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PROBING CHEMICAL ENVIRONMENT USING POLYMERIC REVERSE MICELLAR SOLUTIONS WHICH SEQUESTER INORGANIC COORDINATION COMPLEX FLUOROPHORES[1]

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ABSTRACT

A series of tris(bipyridine)ruthenium(II) chloride ($[Ru(bpy)_3]Cl_2$)-sequestered reverse micellar solutions of variable surfactant concentration were examined using fluorescence spectroscopy before and after thermal radical polymerization of the nonpolar phase. The $[Ru(bpy)_3]Cl_2$ emission spectra simulated aqueous solution chemical environments irrespective whether the nonpolar phase is liquid or polymerized into a solid. A range of surfactant concentrations were examined. Emission maxima of the reverse micelle solution-sequestered $[Ru(bpy)_3]Cl_2$ species are red-shifted with respect to aqueous $[Ru(bpy)_3]Cl_2$. The red-shift can be interpreted in the context of increasing chemical environment polarity. Emission maxima of the $[Ru(bpy)_3]Cl_2$ species of polymerized nonpolar phase at approximately 600 nm were consistent with $[Ru(bpy)_3]Cl_2$ aqueous species. The work represents a pathway to preserve solution-dependent chemical processes of molecular sensors. [J PA Acad Sci 90(1): 1-6, 2016]

INTRODUCTION

Chemical sensors produce a detectable signal pertaining to the presence of an external stimulus or an amount of an analyte (Banica 2014; Hulanicki *et al.* 1991; Madou *et al.* 1989). For instance, gas, optical, and electrochemical sensors find use in environmental and biomedical professions (Janata 1992). Yet, the chemical species working as a sensor must often be maintained in a liquid solution environment to preserve sensing capabilities amenable to its chemical reactions or physical properties (Rondi *et al.* 2015). If that chemical species is immobilized in a glass or frozen in a crystal, then its chemical sensing capabilities can become deactivated (Wang *et al.* 2014). As an example, certain transition metal coordination complex excited states are highly sensitive to oxygen quenching in solution, and in contrast the excited states of the solid phase do not show such a response (McDonagh *et al.* 2008). Accordingly, fabrication of sensors gains importance, especially for optical sensors which can serve as transducers of concentration, magnetic field strength, and even thermocouples (Kumara *et al.* 2014).

The ability to engineer a composite material which contains pools of liquid encased in a solid material would enable a wider use of chemical sensors in the field to the advantage of environmental protection. Glass cuvettes accomplish this task. However, borosilicate glass retains modest chemical reactivity and of course glass is breakable putting the expensive chemical sensor molecules at risk of loss (Madou *et al.* 1989). Also, the liquid in the cuvettes can evaporate over time. Biological cells encase liquid solutions, but the surrounding milieu can pose interferences. Considering that cell organelles like vesicles and vacuoles are composed of phospholipid molecules, ternary micellar solutions can host chemical sensor species. Micellar solutions are composed of lipid-like surfactant molecules which sequester pools of nonpolar liquids containing nonpolar chemical sensors from an aqueous solvent (Luisi *et al.* 2014). Reverse micellar solutions can be made if the solvent becomes a nonpolar phase (Khoshnood *et al.* 2015). An especially-chosen nonpolar phase, such as styrene, can be transformed into a solid (polystyrene) under radical polymerization conditions to yield a solid-state chemical sensor wherein the chemical sensors are maintained in nanoscopic-sized liquid pools of water (figure 1) (Arai *et al.* 2015; Xue *et al.* 2014). If the solidified polymer-surfactant composite materials remain clear, then that is an indication that the reverse micelles containing the chemical sensor in liquid environments are intact.

Experiments were undertaken to test the feasibility of this sensor fabrication strategy for solidified reverse micelle composite materials and to assess the chemical environment about the chemical sensor molecular species. The inorganic complex tris(bipyridine)ruthenium(II) chloride $[Ru(bpy)_3]Cl_2$ was the chemical sensor chosen for this study, because it finds use in commercial applications (Castellano *et al.* 2015; Gaines 1980). Its chemical structure is presented in figure 2. This dye shows a strong and broad absorption maximum at 450 nm, as well as a strong and broad emission maximum at 600 nm in aqueous solution (Gaines 1980).

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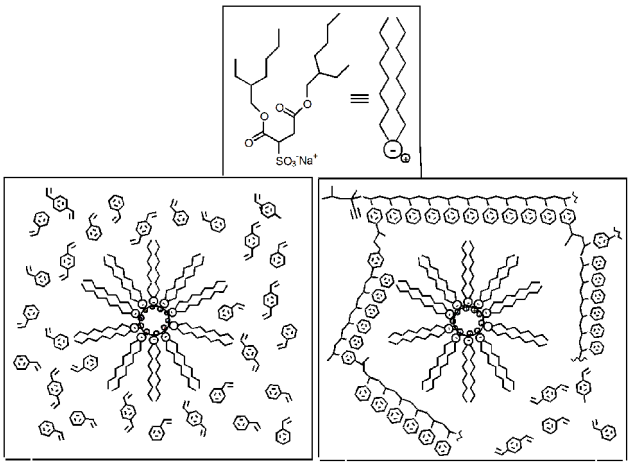


Figure 1. Schematic cross-sectional representations of a composite precursor reverse micellar solution (CPRMS) at the bottom left and a polymer encapsulated reverse micelle composite material (PERMC) at the bottom right. The chemical structure of the Aerosol-OT (AOT) surfactant molecules composing the micelles in the CPRMSs and PERMCs is represented by the crosshatched drawing (upper center). The 2:1 styrene:divinylbenzene nonpolar phase of the CPRMS (bottom left) is polymerized after the addition of 2,2'-azobis(2,4-dimethylpentanenitrile) (ADPN) and subsequent sample heating to 38 °C for ca. 96 hrs. The polymerization front proceeds leaving the micelle(s) and the sequestered polar phases intact inside the PERMC (bottom right).

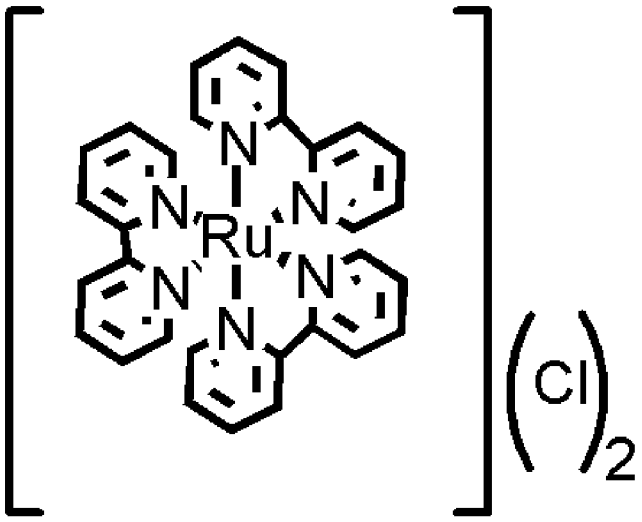


Figure 2. The structure of [tris(bipyridine)ruthenium(II)] chloride, $[Ru(bpy)_3]Cl_2$. A metal-to-ligand electron transfer occurs upon the absorption of blue light at 450 nm upon promotion to an excited state. The compound emits orange light at 600 nm to relax to the ground state.

These spectral band shapes would narrow if the dye became immobilized in between strands of polymer or in between nonpolar surfactant chains (Drago 1992). This narrowing results from fewer vibrational states upon immobilization in a solid matrix. The purpose of this work is to determine a range of surfactant concentrations amenable to sensor material synthesis.

MATERIALS AND METHODS

The synthesis of the chemical sensor tris(bipyridine) ruthenium(II) chloride $[Ru(bpy)_3]Cl_2$ has been reported previously (Burstall 1936). Ruthenium(III) chloride, 2,2'-bipyridine, and hypophosphorous acid were purchased from Aldrich and used without further purification. Dioctyl sulfosuccinate sodium salt, surfactant, 98% (also called Aerosol-OT or AOT), styrene, nonpolar phase, 99%, divinylbenzene, nonpolar phase, 80% (with the main impurity as 4-ethylstyrene), 2,2'-azobis(2-methylpropionitrile), radical polymerization initiator, 98%, Supelco 4 mL graduated screw top vials, and septa were purchased from Aldrich. Doubly-deionized water was used. De-Hibit 200 was purchased from Polysciences Inc. and used as received. Table 1 presents the volumes and concentrations selected.

The reverse micellar solution nonpolar phase consisted of a 2:1 styrene:divinylbenzene. The mixture was eluted from a gravity column packed with De-Hibit 200. The eluate was foil-wrapped and stored at 2 – 8 °C. Stock solutions of $[Ru(bpy)_3]Cl_2$ in doubly-deionized water and AOT in 2:1 styrene:divinylbenzene were made. Reverse micellar solutions were prepared using a 5 mL leur lock glass syringe and a Hamilton Gastight 250 μL glass syringe. Hydration ratio (W_o) values for each reverse micellar solution are derived from the expression: $W_o = [V_{plr}C_{plr}] \div [V_{tot}C_{surf}]$ where V_{plr} is the volume in L of the polar phase, C_{plr} is the concentration of the polar phase solvent in units of M, V_{tot} is the total volume of the reverse micellar solution, and C_{surf} is the concentration of the AOT surfactant in M (Luisi *et al.* 2014).

The reverse micellar solutions were subjected to fluorescence analysis on a Perkin Elmer LS45 spectrophotometer. Upon the completion of these assays, trace 2,2'-azobis(2-methylpropionitrile) thermal radical polymerization was added to each reverse micellar solution (Bevington 1987). The reverse micellar solutions were septum-capped and placed in a 40.0 °C sand bath to solidify the 2:1 styrene:divinylbenzene nonpolar phase with an understanding of the effect of temperature on reverse micelle aggregation (Minchor *et al.* 2015). Upon solidification, the glass vials were cracked to retrieve the cylindrical solidified composite materials. The composite materials were ground and polished to orthogonal dimensions amenable to fluorescence spectroscopy, and an example is listed

Table 1. Concentrations and volumes of chemicals used to prepare chloride $[Ru(bpy)_3]Cl_2$ – sequestered reverse micellar solutions and the respective hydration ratio (Wo) values.

