% of the body. B. Photomicrograph of haired skin of from a black bear infested with *Sarcoptes scabiei*. There is moderate epithelial hyperplasia overlain by orthokeratotic and parakeratotic keratin. Multiple cross sections of mites (asterisks) are present burrowing within the surface keratin associated with serocellular crusts and cellular debris. H&E. Bar=200 µm. C. Photomicrograph of a higher magnification of a single *Sarcoptes scabiei* within a "mite tunnel" (asterisk) within parakeratotic keratin of the haired skin of a black bear. There is partially chitinized cuticle with prominent refractile cuticular spines (arrow), striated muscle, and a hemocoel (body cavity). H&E. Bar=30 µm. D. Photomicrograph of adnexa surrounding a hair follicle within the skin of a black bear. There are multiple cross sections of nematodes (arrows) identified as *Pelodera* spp. with paired cuticular spines. H&E. Bar=60 µm.

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Histology

Formalin-fixed skin samples were embedded in paraffin, sectioned, and stained with H&E. Slides were reviewed by a board-certified veterinary pathologist.

Serology

We analyzed sera for immunoglobulin G antibodies against *S. scabiei* using a commercially available indirect ELISA (*Sarcoptes*-ELISA 2001® Dog, Afosa, Berlin, Germany). Controls in the kit included a serum sample from *S. scabiei* var. *canis*-infested dogs as a positive control and a known negative canine serum sample for a negative control. Additional negative controls tested included serum samples collected from black bear cubs. Testing was performed following manufacturer's instructions. Optical densities (OD) were read at 450 nm, 15 min after substrate addition, and were expressed as a percentage (OD sample–OD negative control)/(OD positive control–OD negative control). Converted OD values >15% were considered positive, those <10% were considered negative, and those between 10–15% were considered equivocal.

Statistical analyses

We used a Fisher's exact test to determine if the prevalence of antibodies to *S. scabiei* was different between adult and yearling bears with mange compared to cubs with no gross lesions suggestive of mange. We also used the Fisher's exact test to compare detection rates of *S. scabiei* with the different PCR assays.

RESULTS

We tested samples from 72 black bears including 65 adults (39 females, 26 males) and seven yearlings (four females, three males) with severe mange, although not all diagnostic samples were collected from each bear (see Supplementary Material Table S1). Morphologic identification of mites present in skin scrapes revealed that *S. scabiei* was the only

TABLE 1. Results of PCR testing of skin scrape samples from black bears (*Ursus americanus*) from Pennsylvania, USA for detection of *Sarcoptes scabiei* using the internal transcribed spacer (ITS)-2 and cytochrome *c* oxidase I (*cox*1) protocols, with and without grinding.

PCR protocol	No.	PCR positive (%)
ITS-2 without grinding	69	60 (87)
ITS-2 with grinding	69	56 (81)
*cox*1 with grinding	69	50 (72)

TABLE 2. Results of PCR testing of three, full-thickness skin samples from black bears (*Ursus americanus*) from Pennsylvania, USA using the internal transcribed spacer (ITS)-2 and cytochrome *c* oxidase I (*cox*1) protocols for *Sarcoptes scabiei*.

Sample	PCR protocol	No.	PCR positive (%)
1	*ITS-2*	56	40 (71)
2	*cox*1	56	30 (54)
3	*cox*1	56	18 (32)

mite species present in 66/72 (92%) of bear skin samples. Mites were not identified in skin scrapings from the remaining six bears, but *S. scabiei* was detected in five of these by PCR testing of at least one sample type or by one assay. Of the 66 skin scrape samples with mites identified, an average of 24 mites was observed per slide (range 1–113 mites).

The detectability of *S. scabiei* in skin scrapes using the *ITS-2* PCR protocol was similar (P=0.486) with or without grinding of the skin sample (Table 1). Also, no significant difference (P=0.313) was noted in detection using the *ITS-2* or *cox1* protocols on skin scrapes (Table 1). A total of 56 full-thickness skin samples were tested by PCR using both *ITS-2* and *cox1* protocols. Generally, the PCR detection of *S. scabiei* in full-thickness skin samples was lower compared with skin scrapes (Tables 1, 2). Forty of 56 (71%) full-thickness skin samples tested with the *ITS-2* protocol were positive while only 30 (53%) and 18 (32%) of the two full-thickness samples tested using the *cox*1 protocol were positive (Table 2). However, the *ITS-2* protocol did not detect all *S. scabiei*-positive samples because four samples found negative using the *ITS-2* protocol were positive using the *cox*1 protocol. All 36 fecal samples tested negative using both *ITS-2* and *cox*1 PCR assays.