Reverse Micellar Solution	[AOT] (mM)	Vsty:dvb (mL)	$[[Ru(bpy)_3]Cl_2]$ (mM)	V$[Ru(bpy)_3]Cl_2$ in DDI (µL)	Wo
1	10	4.0	10.0	10.0	13.8
2	20				6.9
3	30				4.6
4	40				3.4
5	50				2.7
6	60				2.3
7	70				1.9
8	80				1.7
9	90				1.5

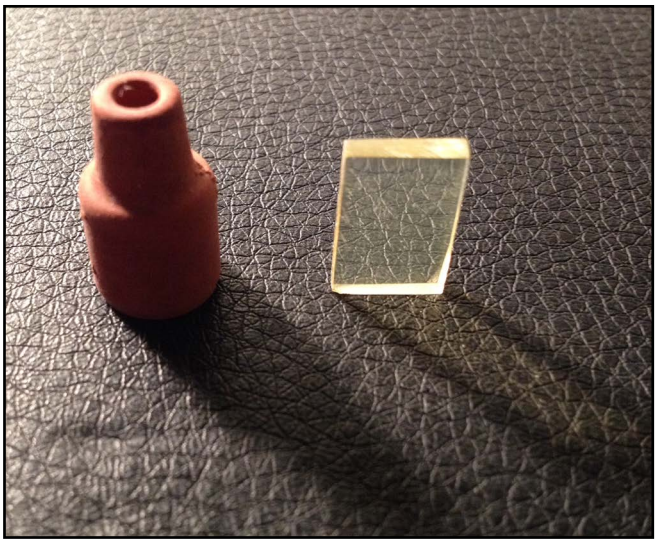


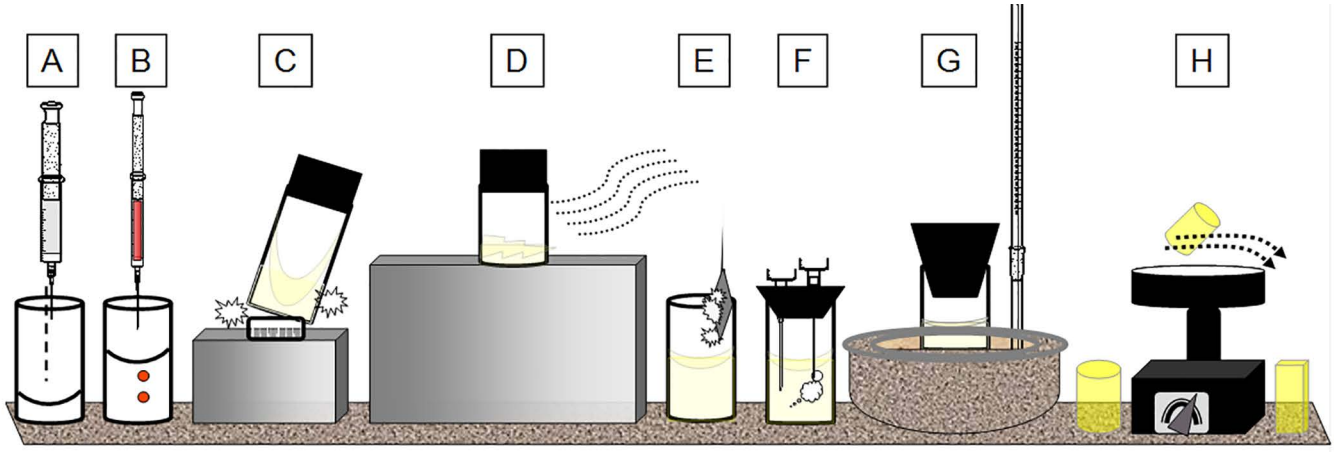
Figure 3. Image of an orthogonal solidified reverse micelle composite material of good optical quality pictured with a septum for scale.

in figure 3. Any light-scattering analytes of poor optical quality (translucent or opaque) due to incomplete micelle formation or crystalline regions in the solidified polymer nonpolar phase were not subjected to spectroscopic assays. Spectral intensities were normalized. Figure 4 summarizes the composite preparation methodology.

RESULTS AND DISCUSSION

Emission fluorimetry data on the $[Ru(bpy)_3]Cl_2$ molecular sensor was obtained for the reverse micellar solutions prior to the solidification (polymerization) of the nonpolar styrene:divinylbenzene phase and is presented in table 2 and figure 5. The fluorimetry data was compared to that of an aqueous solution of $[Ru(bpy)_3]Cl_2$ for which the emission spectrum consists of a broad band centered at 600 nm (Rajkumar *et al.* 2014). The 10 mM AOT surfactant reverse micellar solution shows a 600 nm emission maximum and the peak is not as broad, because the critical micelle concentration is not met causing incomplete micelle formation. The remaining $[Ru(bpy)_3]Cl_2$ – sequestered reverse micellar solutions show broad peaks in their emission spectra, yet the emission maxima red-shifted to 630 nm compared to aqueous $[Ru(bpy)_3]Cl_2$ appearing at 600 nm. The peak broadness corroborates that the $[Ru(bpy)_3]Cl_2$ dye is subject to one averaged chemical environment from different vibrational energy states afforded by solution (Minchor *et al.* 2014; Shirota *et al.* 1999). The red-shift is due to an increase in chemical environment polarity about the $[Ru(bpy)_3]Cl_2$ dye stemming from Coulombic association with the AOT surfactant polar head groups within the reverse micelles (Yam *et al.* 2015; Che-Sheng *et al.* 2014).

Trace 2,2'-azobis(2-methylpropionitrile) radical polymerization initiator was added to the $[Ru(bpy)_3]Cl_2$ – sequestered reverse micellar solutions composed of 2:1 styrene:divinylbenzene nonpolar phase, AOT surfactant, and water polar phase. Heating the septum-capped reverse micellar solutions to 40.0 °C sand bath over 104 hours yielded solidified composites. If optical quality is low, then the light scattering due to crystalline regions or disrupted micelles is not amenable to spectroscopic assays and such composites were not investigated. Still, composites of 50, 60, and 70 mM AOT surfactant concentrations were of high optical



A	Injection of 4 mL of 50 mM AOT in 2:1 styrene:divinylbenzene
B	Injection of 10 µL of 10 mM $[Ru(bpy)_3]Cl_2$ in water
C	Agitation for dissolution
D	Sonication for dissolution
E	Addition of trace 2,2'-azobis(propionitrile) radical polymerization initiator
F	Nitrogen gas purging
G	Solidification of reverse micellar solution nonpolar phase over 106 hours at 40 °C
H	Grounding and polishing cylindrical composites into orthogonal composites

Figure 4. Schematic diagram and tabular information for synthesis of solidified reverse micellar solutions.

Table 2. Fluoresence emission wavelength maxima for a series of reverse micelle-sequestered $[Ru(bpy)_3]Cl_2$ chemical sensors of variable AOT surfactant concentration prior to styrene:divinylbenzene nonpolar phase polymerization.

Reverse Micellar Solution	[AOT] (mM)	Vsty:dvb (mL)	$[[Ru(bpy)_3]Cl_2]$ (mM)	V$[Ru(bpy)_3]Cl_2$ in DDI (µL)	Emission Wavelength For Reverse Micellar Solutions (nm)
1	10	4.0	10.0	10.0	600.0
2	20				626.5
3	30				626.0
4	40				628.0
5	50				627.5
6	60				627.5
7	70				628.5
8	80				629.0
9	90				625.0

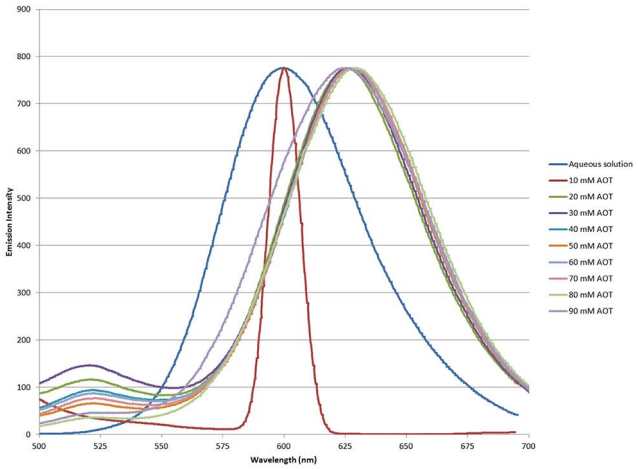


Figure 5. Fluorescence emission spectra of an aqueous solution of $[Ru(bpy)_3]Cl_2$ and a series of reverse micelle-sequestered $[Ru(bpy)_3]Cl_2$ chemical sensors of variable AOT surfactant concentration prior to styrene:divinylbenzene nonpolar phase polymerization.

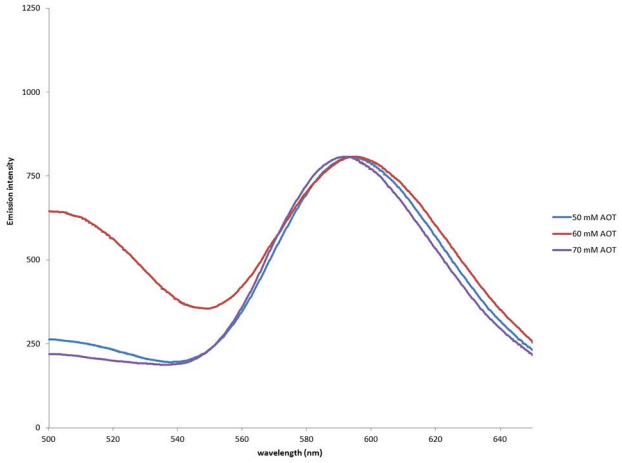


Figure 6. Fluorescence emission spectra of micelle-sequestered $[Ru(bpy)_3]Cl_2$ chemical sensors of variable AOT surfactant concentration after styrene:divinylbenzene nonpolar phase solidification (polymerization).

quality. The cylindrical composites were shaped by sanding to orthogonal dimensions and then polished. Emission fluorimetry data on the $[Ru(bpy)_3]Cl_2$ – sequestered chemical sensors for these solidified composites of polymerized nonpolar phases was obtained. The $[Ru(bpy)_3]Cl_2$ emission spectra again showed broad bands centered at 595 nm. This data indicates that the $[Ru(bpy)_3]Cl_2$ is surrounded by micellar aqueous water thereby allowing the sensor to retain its solution state chemistry essential to its molecular sensor qualities (Tummala *et al.*2015). This is a very important result and shows that the micelles withstood the 40 °C temperature, the heating time, and and the polymerization hardening of the outer nonpolar phase. If the micelles would have broken apart, then the emission maxima peaks would be sharp, not broad. The $[Ru(bpy)_3]Cl_2$ emission spectra bands are blue-shifted for the solidified composites compared to the reverse micellar solutions and in so doing approach the emission maximum wavelength of $[Ru(bpy)_3]Cl_2$ in water at 600 nm. Surfactant concentrations of 50 – 70 mM proved successful for sensor materials synthesis as presented in table 3 and figure 6.

Table 3. Fluorescence emission wavelength maxima for a series of reverse micelle-sequestered $[Ru(bpy)_3]Cl_2$ chemical sensors of variable AOT surfactant concentration after styrene:divinylbenzene nonpolar phase solidification (polymerization) measured for non-light scattering composite materials of high optical quality.

Reverse Micellar Composite	[AOT] (mM)	Vsty:dvb (mL)	$[[Ru(bpy)_3]Cl_2]$ (mM)	V$[Ru(bpy)_3]Cl_2$ in DDI (µL)	Emission Wavelength For Reverse Micellar Composites (nm)
10/10	10	4.0	10.0	10.0	
20/10	20				
30/10	30				
40/10	40				
50/10	50				594.0
60/10	60				595.5
70/10	70				592.0
80/10	80				
90/10	90				

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A CHECKLIST AND DISTRIBUTIONAL SYNTHESIS OF AMPHIBIAN AND REPTILE SPECIES IN COLUMBIA COUNTY, PENNSYLVANIA[1]

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ABSTRACT

Species inventories and distributional analyses at fine resolutions, such as at the county level, can provide a wealth of data to inform biological research and management as well as provide important baseline data for long-term biological monitoring. This work represents the first herpetological species and distributional inventory for Columbia County in northeastern Pennsylvania. A review of literature, museum records, and vouchered submissions on the Pennsylvania Amphibian and Reptile Survey (PARS) database confirmed the occurrence of 39 species of amphibians and reptiles within the county. The Eastern Musk Turtle (*Sternotherus odoratus*) is reported herein as a new county record. Eleven species confirmed within Columbia County are of conservation concern. Known township occurrence data, compiled from museum records, published literature, and field observations are presented for 28 non-imperiled species and (with township names omitted) for seven imperiled species. This work contributes to the understanding of Pennsylvania's herpetofauna and provides important baseline data for long-term monitoring and future research on amphibian and reptile species within Columbia County. [J PA Acad Sci 90(1): 7-12, 2016]

INTRODUCTION

The occurrence and distribution of amphibians and reptiles within the commonwealth of Pennsylvania was initially compiled by McCoy (1982) and updated by Hulse *et al.* (2001). More recently, the Pennsylvania Amphibian and Reptile Survey (PARS), an online atlas project (www.paherpsurvey.org), is currently expanding upon the distribution of Pennsylvania's herpetofauna via the submission of voucher photographs by volunteers. However, species inventories focused upon smaller geographic regions such as at the county level can provide data at a finer resolution to inform biological research, wildlife management, and conservation work implemented on both small and large geographic scales. Furthermore, these can provide important baseline data for long-term species monitoring (Chambers, 2006). Within Pennsylvania, county herpetological inventories have been compiled and published historically for several counties (e.g., Lehigh County, Mattern and Mattern 1917; Union County, Pawling, 1939; and Venango County, Swanson, 1948; 1952). More recently, exhaustive inventories have been compiled for several counties from extensive review of museum holdings and literature in combination with field observations (e.g., Indiana County, Chambers, 2006; Erie County, Gray and Lethaby 2008; 2012).

No effort has been conducted focusing exclusively upon the occurrence and distribution of amphibian and reptile species within Columbia County, Pennsylvania. Columbia County, located in the northeastern portion of the commonwealth, covers approximately 1,300 km^2 of area including ca. 18 km^2 of water and is divided into 24 townships (Figure 1.) The county is bordered by Montour and Northumberland counties (west), Luzerne County (east), Schuylkill County (south) and Lycoming and Sullivan counties (north) and bisected approximately through the center by the north branch of the Susquehanna River. The majority of the county is encompassed within the Valley and Ridge physiographic province, with the northernmost portion reaching into the Alleghany Plateau (Columbia County NAI, 2004). Many sections of Columbia County have been historically timbered, are agricultural or developed areas; however, the county contains many large tracts of forested land and several wetland areas, which provide habitat for many organisms, including amphibian and reptile species (Columbia County NAI, 2004). This paper aims to 1) compile a comprehensive species inventory of confirmed amphibian and reptile species occurring within Columbia County via examination of published literature, museum records, and vouchered submissions on PARS; 2) of these, identify species of conservation concern that occur within the county; and 3) compile an inventory of township-level occurrence for each species confirmed within the county via examination of available museum records (spanning from the late 1950s to the early 1990s), published literature, and field observations.

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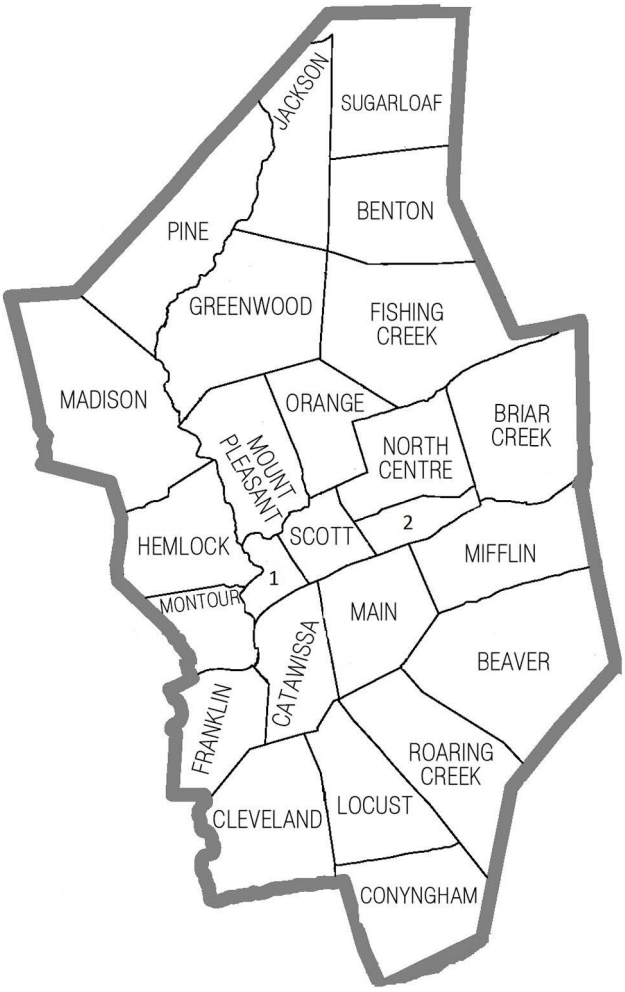

Figure 1. Township map of Columbia County, Pennsylvania. (1 = Bloomsburg; 2 = South Centre Township).

METHODS

Records concerning amphibian and reptile species occurrence within Columbia County were compiled from literature review (e.g., McCoy, 1982; Hulse *et al.* 2001; and other references reported herein), examination of museum holdings on VertNet (2015) and specimens held within the herpetology collection at Bloomsburg University. Additionally, vouchered submissions (i.e., verified photographs) were searched on the Pennsylvania Amphibian and Reptile Survey (PARS) website (www.paherpsurvey.org) to confirm the occurrence of species within Columbia County not represented by museum specimens or reported in the literature. The conservation status of amphibian and reptile species in Pennsylvania occurring in Columbia County was noted based on species descriptions on PARS (2015). Data regarding species occurrence in townships were compiled from examination of museum collection records, published literature, and from unvouchered field observations conducted opportunistically by the author from summer 2005 through fall 2015. Records of species occurrence in the municipality of Bloomsburg (considered a town), while not technically a township, were considered as such in this analysis, as the Bloomsburg municipal zone covers a geographic area comparable in size to that of a township (Figure 1). Records occurring in municipalities below the township level (i.e., boroughs) were considered as occurring within the township that encompassed that municipality. Scientific and common names herein follow Crother (2012).

RESULTS

Thirty-nine amphibian and reptile species are confirmed to occur within Columbia County (11 salamanders, 9 anurans, 6 turtles, 1 lizard, and 12 snakes). Eleven species (1 salamander, 2 anurans, 3 turtles, 1 lizard, and 4 snakes) confirmed to occur within Columbia County are considered species of conservation concern within Pennsylvania (according to information on PARS, 2015). Township occurrence data compiled from museum holdings and field observations are available for all non-imperiled species and seven imperiled species (township names are omitted for imperiled species to discourage unauthorized collecting).

Salamanders

Ambystoma jeffersonianum (Jefferson Salamander). Reference: PARS (2015). Townships: no data. Status: PA species of special concern.

Ambystoma maculatum (Spotted Salamander). Reference: Surface (1913). Townships: Beaver, Conyngham, Locust, Mifflin, Roaring Creek, Sugarloaf.

Desmognathus fuscus (Northern Dusky Salamander). Reference: Hulse *et al.* (2001) Townships: Bloomsburg, Greenwood, Hemlock, Madison, Main, Mt. Pleasant, Scott, South Centre, Sugarloaf.

Desmognathus ochrophaeus (Allegheny Mountain Dusky Salamander). Reference: Hulse *et al.* (2001). Townships: Bloomsburg, Greenwood, Hemlock, Madison, Mt. Pleasant, Scott.

Eurycea bislineata (Northern Two-lined Salamander). Reference: Hulse *et al.* (2001). Townships: Conyngham, Greenwood, Fishing Creek, Hemlock, Locust, Madison, Mt. Pleasant, Orange, South Centre.

Eurycea longicauda (Long-tailed Salamander). Reference: Hulse *et al.* (2001). Townships: Bloomsburg, Greenwood, Mt. Pleasant, Scott.

Gyrinophilus porphyriticus (Spring Salamander). Reference: Hulse *et al.* (2001). Townships: Bloomsburg, Greenwood, Scott, South Centre.

Notophthalmus viridescens (Eastern Newt). Reference: Hulse *et al.* (2001). Townships: Beaver, Benton, Bloomsburg, Catawissa, Cleveland, Conyngham, Locust, Scott, Sugarloaf.

Plethodon cinereus (Eastern Red-backed Salamander). Reference: Hulse *et al.* (2001). Townships: Benton, Bloomsburg, Cleveland, Conyngham, Fishing Creek, Hemlock, Madison, Main, Mt. Pleasant, North Centre, Scott, South Centre.

Plethodon glutinosus (Northern Slimy Salamander). Reference: Hulse *et al.* (2001). Townships: Beaver, Catawissa, Greenwood, Hemlock, Locust, Main, Mt. Pleasant, Pine, Roaring Creek, South Centre.

Pseudotriton ruber (Red Salamander). Reference: Hulse *et al.* (2001). Townships: Bloomsburg, Greenwood, Locust, Madison, Main, Mt. Pleasant, Roaring Creek.

Frogs and Toads

Anaxyrus americanus (American Toad). Reference: Hulse *et al.* (2001). Townships: Beaver, Benton, Bloomsburg, Cleveland, Conyngham, Fishing Creek, Greenwood, Locust, Main, Mifflin, Montour, North Centre, Orange, Roaring Creek, Scott, South Centre.

Anaxyrus fowleri (Fowler's Toad). Reference: PARS (2015). Townships: no data. Status: PA species of special concern.

Hyla versicolor (Gray Treefrog). Reference: PARS (2015 [audio & photo vouchers]). Townships: Conyngham, Locust, Roaring Creek.

Lithobates catesbeianus (American Bullfrog). Reference: Hulse *et al.* (2001). Townships: Catawissa, Montour, North Centre, Scott.

Lithobates clamitans (Green Frog). Reference: Hulse *et al.* (2001). Townships: Bloomsburg, Cleveland, Conyngham, Fishing Creek, Hemlock, Locust, Madison, Montour, Mt. Pleasant, North Centre, Scott.

Lithobates palustris (Pickerel Frog). Reference: Hulse *et al.* (2001). Townships: Cleveland, Greenwood, Locust, Scott.

Lithobates pipiens (Northern Leopard Frog). Reference: PARS (2015). Townships: no data. Status: PA species of special concern.

Lithobates sylvaticus (Wood Frog). Reference: Hulse *et al.* (2001). Townships: Beaver, Benton, Cleveland, Conyngham, Fishing Creek, Locust, Roaring Creek, Sugarloaf.

Pseudacris crucifer (Spring Peeper). Reference: Hulse *et al.* (2001). Townships: Cleveland, Conyngham, Locust, Scott.

Turtles

Chelydra serpentina (Snapping Turtle). Reference: Hulse *et al.* (2001). Townships: Benton, Bloomsburg, Catawissa, Franklin, Hemlock, Main, Mifflin, Mt. Pleasant, North Centre, Scott, South Centre.

Chrysemys picta (Painted Turtle). Reference: Surface (1908). Townships: Bloomsburg, Briar Creek, Catawissa, Mifflin, Mt. Pleasant, Montour, North Centre, Scott, South Centre.

Clemmys gutatta (Spotted Turtle). Reference: Columbia County NAI (2004). Townships: confirmed in three townships. Status: PA species of special concern.

Glyptemys insculpta (Wood Turtle). Reference: Hulse *et al.* (2001). Townships: confirmed in three townships. Status: PA species of special concern.

Sternotherus odoratus (Eastern Musk Turtle). Reference: none (previously unreported in the county). Townships: Montour.

Terrapene carolina (Eastern Box Turtle). Reference: Surface (1908). Townships: confirmed in seven townships. Status: PA species of special concern.

Lizards

Sceloporus undulatus (Eastern Fence Lizard). Reference: PARS (2015). Townships: confirmed in one township. Status: PA species of special concern.

Snakes

Agkistrodon contortrix (Copperhead). Reference: McCoy (1982). Townships: confirmed in two townships. Status: PA species of special concern.

Coluber constrictor (North American Racer). Reference: Surface (1906). Townships: Catawissa, Main, North Centre, Scott.

Crotalus horridus (Timber Rattlesnake). Reference: Hulse *et al.* (2001). Townships: confirmed in three townships. Status: PA candidate species for listing.