Histologically, mites were identified within the epidermis of 38/40 (95%) black bears with severe mange (Fig. 1B, C). Lesions consisted of moderate to severe acanthosis and neutrophilic epidermitis with large serocellular crusts with marked parakeratotic hyperkeratosis. Evidence of secondary bacterial colonization within the serocellular crust was present in 37/40 (93%) bears and superficial yeast (presumably *Malassezia* spp.) was present in 23/40 (58%). Yeast spores, morphologically consistent with *Candida* spp., were observed within the serocellular crust of one bear. Nematodes morphologically consistent with *Pelodera strongyloides*, a free-living saprophytic nematode, were present within hair follicles of 4/40 (10%) bears. Of the 40 bears with gross lesions suggestive of mange that were examined histologically, only one had histologic lesions not consistent with sarcoptic mange. Lesions in this bear were characterized by orthokeratotic hyperkeratosis and inflammatory cells and debris present within dilated hair follicles. No intralesional mites were identified histologically in this bear. Cytologic examination of skin scrapes and molecular testing on skin scrapes and full-thickness skin samples from this bear were also negative. Collectively, these data suggest the grossly apparent skin lesions in this bear were not a result of mange; however, the cause was not determined.

Of the 49 serum samples from bears with confirmed *S. scabiei* infestation, 45 (92%) were positive for antibodies to *S. scabiei* and one (2%) was equivocal (Table 3). There was no difference in antibody prevalence between females (36/38; 95%) and males (9/11; 82%) with confirmed sarcoptic mange (P=0.214). Although adults (95%) had a higher seroprevalence than did yearlings (67%), the difference was not significant (P=0.068). The 62

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TABLE 3. Serologic testing of adult and yearling black bears (*Ursus americanus*) from Pennsylvania, USA with sarcoptic mange, and of cubs with no lesions suggestive of mange, using a commercially available indirect enzyme-linked immunosorbent assay for *Sarcoptes scabiei* of domestic dogs (*Canis lupus familiaris*).

Mange status	Age	Sex	No.	Antibody presence (%) Positive	Equivocal
Yes	Adults	Female	35	34 (97)	0
		Male	8	7 (88)	1 (13)
		Total	43	41 (95)	1 (2)
	Yearling	Female	3	2 (67)	0
		Male	3	2 (67)	0
		Total	6	4 (67)	0
No	Cubs	—[a]	62	0	0

[a] Dash (—) = sex not known.

serum samples from black bear cubs were negative for antibodies to *S. scabiei*.

DISCUSSION

The results of this study indicate that *S. scabiei* is currently the primary cause of mange in black bears in Pennsylvania. Previous reports of mange from any of the three species of mite in free-ranging black bears are rare and sporadic (Desch 1995; Bornstein et al. 2001; Joyner et al. 2004; Peltier et al. 2017). Aside from clinical demodectic mange in black bears from Florida, none of the prior mange cases in black bears were considered significant at the population level.

Cytology was an effective diagnostic tool for black bears with mange. Mites were readily identified in skin scrapes from most bears and most samples had high mite burdens relative to other wild species; for example, red fox and coyote (*Canis latrans*). Although less sensitive than cytology, both PCR tests were able to detect *S. scabiei* in the majority of skin samples, with the *ITS-2* protocol being more sensitive than *cox*1. A previous study comparing different PCR assays for detection of *S. scabiei* in humans also found that the *ITS-2* protocol was more sensitive than elongation factor 1α and myosin heavy chain gene targets (Fukuyama et al. 2010). Sample collection procedures influenced detection success. Skin scrapes were generally more sensitive than skin biopsies, grinding prior to extraction did not increase sensitivity, and testing of fecal samples was unsuccessful. The relative success of these procedures was presumably dependent on the number of mites in the tested sample. Skin scrapes sampled a larger area of infested epidermis than did a full-thickness biopsy, which contained additional tissue layers, and presumably obtained more mites. Bears with sarcoptic mange confirmed by cytology that were negative by PCR all had low numbers of mites (range 1–9 mites). While additional research is needed to evaluate ways to improve diagnostic efficiency for mange in black bears, our results suggested the best sampling approach involved collection of skin scrapes from several affected locations of the body. Molecular testing of feces would presumably have provided an additional noninvasive surveillance tool for mange in black bears; however, our results indicated it was not a viable approach. The poor detection of *S. scabiei* in feces was likely a result of insufficient numbers of mites ingested during the grooming process, the larger volume of bear feces diluting the mites, or the presence of PCR inhibitors.