Diadophis punctatus (Ring-necked Snake). Reference: Surface (1906). Townships: Benton, Greenwood, Orange, Scott, Sugarloaf.

Heterodon platirhinos (Eastern Hog-nosed Snake). Reference: PARS (2015). Townships: no data. Status: PA species of special concern.

Lampropeltis triangulum (Milksnake). Reference: Surface (1906). Townships: Benton, Catawissa, Greenwood, Main, Pine, Sugarloaf.

Nerodia sipedon (Common Watersnake). Reference: Hulse *et al.* (2001). Townships: Bloomsburg, Briar Creek, Catawissa, Greenwood, North Centre, Scott, South Centre, Sugarloaf.

Opheodrys vernalis (Smooth Greensnake). Reference: Hulse *et al.* (2001). Townships: confirmed in one township. Status: PA species of special concern.

Pantherophis alleganiensis (Eastern Ratsnake). Reference: Hulse *et al.* (2001). Townships: Conyngham, Locust, Jackson.

Storeria dekayi (Dekay's Brownsnake). Reference: Hulse *et al.* (2001). Townships: Jackson, Locust.

Storeria occipitomaculata (Northern Red-bellied Snake). Reference: Hulse *et al.* (2001) Townships: Jackson, Sugarloaf.

Thamnophis sirtalis (Common Gartersnake). Reference: Surface (1906). Townships: Beaver, Bloomsburg, Briar Creek, Catawissa; Jackson, Madison, North Centre, Scott, South Centre.

DISCUSSION

This work represents the first consolidation of reptile and amphibian species records for Columbia County, Pennsylvania. Surface (1906; 1908; 1913), McCoy (1982) and Hulse *et al.* (2001) collectively reported the occurrence of 31 reptile and amphibian species within Columbia County. Review of museum records, PARS records, and other literature in this work confirmed the occurrence of eight additional species (1 salamander, 3 anurans, 2 turtles, 1 lizard, and 1 snake) in Columbia County, for a total county occurrence of 39 confirmed species. Six of these additional species confirmed within Columbia County fall within their expected species range in Pennsylvania (Hulse *et al.* 2001). Two species (*Sceloporus undulatus* and *Sternotherus odoratus*) confirmed within Columbia County fall outside the expected range for these taxa in Pennsylvania (Hulse *et al.* 2001). Hence, occurrence of these species within Columbia County represent range extensions for these species in Pennsylvania. The *Sternotherus odoratus* specimen reported herein from the herpetology collection at Bloomsburg University represents the first vouchered record and the first report in the literature for this species within Columbia County.

Potentially, other amphibian and reptile species occur in the county but have yet to be confirmed. For instance, several species that have not been reported in Columbia County, including the Northern Map Turtle (*Graptemys geographica*), Common Five-lined Skink (*Plestiodon fasciatus*) and Eastern Cricket Frog (*Acris crepitans*) have expected ranges within Pennsylvania that encompass Columbia County and are known to occur in adjacent counties (Hulse *et al.* 2001; PARS, 2015). It may be possible that intensive field surveys will reveal these and other species within the county. Notably, Columbia County contains eleven species of conservation concern. Of these, ten are considered "species of special concern" in Pennsylvania and one species (*Crotalus horridus*) is currently a Pennsylvania candidate species for listing. This may warrant more intensive monitoring of the county's herpetofauna.

Species with confirmed occurrence in the greatest number of townships (i.e., the broadest confirmed township distribution; > 10 confirmed townships) include *Anaxyrus americanus*, *Chelydra serpentina*, *Lithobates clamitans*, and *Plethodon cinereus*. Notably, these species are considered relatively common and abundant within Pennsylvania (Hulse *et al.* 2001). Conversely, species with few confirmed township occurrences, (i.e., *Ambystoma jeffersonianum*, *Anaxyrus fowleri*, *Heterodon platirhinos*, *Lithobates pipiens*, *Storeria occipitomaculata*, and *Sceloporus undulatus*) primarily consist of species of conservation concern and/or cryptic species (Hulse *et al.* 2001; PARS, 2015). In a survey of the herpetofauna of Indiana County, Pennsylvania, Chambers (2006) located few species known to occur in the county that were of conservation concern. The discrepancy of township records reported herein may be due in part because of a contrast in abundance between imperiled and common species (Chambers, 2006). Additionally, this discrepancy may be influenced by other factors such as a bias in historic museum collecting and field observations conducted within public or easily accessible areas (Gray and Lethaby, 2008).

Further, long-term field sampling, as well as examination of herpetological collections at smaller institutions (i.e., collection records unavailable on VertNet and other databases) may reveal the occurrence of additional species and broaden the known township-level distribution of species within Columbia County. However, this study provides a relatively robust baseline dataset regarding township-level occurrence for most species occurring within the county. Such data are valuable for understanding the abundance and distribution of species and provide an important baseline for monitoring the status of species at the county and state level (Chambers, 2006) and are especially pertinent in the wake of global amphibian and reptile declines (e.g., Gibbons *et al.* 2000; Houlahan *et al.* 2000; Stuart *et al.* 2004).

ACKNOWLEDGEMENTS

Data reported herein from the author's field notes were primarily the result of opportunistic observations, in which amphibians and reptiles were incidentally encountered and observed visually. Additional field observations from 2014-2015 were conducted under Bloomsburg University IACUC protocol # 120 and 131. I am very grateful to Michael and Sharon Hartzell for all of their support, and to three anonymous reviewers for comments which greatly improved this manuscript. Additionally, I thank Clay Corbin for allowing me to examine herpetology specimens in the Bloomsburg University Collection.

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APPENDIX

Museum Specimen Records Used

Specimens reported from the uncatalogued herpetology collection at Bloomsburg University are listed as "BUHERPS (number of specimens)." All other collection abbreviations follow Perez (2014).

Salamanders

Ambystoma maculatum: BUHERPS (3); *Desmognathus fuscus*: CM 54725 – 54727, CM 68605, USNM 3905, USNM 388657; *Desmognathus ochrophaeus*: CM 116208 – 116217, CM 68607 – 68633, CM 68652 – 68654; *Eurycea bislineata*: BUHERPS (3), CM 116218 – 116221, CM 37196, CM 68599 – 68602, OSUM 4483, OSUM 4487; *Eurycea longicauda*: BUHERPS (1), CM 68603; *Gyrinophilus porphyriticus*: CM 37202, CM 37203, CM 68634, FMNH 208274, FMNH 208275; *Notophthalmus viridescens*: BUHERPS (2), USNM 3810, USNM 388619, USNM 277508; *Plethodon cinereus*: USNM 388622 – 388652, USNM 388658 – 388696; *Plethodon glutinosus*: BUHERPS (2), CM 37199, CM 69635 – 68636, UMMZ 53233, USNM 388625, USNM 388654 – 388655, USNM 388618, USNM 388689 – 388696; *Pseudotriton ruber*: CM 144563, USNM 388656.

Frogs and Toads

Anaxyrus americanus: CM 37204; *Lithobates clamitans*: BUHERPS (1), CM 68637 – 68640, FMNH 208308; *Lithobates palustris*: CM 37205, CM 68647 – 68651; *Lithobates sylvaticus*: USNM 388688.

Turtles

Chelydra serpentina: BUHERPS (1); *Clemmys gutatta*: BUHERPS (1); *Sternotherus odoratus*: BUHERPS (1); *Terrapene carolina*: BUHERPS (2).

Snakes

Coluber constrictor: CM 37194; *Crotalus horridus*: BUHERPS (1), USNM 16283; *Diadolphus punctatus*: CM 68641 – 68645, CM 144490 – 144491, CM 144500 – 144503, CM 144512, CM 144548, CM 144551 – 144554, CM 144556 – 144557, CM 144559, CM 144563 – 144564; *Lampropeltis triangulum*: CM 37193, CM 144492, CM 144513 – 144514, CM 144558, CM 144560, CM 144594; *Nerodia sipedon*: CM 37201, CM 144549, CM 68646; *Opheodrys vernalis*: CM 123169; *Pantherophis alleganiensis*: CM 123171; *Storeria dekayi*: BUHERPS (1), CM 123174; *Storeria occipitomaculata*: CM 123170, CM 144550; *Thamnophis sirtalis*: CM 37200, CM 144555, CM 144561, CM 144587.

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THE USE OF A BRINE SHRIMP ASSAY TO DETECT BIOACTIVITY IN THE ENDOPHYTE-INFECTED GRASS, *AGROSTIS HYEMALIS*[1]

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ABSTRACT

Endophytic fungi of grasses in the genus *Epichloë* produce protective alkaloids that are known to deter herbivory. In addition to direct measurement of alkaloids, various feeding tests are used to assess bioactivity. Brine shrimp (*Artemia salina*) assays have been used to detect bioactivity in plants and fungi, including endophytic fungi of medicinal plants. We tested the utility of a brine shrimp assay to detect bioactivity in the endophyte-infected (symbiotic) grass *Agrostis hyemalis*. We exposed brine shrimp larvae (nauplii) to whole plant aqueous extracts as well as to fermentation cultures of the isolated fungus and observed nauplii survival after 24 hours. Whole plant extracts of symbiotic grasses reduced survival, compared to brine shrimp survival in the extracts of grass lacking the endophyte. Furthermore the effect of symbiotic grass extracts was dose-dependent, with reduced survival in the higher concentration compared to lower. These results suggest that the brine shrimp assay detects bioactivity of endophyte-produced toxins. This short-term assay is simple, inexpensive, requires no specialized equipment, is suitable for student research projects, and could potentially serve as an initial screening of symbiotic grasses for alkaloid production. [J PA Acad Sci 90(1): 13-20, 2016]

INTRODUCTION

Fungal endophytes are ubiquitous plant symbionts that live within their host tissues (Carroll 1988; Clay 1990; Strobel & Daisy 2003; Rodriguez *et al.* 2009). They may be systemic or localized within the host; some are completely asymptomatic, while others exist epiphytically at certain stages of their life cycles. These fungi typically produce a range of biologically active molecules, some of which have the potential for agricultural and pharmaceutical applications (Gunatilaka 2006; Kaul *et al.* 2012; Petrini *et al.* 1993; Schultz *et al.* 2002).

Endophytic fungi in the family Clavicipitaceae, which infect cool-season grasses, are systemic to aboveground plant parts, growing asymptomatically in the intercellular apoplasm of their hosts during all or most of their life cycles (Clay 1990; Schardl *et al.* 2004). These endophytes belong to the genus *Epichloë*, which has recently been realigned to include the anamorphic *Neotyphodium* species (Leuchtmann *et al.* 2014). The associations (symbiota) of epichloid endophytes and their grass hosts have been studied fairly intensely, especially after the connection was made between endophyte infection of forage grasses and livestock toxicity when grazing on infected grasses (Bacon 1995). The grass-endophyte association is often mutualistic, resulting in both the grass and endophyte benefitting from the association. The endophyte is assumed to benefit because it gains all of its nutrients from the host, as well as protection from desiccation within the host (White *et al.* 2000). Benefits gained by the symbiotic grass include protection from herbivores and other biotic stresses, increased competitive ability, as well as greater drought tolerance and resistance and/or tolerance to other abiotic stresses (reviewed by Kuldau and Bacon 2008; Malinowski & Belesky 2000; Schardl *et al.* 2007; Schardl *et al.* 2004). But perhaps the most important benefit conferred to grasses by epichloid endophytes is the production of bioprotective alkaloids, which serves as the basis for classifying these associations as defensive mutualisms (Clay 1988; Panaccione *et al.* 2014). Epichloid endophytes are known to produce four main classes of alkaloids: ergot alkaloids, indole-diterpenes, lolines, and peramine (Panaccione *et al.* 2014). The majority of these endophytes produce only one or two of the alkaloid classes, while a few produce three; although some symbiotic grasses lack alkaloids altogether (Leuchtmann *et al.* 2000; Schardl *et al.* 2012; Siegel *et al.* 1990; TePaske *et al.* 1993).

The organisms affected by each alkaloid class and the mechanisms of bioactivity of the alkaloid classes differ. All classes of alkaloids have some activity against invertebrates (Schardl *et al.* 2013). Ergot alkaloids and indole-diterpenes are toxic to grazing mammals and other vertebrates. Lolines and peramine have little, if any, negative effects on vertebrates. Rather, they provide resistance to insects and

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nematodes. Studies suggest that peramine is effective as a feeding deterrent and also impedes growth and development, whereas lolines have broad insecticidal activity (reviewed by Bush *et al.* 1997; Panaccione *et al.* 2014; Schardl *et al.* 2013).

There are several methods for determining the levels of bioprotective alkaloids produced by symbiotic grasses. The genes involved in alkaloid production have been identified (Tanaka *et al.* 2005; Spiering *et al.* 2005; Young *et al.* 2006). Typically, alkaloids are directly detected and measured typically utilizing HPLC or TLC separation, UV detection, mass spectrometry, gas chromatography (reviewed by Tapper and Latch 1999), as well as ELISA techniques (Agrinostics n.d.; reviewed by Barker *et al.* 2009). Direct measurement can be somewhat limited when certain compounds may be present below the limits of detection. In addition, methods are often limited to the measurement of the target alkaloids, though other unknown bioactive metabolites may be present (Brem & Leuchtmann 2001; Rasmussen *et al.* 2009). Furthermore, the direct measurement of alkaloids provides no information on possible synergistic effects of combinations of alkaloids on herbivores.

Thus bioassays remain a valuable research tool. Survival and/or choice tests using a number of different organisms have been used, including typical pests of grasses as well as naïve herbivores (reviewed by Kuldau & Bacon 2008). The effects of endophyte-infection of grasses on herbivores typically employ feeding tests, which may require rearing facilities to maintain test organisms, permits to work with those that are agricultural pests, often several weeks to months to run the tests, and relatively large amounts of plant material depending on the test organism and length of the assay.

A potential screening for endophyte bioactivity that overcomes many previously mentioned drawbacks is a brine shrimp assay (BSA). The assay is simple, relatively rapid, inexpensive, and requires relatively small amounts of plant material (McLaughlin *et al.* 1998; Meyer *et al.* 1982). Brine shrimp, *Artemia salina* L, have proven useful to screen for general bioactivity and have been widely used for bioassays in other contexts (McLaughlin *et al.* 1998; Miller 2011). Colonies of test organisms do not have to be maintained, as the lifecycle of *A. salina* consists of a dormant cyst stage from which the larvae (nauplii) quickly emerge, within approximately 24 hours, when placed in salt water. And the assay itself requires little time, with results obtained within 24 hours (McLaughlin *et al.* 1998). A brine shrimp lethality assay involves exposing brine shrimp nauplii to the chemical or substance in question and measuring the proportion that survive exposure (McLaughlin *et al.* 1998). Conducting the assay at several concentrations allows the calculation of the concentration resulting in 50% mortality of the test organisms (LC50), thus providing a method to quantify the toxicity or pharmacological activity of the substance.

Several applications of the BSA suggest its utility in screening epichloid symbiota for bioactivity. It is widely used to screen both plants and fungi for the presence of bioactive and toxic molecules; examples include traditional medicinal plants (Krishnaraju *et al.* 2005; Moshi *et al.* 2006; Olowa & Nuñeza 2013; Pimental Montanher *et al.* 2002 and others), endophytes isolated from medicinal plants (Eyberger *et al.* 2006; Lu *et al.* 2011; Shoeb *et al.* 2014), and fungal contaminants of foods for humans and livestock (Davis, Wagener, Dalby, Morgan-Jones & Diener 1975; Davis, Wagener, Morgan-Jones & Diener 1975; Harwig & Scott 1971; but see Prior 1979). As a test for general bioactivity, brine shrimp are ideal because as a naïve test subject they have no recent evolutionary history with grass/endophyte symbiota, thus evolved resistance is not a factor and general toxicity is measured.

As a first step in determining the utility of the BSA in testing the toxicity of epichloid symbiota, we tested the sensitivity of brine shrimp to whole plant aqueous extracts and fermentation cultures of the isolated fungus of the symbiotic grass *Agrostis hyemalis* (Walter) Britton, Sterns & Poggenb.

MATERIALS AND METHODS

Plant Material

Agrostis hyemalis is naturally infected with *Epichloë amarillans* J. F. White (Craven, *et al.* 2001), a fungal endophyte that produces bioactive alkaloids (Schardl *et al.* 2012). *Agrostis hyemalis* plants were grown and maintained in the King's College, Wilkes-Barre, PA, greenhouse using seeds collected from one plant in a small population in Luzerne County, Pennsylvania (41° 19' 53" N, 75° 47' 54" W). We confirmed endophyte presence in these plants by several established techniques: staining followed by microscopic examination of leaf sheaths, pith, and seeds as well as culture of the endophyte in cornmeal-malt agar from nodes and seeds (Clark *et al.* 1983). The growth and morphology of the endophyte in culture was consistent with *Epichloë* endophytes. All plants established from the collected seeds were determined to be endophyte-infected. The established plants flowered in the greenhouse and we harvested seeds. To generate uninfected plants, we heat-treated the harvested seeds in a 60-degree drying oven for 0, 2, and 4 days and used those seeds to establish additional *A. hyemalis* plants. Previous studies have shown heat treatment of seeds is an effective method for eliminating endophyte infection in grasses (Davitt *et al.* 2010). We confirmed absence of infection on a subset of the plants from heat-treated seeds and presence in the 0-day heat-treated seeds.

Fungal Cultures

We allowed the fungal cultures of the endophyte isolated on cornmeal-malt agar to proliferate before transferring to liquid media. Media used were M102 (pH 5.79) and M43 (pH 6.0) as described by Bacon and White (1994). We transferred ~0.5 cm^2 blocks of fungal mycelium with associated agar to the liquid media and allowed to incubate at 26° C for three weeks.

Preparation of Plant Extracts

We clipped vegetative plant material (including leaves, sheaths, and pseudostems) from a single plant per trial. Three grams (wet weight) of leaves were blended for approximately 5 minutes with 100 mL of 1% marine salt solution (distilled water and Instant Ocean® Sea Salt, Blacksburg, VA). The blended mixture was first strained through a piece of 1 mm^2 nylon mesh, which removed the largest pieces of grass that remained after blending. The extract was then filtered with a Buchner funnel and #4 Whatham filter paper. We conducted a preliminary brine shrimp assay (using the protocol described below) with plant material from symbiotic plants to determine which of the plant extract components would cause the greatest reduction in brine shrimp survival. Three treatments using the final filtrate and the plant material filtered by the mesh and the filter paper were preliminarily tested. In this preliminary trial, the treatments differed in their effect on brine shrimp survival (χ^2= 17.22, df= 2, $p<0.001$). The filtrate treatment resulted in substantially reduced brine shrimp survival (Table 1).

For the trials we used the filtrates of the extracts, which were prepared from the following plants: symbiotic *A. hyemalis* plants from 0-day heat-treated seeds (E+) and plants from 2-day and 4-day heat-treated seeds (2-day and 4-day) presumed to have little to no endophyte surviving though this was not tested directly. With each of the 0-day, 2-day and 4-day plant extract treatments we conducted two trials at a 0.03 g/ml concentration, each using a different plant. We conducted an additional trial of an E+ and a 4-day plant that was confirmed uninfected by microscopic examination of leaf sheaths. In this trial we had less plant material available so we ground 1.5 g (wet weight) of plant material and 50 ml of 1% saltwater solution (prepared as above) with a mortar and pestle. We created a reduced plant extract concentration of 0.006 g/ml for this trial by diluting the filtrate 1:4 with 1% salt solution.

Preparation of Fungal and Media Treatments

We prepared fungus grown in M102 and M43 media for assay in a similar manner as the plant extracts, i.e. we blended fungal mycelium in a blender with 1% salt solution, filtered this through #4 Whatham filter paper in a Buchner funnel, and added 5 mL of the filtrate to 10 ml glass vials.

As a control, we also tested the effect of fungal culture media (alone and with growing fungal cultures) on brine shrimp survival. We adjusted the pH of the media to 6.5 and then added 1 ml of each liquid media to 4 ml of 1% salt solution in glass vials. Media without fungal growth and media after three weeks of fungal growth were compared to extracts from 0-day, 2-day, and 4-day plant extracts.

Brine Shrimp Assay

Brine shrimp nauplii (Carolina Biological Company, Burlington, NC) were hatched from cysts in a 1% salt solution (prepared as above) in a 2.5 L plastic aquarium under artificial light (12/12 hour light/dark cycle) with aeration. The brine shrimp were not fed, as they are known to consume their yolk sack in the first stage of development (Sorgeloos *et al.* 1978). For all assays, 48-hour (post initiation of hatching) brine shrimp nauplii were used, as this age had been previously determined to be optimal age for sensitivity in bioassays (M. Kolbeck, unpublished data). The BSA consisted of 10 brine shrimp per 5 ml of plant extract or 1% salt solution control in 10 ml uncovered glass vials, with 5 replicate vials per treatment and trial. The assays were conducted at room temperature for 24 hours, after which we assessed brine shrimp survival by pouring the contents of the vials, individually, to a watch glass and counting the number alive under a dissecting microscope (80x). We considered a brine shrimp nauplius alive if it was actively moving.

Table 1. The results of preliminary tests to determine plant extract components with the greatest activity against brine shrimp survival. These treatments were the coarse, fibrous plant tissue that did not pass through the mesh (coarse treatment); solid matter that did not pass through the filter paper (fine treatment); and the liquid filtrate that passed through both the mesh and the filter paper (filtrate treatment). The coarse and fine materials were each added to a 1% salt solution.

	Coarse treatment	Fine treatment	Filtrate treatment
Mean percent brine shrimp survival (+/- SEM)	92% (3.7%)	100% (0%)	34% (8.7%)

Data Analysis

We used a chi-square goodness of fit test to test the effect of seed treatment on brine shrimp survival. Specifically we compared the 0.03 g/ml extracts from plants grown from seeds subjected to the three levels of seed heat treatment: 0, 2, and 4 days (E+, 2-day and 4-day plants). We used a PROBIT analysis to test for the effect of extract concentration and to calculate the extract LC50 (Minitab 16, Minitab, Inc.). We tested brine shrimp survival in 0-day plant extracts at 0.006 and 0.03 g/ml concentrations.

RESULTS

Extracts differed in their effect on brine shrimp survival (Figure 1). The heat treatment of seeds prior to planting significantly affected brine shrimp survival in the 0.03 g/ml plant extracts (χ^2= 41.52, df=2, p<0.0001), with longer heat treatment resulting in greater brine shrimp survival compared to shorter and no heat treatment (mean survival in 4-day= 97%, 2-day= 67%, 0-day= 25%). Trials within treatments were similar, except the trials with grass extracts from the 2-day treated seeds (96% & 38% survival) (Table 2).