Histopathologic lesions from the black bears with mange were consistent with those previously described in free-ranging carnivores (Schmitt et al. 1987; Deem et al. 2002; Nimmervoll et al. 2013). Consistent with previous reports of mange in bears, the affected skin was frequently coinfected with other microorganisms such as the nematode *P. stronglyoides*, yeast, or bacteria (Fitzgerald et al. 2008; Dykstra et al. 2012). Presumably, the damage to the epidermis from *S. scabiei* predisposes bears to secondary infections from these microorganisms which are common in the environment or normal skin flora. Further research is needed to better understand the role these coinfections may play in the risk of initial *S. scabiei* infestation, progression of disease, prognosis, and treatment of free-ranging black bears with mange.

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Serologic testing for *S. scabiei* provides many benefits for characterizing the epidemiology of mange in black bear populations. The commercial ELISA evaluated was developed for the detection of *S. scabiei* in domestic dogs but had not been validated in any wild species (Nimmervoll et al. 2013). Based on our results, the ELISA was an accurate tool to detect antibodies to *S. scabiei* in bears with active infestations. Only 6% (3/49) of bears with gross signs of severe mange were seronegative, one of which was a yearling not confirmed to be infested with *S. scabiei* through any other diagnostic test. The other two bears, an adult and a yearling, were both positive for *S. scabiei* by PCR but had either low numbers or no mites in skin scrapes. These bears, along with the adult bear with an equivocal serologic result, may have been in the early stages of infection and had not yet developed a high antibody titer. Alternatively, bears with sarcoptic mange may only develop a transient increase in immunoglobulin G antibodies during challenges with *S. scabiei*, similar to sheep and goats (Bovidae; Tarigan and Huntley 2005; Rodriguez-Cadenas et al. 2010). Also, Davidson et al. (2008) identified two red foxes that were positive for sarcoptic mange but remained seronegative, suggesting that not all individuals with infections will seroconvert, possibly due to delayed seroconversion or to immunosuppression resulting in no detectable antibody reaction. We did not note a difference in antibody prevalence between male and female bears, which is similar to previous serologic studies on wild foxes (Bornstein et al. 2006; Davidson et al. 2008). Currently, surveillance for mange in black bears in Pennsylvania is syndromic, which has limitations that may be overcome by serologic testing that can detect previous exposures and mild infestations. Future work needs to investigate the likelihood that a bear seroconverts following infestation or during different extents of clinical disease (i.e., mild vs. severe mange), antibody persistence following clinical mange, lower immune responses following subsequent periods of clinical mange, maternal antibody production, and possible serologic cross-reactivity with *U. americanus*. Although there are many unknowns, our serologic data suggest that this approach may be useful in understanding the extent of *S. scabiei* infestation and mange in bears.

Overall, our data in this study and a previously published study (Peltier et al. 2017) indicate that the unprecedented mange expansion in black bears in Pennsylvania and surrounding states is due primarily to *S. scabiei*. The risk factors involved in this epizootic remain unknown. One possibility is the emergence of a novel *S. scabiei* strain; however, no single genetic type was recently detected (Peltier et al. 2017). Other possible reasons include changes in bear behavior or density, coinfecting pathogens, habitat changes, or other undefined factors. Thus, more research is warranted to define *S. scabiei* epidemiology and ecology in bears. Although *S. scabiei* appears to be the predominant mite species associated with the current severe mange event occurring in black bears, the relatively low sample size of our study may have missed other mite species (e.g., *Ursicoptes* spp.) that may be circulating and contributing to disease.

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SUPPLEMENTAL MATERIAL

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