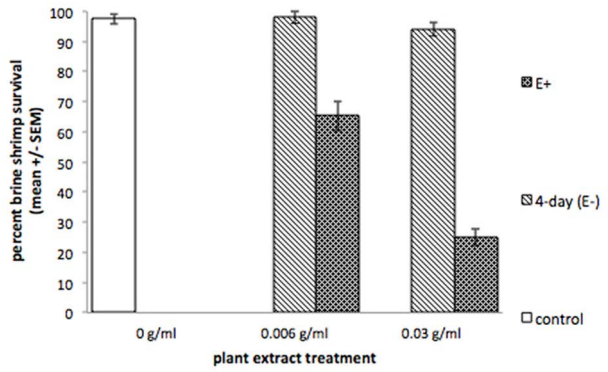

Figure 1. Mean percent brine shrimp survival in two concentrations with symbiotic *Agrostis* extracts (E+), extracts from plants grown from 4-day heat-treated seeds and in 1% salt solution control. Error bars show standard error of the mean.

Increased concentration of 0-day (E+) plant extracts decreased brine shrimp survival (coefficient = -119.643, p<0.0001). The estimated plant extract lethal concentration of 50% of the brine shrimp nauplii (LC50) was 0.020 g/ml (95% CI 0.017 – 0.023 g/ml).

Table 2. Mean percent survival per trial (standard error of the mean), where each trial was conducted on a different plant. *This trial was conducted with three brine shrimp replicates, instead of the usual five.

	Percent survival (+/- SEM)	
Extract Treatment (0.03 g/ml)	Trial 1	Trial 2
E+	24 (4.0)	26 (4.0)
2-day	96 (4.0)	38 (15.3)
4-day	94 (3.3)	100 (0)
Control	100 (0)	93 (3.3)*

Neither the cultured fungus nor the culture media after fungal growth caused brine shrimp mortality (100% survival in all treatments).

DISCUSSION

The brine shrimp assay (BSA) proved useful to detect the bioactivity of symbiotic *Agrostis hyemalis*, presumed to be naturally infected by *Epichloë amarillans* (Craven *et al.* 2001). This bioactivity observed in infected grass was not detected in uninfected grass or in grass that was presumed to be uninfected following heat-treatment of the seeds. We detected no bioactivity with the BSA in any of the fungus and media treatments, thus demonstrating that observed effects were not due to the fungus in culture.

Unfortunately we were only able to confirm the absence of the endophyte in one of the three plants from 4-day heat-treated seeds in our experiment, due to death of plants in the greenhouse. However, the results of the BSA suggest that all of the tested plants that originated from the 4-day heat treatment were free from infection; in those treatments brine shrimp survival was near 100% and similar to the survival in the 1% salt solution controls leading us to conclude that the endophyte infection was eliminated by the heat treatment. The BSA results from extracts from the 2-day heat-treated plants suggest that one plant remained infected and the other was free from infection.

Endophyte-produced alkaloids are a possible source of the bioactivity detected by the BSA. In previous surveys of alkaloid production that included the *A. hyemalis*/*E. amarillans* symbiotum, three of the main classes of alkaloids have been detected, but not consistently in all samples and not all three in any one sample. This symbiotum has been reported to produce loline, N-Acetylnorloline (NANL) (Schardl *et al.* 2007; Schardl *et al.* 2013), peramine (Siegel *et al.* 1990; Schardl *et al.* 2013), and in one study the ergot alkaloid, ergovaline (Siegel *et al.* 1990).

The genes responsible for alkaloid production have been identified and sequenced and products confirmed (Schardl *et al.* 2012). The isolate of *E. amarillans* that was analyzed possessed the specific genes that code for the loline, NANL, but not for lolines that are formed later in the biosynthetic pathway, such as N-Acetyloline (NAL) and N-Formylloline (NFL). Peramine production depends on the presence of a single gene (PerA), which was also present in *E. amarillans* (Schardl *et al.* 2013).

Peramine, lolines, and ergot alkaloids all have documented anti-insect activity making them candidates for the observed activity against brine shrimp. Furthermore, evidence from several studies suggests that these alkaloids are likely to be present in the aqueous extracts of the plant material we used. These alkaloids have been detected in cut leaf and guttation fluid of grasses (Koulman *et al.* 2007). Lolines are active against various aphids, which feed on the sugars in phloem sap, suggesting their presence in plant fluids (Siegel *et al.* 1990; Wilkinson *et al.* 2000). And that lolines have been detected in plant roots (Burhan 1984, as cited in Wilkinson *et al.* 2000) where the endophyte is not present, suggests that lolines are translocated via phloem sap.

Loline alkaloids have been shown to be directly toxic to insects in addition to acting as a feeding deterrent (reviewed by Schardl *et al.* 2007). A study using *Schizaphis graminum* to assess loline's bioactivity suggests that it is directly toxic, with activity comparable to the insecticide nicotine sulfate (Riedell *et al.* 1991). Peramine, on the other hand, primarily acts as a feeding deterrent (Rowan *et al.* 1986). The bioactivity detected in the BSA is unlikely to be caused by a feeding deterrent, as food was not provided or necessary during the assays.

The BSA could be used to test for differences in the bioactivity of endophyte-infected grasses over the course of plant development as well as the effect of abiotic growing conditions on bioactivity. When more than one class of alkaloid is present in a symbiotum and the alkaloids differ in within plant expression over time or in response to abiotic factors, alkaloid profiles are likely to also vary. For example, in one study lolines were found to consistently increase between the May and August harvests and while peramine generally increased, but the change was more variable (Leuchtmann *et al.* 2000). Roylance *et al.* (1994) found that peramine and ergovaline vary independently of each other. Further, if alkaloids are synergistic in their effect on herbivores a bioassay, such as the BSA, could be used to screen for these possible synergistic effects (e.g. Yates *et al.* 1989).

A bioassay, such as the BSA, that requires relatively small amounts of plant material could be used to test for differences in bioactivity between plant parts that are expected based on reports of differences in the various alkaloids. Loline alkaloids were found to differ between plant parts in symbiotic tall fescue, with the highest levels in seeds and decreasing amounts in stem, leaf sheath and blade (Bush *et al.* 1993). Levels of peramine also differed in symbiotic tall fescue and perennial ryegrass, with higher levels in the stem and blade and little or none in the roots (Fannin *et al.* 1990). Higher amounts of ergot alkaloids were found in the leaf sheath than in the blades (Lyons et al 1986). And some ergot alkaloids vary greatly between individual tillers of the same plant (Mace *et al.* 2014). Our technique requires less than one gram of fresh plant per 5-replicate trial. And with modifications to the BSA, the test may potentially require even less plant material. For example, a 96-well microplate brine shrimp assay has been developed that uses just 200 µl of test substance per replicate compared to 5 ml in our application of the assay (Molina-Salinas & Said-Fernández 2006).

While *Epichloë* endophyte infection appeared necessary for the bioactivity of plant extracts detected by the BSA, we did not detect activity against brine shrimp in the fungus growing outside of the host grass. Neither the fungus alone nor the media of the fermentation cultures resulted in brine shrimp mortality, although activity against brine shrimp in the spent broths of different endophytes has been reported previously (Lu *et al.* 2011). There are several possible reasons for lack of activity in the present study. It is possible that the isolated endophytes were not responsible for the observed bioactivity. Or perhaps the active compounds are only produced or produced at levels necessary to cause brine shrimp mortality *in planta* or the culture conditions necessary to produce the bioactive compounds were not provided. While all alkaloid classes have been detected in fermentation cultures of the various epichloid endophytes, the culture conditions necessary for their *in vitro* production differs (Blankenship *et al.* 2001). In a previous study, Bacon *et al.* (1977) found that methanol extracts of the endophyte from *Agrostis perennans* grown on the defined media (M96 and M102) were toxic in a chicken embryo bioassay, but M43 was not. We saw no difference in brine shrimp mortality in the two media treatments, M43 compared with M102, but our study differed in that we tested the culture media itself and not methanol extracts. Though alkaloid production has been detected in fungal cultures, the levels may not be as great as those produced by the symbiota. One study found that genes from the ergovaline gene cluster were only highly expressed *in planta* (Fleetwood *et al.* 2007). Given the success of this preliminary assessment of the brine shrimp bioassay to detect bioactivity in a symbiotic grass, there remain a number of areas that we feel should be explored. To determine the broader applicability of this assay, additional tests are necessary, including trials that compare the results of the BSA with those of other bioassays and with the responses of common grass pests and testing additional grass-endophyte symbiota, especially those with known alkaloid profiles. Future tests of BSA with known quantities of individual alkaloids will allow the determination of brine shrimp LC50 for each. Testing artificially infected grasses will help to confirm the role of *Epichloë* endophytes in brine

shrimp toxicity rather than another unknown symbiont. Finally this assay applied to endophyte-infected grasses, and more generally to defensive mutualisms, would be ideal as part of an undergraduate teaching laboratory focused on symbiotic interactions.

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RESEARCH NOTE: MULTIPLE PATERNITIES IN AMERICAN BLACK BEARS FROM NEW JERSEY[1]

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ABSTRACT

The American black bear (*Ursus americanus*) was nearly extirpated from New Jersey in the early to mid-twentieth century. The black bear is the largest land mammal in New Jersey and occupies the northwestern region of the state. Both sexes of black bears are known to practice multiple mating. In this study, we assessed paternity among bears from New Jersey. All analyses were performed using eight microsatellite loci: G10L, Mu50, G10P, G10H, G10O, G10J, G10C, and Mu59. We analyzed fifteen family units (sow and offspring) for a total of 50 cubs and yearlings for paternity. Data from eight microsatellite loci allowed assignment of multiple paternities for cubs in three out of 15 (20%) litters. [J PA Acad Sci 90(1): 21-24, 2016]

INTRODUCTION

American black bears are widely distributed and their historic range was once all of the forested regions of North America, including much of New Jersey (Hall 1981). Habitat destruction and predator control programs led to their extirpation in many areas (Hall 1981; Pelton 1982). Currently, black bears are found across Canada, the United States, and into Mexico, although in mostly localized populations. In New Jersey, black bears have been sighted in all 21 counties, but the highest densities are found in the northwest region of the state (New Jersey Division of Fish and Wildlife 2010).

Both sexes of black bears are known to practice multiple mating (Schenk and Kovacs 1995) and during the mating period males tend to roam large areas looking for receptive females. More than one male may be present with a female; however, dominant males may remain with and guard a female during this time (Barber and Lindzey 1986). Black bears also exhibit induced ovulation and delayed implantation (Wimsatt 1963). Black bears on the Chapleau Crown Game Preserve in Ontario, Canada were shown to have dual paternities within a single litter (Schenk and Kovacs 1995). Multiple paternities in black bears have also been shown in the Pisgah Bear Sanctuary in western North Carolina (Kovach and Powell 2003) and in Big Bend National Park, Texas (Onorato *et al.* 2004). Other bear species, such as grizzlies (*U. arctos horribilis*; Craighead *et al.* 1995) and polar bears (*U. maritimus*; Zeyl *et al.* 2009), also exhibit multiple paternities.

The objective of this study was to evaluate paternities in fifteen family units in New Jersey using eight microsatellite loci.

MATERIALS AND METHODS

In collaboration with NJDFW personnel, dens of radio and satellite collared sows were located in February and March of 2010. The sow was immobilized using a combination of ketamine hydrochloride (Ketaret®, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa) and xylazine hydrochloride (Rompun®, Mobay Corporation Animal Health Division, Shawnee, Kansas). Cubs were approximately 2 months of age and were not anesthetized. Tissue samples collected were in the form of ear punches using a standard leather punch measuring 4 mm in diameter. Tissue samples were collected from sow and cubs for paternity testing. Collected samples were stored in 95 percent alcohol in the field and transported back to the laboratory where they were stored at -20 °C until DNA extraction.

DNA was extracted from bear tissue using the MO BIO UltraClean Tissue & Cells DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) following standard protocols, with incubation time extended to three hours. Extraction products were quantified using a Qubit Fluorometer (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

The Polymerase Chain Reaction (PCR) was performed in a 10 µL total solution containing 5 µL Promega Master Mix (Promega, Madison, WI), 0.25 µL each of 10 µM forward and reverse primers, 1.0 µL nuclease free water and 3.5 µL

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of extracted whole genomic DNA. The eight PCR primers used included G10L, Mu50, G10P, G10H, G10O, G10J, G10C and Mu59 (Applied Biosystems, Foster City, CA) (Paetkau and Strobeck 1994, 1995, 1998; Taberlet *et al.* 1997; Paetkau *et al.* 1998). Three individual PCR protocols were utilized for these eight loci. The PCR conditions for locus G10L involved an initial denaturing step of 95 °C for 15 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 1 minute 30 seconds, and an extension at 72 °C for 1 minute 30 seconds, followed by a final extension time of 10 minutes at 72 °C. The profile for locus G10J involved an initial denaturing step of 95 °C for 15 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 57 °C for 1 minute 30 seconds, and extension at 72 °C for 1 minute 30 seconds, followed by a final extension time of 10 minutes at 72 °C. The profile for the remaining microsatellite loci (Mu50, G10P, G10H, G10O, G10C and Mu59) included an initial denaturation of 95 °C for 15 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 58 °C for 1 minute 30 seconds, and extension at 72 °C for 1 minute 30 seconds, followed by a final extension time of 10 minutes at 72 °C (Ombrello 2011). PCR products were pooled and analyzed on an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Results were analyzed using the program GeneMapper 3.7.

The genotypes of cubs and yearlings were compared to their respective sows for paternity analysis. If more than two paternal alleles were identified at one locus in a litter, then multiple paternities was confirmed. Only litters containing three or more offspring were utilized for the paternity analysis.

RESULTS

Fifteen family units (sow and offspring) containing 50 cubs and yearlings were analyzed for paternity. Multiple paternities were found in three (20%) of the family units. The family unit with sow 5959 showed multiple paternities (three paternal alleles) at locus G10P. Multiple paternities were found at loci G10O and G10J for the family unit of sow 3403. Three paternal alleles were found at locus G10H in the family unit of sow 4928 (Table 1).

DISCUSSION

The current study provides insight to mating behavior of black bears in New Jersey. Schenk and Kovacs (1995) reported that males were polygynous during a single breeding season and that male black bears do not practice extended mate guarding, but rather roam large areas and compete with other males for access to females. Reproductive success is skewed towards the largest males in a given area (Kovach and Powell 2003). Male reproductive success is further ensured because black bears are induced ovulators (Wimsatt 1963). The first male to mate with a female in estrus triggers ovulation, giving that male the advantage in fertilization (Wimsatt 1963). This type of reproductive behavior does not favor long-term mate guarding by males, but rather promotes the idea of males roaming large areas looking for receptive females.

Table 1. Genetic profiles of family units showing multiple paternities. Loci highlighted illustrate multiple paternities at that locus. Paternal alleles (*p*), maternal alleles (*m*) and paternal and maternal origins not determined (*nd*) are labeled for each cub at each locus.

Family Unit	Bear ID	Age Class	G10L		MU50		G10P		G10H		G10O		G10J		MU59		G10C	
1	5959	Adult	145	157	121	141	175	179	247	249	209	219	83	85	245	247	115	115
	5959-1	Cub	145	147	121	141	171*P*	175*M*	249	249	205	209	85	85	245	247	115	117
	5959-2	Cub	147	157	141	143	179*M*	183*P*	247	249	207	209	83	103	245	245	115	115
	5959-3	Cub	147	157	141	143	177*P*	179*M*	249	261	205	219	85	103	245	245	115	115
2	3403	Adult	147	157	139	143	171	179	243	261	205	205	99	103	243	247	109	115
	3403-1	Cub	137	157	125	143	175	179	243	253	205*M*	205*P*	103*M*	103*P*	243	247	109	119
	3403-2	Cub	145	157	125	139	179	183	243	249	201*P*	205*M*	83*P*	99*M*	245	247	115	117
	3403-3	Cub	145	147	125	143	179	183	249	261	205*M*	209*P*	99*M*	99*P*	245	247	109	117
3	4928	Adult	139	147	125	141	181	183	243	247	209	221	83	85	239	243	115	117
	4928-1	Cub	139	139	121	125	183	185	243*M*	247*P*	205	209	83	83	239	241	115	117
	4928-2	Cub	139	147	125	141	181	185	241*P*	243*M*	205	209	83	83	241	243	115	115
	4928-3	Cub	147	147	121	141	181	183	243*nd*	247*nd*	207	209	83	83	239	241	115	117
	4928-4	Cub	139	147	125	141	181	185	247*M*	255*P*	207	221	83	83	243	249	115	117

Multiple paternities occurred in 3 of 15 (20%) black bear litters sampled in New Jersey. Similar paternity studies in black bears are limited. Dual paternity was found in 25% (1 of 4) of black bear family units analyzed in northern Ontario (Schenk and Kovacs 1995). In western North Carolina multiple paternities were found in 2 of 7 (29%) family units sampled (Kovach and Powell 2003). Kovach and Powell (2003) also combined genetic testing with tracking of radio-collared black bears to determine the amount of multiple mating. Multiple paternities were also found in 1 of 5 (20%) litters examined in Texas (Onorato *et al.* 2004). It should be noted that while multiple paternity does occur in black bears, its quantification is rarely reported. The behavior of large, elusive and solitary carnivores such as these is often difficult to monitor.

Multiple paternity has also been described in other bear species, including brown bears (*U. arctos*) in Alaska (Craighead *et al.* 1995) and polar bears in Norway and Russia (Zeyl *et al.* 2009). White-tailed deer (*Odocoileus virginianus*), pronghorn antelope (*Antilocapra americana*), deer mice (*Peromyscus maniculatus*) and common shrews (*Sorex araneus*) are also known to exhibit multiple paternities (Birdsall and Nash 1973; Tegelstrom *et al.* 1991; DeYoung *et al.* 2002; Carling *et al.* 2003).

Female black bears are also polyandrous (Schenk and Kovacs 1995). Females have been observed mating with more than one male in a 24-hour period (Rogers 1987). By doing so, females are securing their own reproductive success by mating with several males (Schenk and Kovacs 1995). Sperm competition is promoted by this behavior, further enhancing female reproductive success (Parker 1970).

Our study confirmed that sows in New Jersey are mating with multiple boars. Although it cannot be confirmed with this study, it is likely that males are also practicing multiple mating. Promiscuity reduces the intensity of sexual selection, which lessens the need for mate guarding. Males have large home ranges during the breeding season, as they are looking to find receptive females, however, since the home range size of females in New Jersey is small (MacKenzie 2003; Shramko 2005) compared to other areas of the United States, males may not be required to travel as far for contact with several females in estrus.

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RESEARCH NOTE: TICKS AND TICK-BORNE PATHOGENS OF BLACK BEARS (*URSUS AMERICANUS*) IN NEW JERSEY[1]

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ABSTRACT

Information on the role that American black bears (*Ursus americanus*) play in hosting ticks and tick-borne pathogens is limited. In this study, *Dermacentor variabilis*, *Ixodes scapularis*, and blood were collected from black bears (*U. americanus*) in northwestern New Jersey in the summer of 2015. *D. variabilis* was collected from more bears and in greater abundance overall. Two hundred and fifty-six adult *D. variabilis* from 18 bears were tested for *Francisella tularensis* and *Rickettsia* spp. by polymerase chain reaction (PCR). One to five ticks were pooled into 69 groups according to sex, species, life stage, and individual host. *Rickettsia* spp. were detected in 6 of 69 *D. variabilis* pools (8.6%). All *D. variabilis* were negative for *F. tularensis*. Twenty-nine *I. scapularis* (23 adults and 6 nymphs) were collected from 11 bears and were subsequently pooled into 14 groups in the same manner. Fourteen pools of *I. scapularis* from the bears were screened for *Borrelia burgdorferi*, *Babesia* spp., *Anaplasma phagocytophilum* and *Bartonella* spp. by PCR. *B. burgdorferi* was detected in 3 of 14 pools of *I. scapularis* (21.4%), *Babesia* spp. in 2 of 14 (14.2%), *A. phagocytophilum* in 1 of 14 (7.1%), and *Bartonella* spp. in 2 of 14 (14.2%). Fourteen bear blood samples were tested for *F. tularensis*, *Rickettsia* spp., *B. burgdorferi*, *Babesia* spp., *A. phagocytophilum*, and *Bartonella* spp. by PCR. One of 14 bear blood samples was positive for *Babesia* spp. (7.1%). None of the 14 bear blood samples were positive for *F. tularensis*, *Rickettsia* spp., *B. burgdorferi*, *A. phagocytophilum*, or *Bartonella* spp. Although *Babesia* spp. were detected in black bear blood, it remains unclear whether or not this pathogen can be transmitted from infected bears to uninfected ticks. The number of studies on this relationship is limited and these findings warrant further investigation of the black bear's potential role as a reservoir.

[J PA Acad Sci 90(1): 25-30, 2016]

INTRODUCTION

Ticks have been reported to parasitize American black bears, *Ursus americanus*, throughout much of their range. American black bears and ticks removed from them have been previously examined for the presence of tick-borne pathogens, including black bears from New Jersey; however, the number of these studies is limited. In Florida and Georgia, bears were infested with *Amblyomma americanum*, *Amblyomma maculatum*, *Dermacentor variabilis*, *Ixodes scapularis*, and *Ixodes affinis* (Yabsley et al., 2009). Burguess and Huffman (2005) found six species of ticks to infest black bears, including *A. americanum*, *A. maculatum*, *Dermacentor albipictus*, *Dermacentor andersoni*, *D. variabilis*, and *I. scapularis*. However, only three such species are common in New Jersey: the blacklegged tick (*Ixodes scapularis*), the American dog tick (*Dermacentor variabilis*), and the lone star tick (*Amblyomma americanum*) (Schulze et al., 2005).

In recent years, *Borrelia burgdorferi*, *Babesia* spp., *Bartonella* spp., and *Anaplasma phagocytophilum* have emerged as pathogens of growing concern for human health. In New Jersey, Lyme disease (*B. burgdorferi*) incidence was 29 confirmed cases per 100,000 population in 2014 (CDC, 2015b). New Jersey ranked among the highest incidence states for anaplasmosis (*A. phagocytophilum*) with 3.1-136 cases per million in 2010 (CDC, 2013a). As of 2011, the incidence of babesiosis (*B. microti*) in New Jersey, a *Babesia microti*-endemic state, was between 0.5 and 4.9 cases per 100,000 persons in the northwestern counties of the state, and up to 5.0-19.9 cases per 100,000 persons elsewhere in the state (CDC, 2012). Although ticks carry some species of *Bartonella*, there is no direct evidence that they transmit the pathogen to humans and subsequently cause related infections such as cat scratch disease, caused by *Bartonella henselae*, and there is much less known regarding this

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pathogen and black bears (CDC, 2015a). Blacklegged ticks, which are vectors of *A. phagocytophilum*, *Babesia* spp., and *B. burgdorferi*, have recently been associated with black bears, as have American dog ticks (Yabsley et al., 2009; Bove, 2012; Zolnik et al., 2015), which are known vectors of *Rickettsia rickettsii* and *Francisella tularensis*, the causative agents of Rocky Mountain spotted fever and tularemia. While both diseases have been reported in New Jersey, their occurrence in humans is low, with an incidence of Rocky Mountain spotted fever of 1.5-19 cases per million in 2010 and only one case of tularemia in 2014 (CDC, 2013b; CDC, 2015c). However, black bears can be important in maintaining blacklegged and American dog tick populations in forested habitats (Zolnik et al., 2015), possibly facilitating the spread of tick-borne diseases. Of additional interest is the potential for co-infection of multiple pathogens in ticks and wildlife hosts. Co-infection has been documented in multiple tick-borne pathogen studies and can have extreme effects on the symptomology, diagnosis, and treatment of tick-borne diseases in humans (Adelson et al., 2004; Swanson et al., 2006; Aliota et al., 2014). While others have found ticks and tick-borne pathogens to be associated with black bears, it remains unclear what role black bears play as potential hosts of these pathogens (Bove, 2012; Shaw et al., 2015; Yabsley et al., 2009; Zolnik et al., 2015).

The objective of this study was to determine the tick species parasitizing black bears in New Jersey and the presence of pathogens in these bears and in the ticks parasitizing them. We collected and analyzed ticks and blood samples from black bears in northwestern New Jersey in the summer of 2015. Bear blood and tick samples were analyzed by PCR for *A. phagocytophilum*, *Babesia* spp., *Bartonella* spp., *B. burgdorferi*, *Rickettsia* spp., and *F. tularensis*. As tick borne diseases continue to spread throughout the northeastern United States, it is important to evaluate the potential contribution of many factors. While others have recently associated black bears with ticks and tick-borne pathogens, New Jersey has been of particular interest as reforestation and abundant food sources have provided ideal habitat for an increasing number of black bears in this state.

MATERIALS AND METHODS

New Jersey black bears were examined as part of yearly research trapping by the New Jersey Division of Fish & Wildlife (NJDFW) in Warren and Sussex counties during June and September of 2015. Bears were caught in Aldrich foot snares and chemically immobilized. Black bears were anesthetized with a combination of 200 mg/mL ketamine and 45 mg/mL xylazine administered via dart gun. Data collected for each animal included body measurements, weight, and sex. Biologists recorded ear-tag numbers and tattooed the right-ear tag number on the inside of the bear's lip.

Personnel from the NJDFW collected blood samples from the femoral vein of 14 bears using a BD Vacutainer Safety-Lok Blood Collection Set 21G x 3⁄4" x 12" (BD, Franklin Lakes, NJ) and transferred each one into a 7-ml BD Vacutainer K3 containing EDTA. Blood samples were stored in a cooler in the field, delivered to the laboratory, and processed within 12 h of collection. Blood samples were not obtained from all bears due to time constraints. Ticks collected from bears did not represent the entire tick population on each bear. Ticks were stored in Ziploc® bags and later identified to species and development stage using Ward's Guide to North American Ticks key (Ward's, Rochester, NY). Ticks were pooled for DNA extraction. Five or fewer ticks of the same life stage, sex, and species from one individual host were considered one pool. For example, only 4 adult, female American dog ticks removed from one bear were extracted and tested as one pool, but 3 nymph deer ticks from the same bear would be a separate pool. If more than 5 ticks of the same species, sex, and life stage were removed from one host, that group was separated into multiple pools. DNA was extracted from whole blood and ticks using a Qiagen DNeasy Blood & Tissue Kit (Qiagen, Redwood City, CA) according to the manufacturer's protocols, with the addition that ticks were cut in half and mechanically processed by a Mini-Beadbeater (BioSpec Products, Bartlesville, OK) prior to extraction. Samples were stored in 2-ml microcentrifuge tubes at -20 °C.

Ixodes scapularis and all blood samples from the bears were tested for *B. burgdorferi*, *A. phagocytophilum*, *Babesia* spp., and *Bartonella* spp. by polymerase chain reaction (PCR). *Dermacentor variabilis* and all blood samples from the bears were tested for *F. tularensis* and *Rickettsia* spp. by PCR. The PCR reactions for *B. burgdorferi* and *A. phagocytophilum* amplification were carried out in 15 µl nested reactions. The PCR reactions for *Babesia* spp., *Bartonella* spp., *F. tularensis*, and *Rickettsia* spp. amplification were carried out in 20 µl reactions. Each reaction consisted of forward and reverse primer, MgCl buffer, polymerase, dNTPs, nuclease-free water, and DNA template. The primer sequences used are listed in Table 1 and were purchased from Applied Biosystems Custom Oligo Synthesis Service (Waltham, MA).

Borrelia burgdorferi positive controls were obtained from Marten Edwards (Muhlenberg College, Allentown, PA). *Anaplasma phagocytophilum* positive controls were extracted from infected deer blood and sequenced for species confirmation. All negative controls used nuclease free water instead of DNA template. Gel electrophoresis was used to visualize the PCR results. The samples and a 100 base-pair DNA ladder were loaded into a 1.0% agarose gel with Tris-acetate-EDTA buffer and run at 90 V for 35 min. The gels were stained with ethidium bromide and PCR products were visualized under UV light.

Table 1. List of primer sequences for *Anaplasma phagocytophilum*, *Babesia* spp., *Bartonella* spp., *Borrelia burgdorferi*, *Francisella tularensis*, *Rickettsia* spp., and the target site.

Species	Primer Name	Primer Sequence	Base Pairs	Target	Reference
Anaplasma spp.	GE3a	F-CACATGCAAGTCGAACGGATTATTC	932	16S rRNA gene	Massung et al. 1998
	GE10r	R-TTCCGTTAAGAAGGATCTAATCTCC			
Anaplasma phagocytophilum	GE9f	F-AACGGATTATTCTTTATAGCTTGCT	546	16S rRNA gene	Massung et al. 1998
	GE2	R-GGCAGTATTAAAAGCAGCTCCAGG			
Babesia spp.	BAB1	F-CTTAGTATAAGCTTTTATACAGC	238	18S ribosomal gene	Persing et al. 1992
	BAB4	R-ATAGGTCAGAAACTTGAATGATACA			
Bartonella spp.	BHFTSZ965	F-GTATTCGCGAAGAAGTGGATGC	790	ftsZ gene	Ehrenborg et al. 2000
	BHFTSZ1754	R-CGACGTGGAACATAAACAGA			
Borrelia spp.	IGS1	F-GTATGTTTAGTGAGGGGGGTG	1029	16S-23S rRNA genes	Bunikis et al. 2004
		R-GGATCATAGCTCAGGTGGTTAG			
Borrelia burgdorferi	IGS2	F-AGGGGGGTGAAGTCGTAACAAG	812	16S-23S rRNA genes	Bunikis et al. 2004
		R-GTCTGATAAACCTGAGGTCGGA			
Francisella tularensis	MS2	F-CTTCAGCTAAAGATACTGCTGC	194	tul 4 gene	Higgins et al. 2000
	MA2	R-GCACTTAGAACCTTCTGGAGCC			
Rickettsia spp.	TZ15	F-TTCTCAATTCGGTAAGGGC	247	17kDa antigen gene	Tzianabos et al. 1989
	TZ16	R-ATATTGACCAGTGCTATTTC			

RESULTS

Eighteen bears were trapped in June and one in September. Two hundred and fifty-six adult *D. variabilis* were collected from all 18 bears in June and were grouped into 69 pools. A total of 29 *I. scapularis* (23 adults and 6 nymphs) were collected from 10 of the same bears which American dog ticks were removed from in June and from one bear in September and were grouped into 14 pools. Ticks were tested in pools, not as individuals. Blood samples were taken from 13 of the bears in June and the 1 bear in September from which ticks were removed. No other tick species were found on any of these bears.

Prevalence of each pathogen in ticks and blood can be seen in Table 2. The minimum percent positive ticks represents the percentage of pools positive out of total number of ticks tested and demonstrates that one pool testing positive means a minimum of one tick in that pool was positive, however, it is possible that multiple ticks in that pool are also positive. One pool of *I. scapularis*, consisting of 4 adult females, tested positive for both *B. burgdorferi* and *A. phagocytophilum*. Additionally, one *I. scapularis* nymph was co-infected with *Bartonella* spp. and *B. burgdorferi*. There were no other instances of co-infection in ticks.

DISCUSSION

American dog ticks, which vector *R. rickettsii* and *F. tularensis*, were found infesting more black bears in New Jersey and in greater abundance overall than were blacklegged ticks, which are known to vector *A. phagocytophilum*, *B. microti*, *B. burgdorferi*, and some *Bartonella* spp. The low abundance of blacklegged ticks in comparison to the great abundance of American dog ticks is likely in part due to the life cycle seasonality. In the northeastern United States, adult *I. scapularis* are prevalent in fall and these samples were mostly collected in June, when nymph *I. scapularis* are prevalent, but difficult to detect with the naked eye during brief bear examinations due to their small size (Fish, 1993). In northeastern areas such as Massachusetts and Nova Scotia, adult American dog ticks are reported to peak in May and June, when this study was conducted, which explains the high number of American dog ticks in comparison to the low number of blacklegged ticks found on the bears (Campbell, 1979; McEnroe, 1979). These findings support those of other New Jersey black bear studies by Zolnik et al. (2015) and Bove (2012) which also found *D. variabilis* most frequently on bears. While only *Rickettsia* spp. were detected in American dog ticks removed from the bears, *B. burgdorferi*, *Babesia* spp., *Bartonella* spp., and *A. phagocytophilum* all were detected in blacklegged ticks. Although only *Babesia* spp. were detected in bear blood, it is not altogether surprising that *B. burgdorferi* was not detected by PCR. A

Table 2. Results of testing *Ixodes scapularis* for *Borrelia burgdorferi*, *Babesia* spp., *Anaplasma phagocytophilum* and *Bartonella* spp., *Dermacentor variabilis* for *Francisella tularensis* and *Rickettsia* spp., and *Ursus americanus* blood for all pathogens that both species of ticks were tested for. "+ Pools" represents the total positive pools out of total pools. "Min. + Individuals" represents the minimum number of individual ticks that were positive out of total ticks collected, considering that at a minimum at least 1 tick per positive pool must be positive. "+ Individuals" represents the number of positive blood samples out of the total number of blood samples. Prevalence for each result are indicated in parentheses.

Pathogen	*Ixodes scapularis*		*Dermacentor variabilis*		*Ursus americanus* Blood
	+ Pools	Min. + Individuals	+ Pools	Min. + Individuals	+ Individuals
B. burgdorferi	3/14 (21.4%)	3/29 (10.3%)	-	-	0/14 (0%)
Babesia spp.	2/14 (14.2%)	2/29 (6.9%)	-	-	1/14 (7.1%)
A. phagocytophilum	1/14 (7.1%)	1/29 (3.4%)	-	-	0/14 (0%)
Bartonella spp.	2/14 (14.2%)	2/29 (6.9%)	-	-	0/14 (0%)
F. tularensis	-	-	0/69 (0%)	0/256 (0%)	0/14 (0%)
Rickettsia spp.	-	-	6/69 (8.6%)	6/256 (2.3%)	0/14 (0%)

better approach to indicate exposure of the bears to this and other tick-borne pathogens would be to utilize serological methods, which detect antibodies to the pathogen rather than the pathogen itself. *Borrelia burgdorferi* only circulates in the blood for a short time after which it generally resides in the tissues (Steere et al., 2004); so, absence of *B. burgdorferi* from the blood does not indicate that the bear was not exposed to *B. burgdorferi* in its lifetime and this does not suggest that individuals in this area are not at risk of Lyme disease transmission. Serological testing for various tick-borne pathogens would be a valuable addition to evaluate exposure of bears to these pathogens, should funding allow in the future.

In Florida and Georgia, ticks were collected from black bears and tested for various pathogens and putative symbionts (Yabsley et al., 2009). *Ixodes scapularis* were most prevalent on the bears, followed by *D. variabilis*, *A. americanum*, *A. maculatum*, and *I. affinis*. *Anaplasma phagocytophilum*, *F. tularensis*, and *Borrelia* spp. were not detected by PCR in any of the 128 ticks. Forty-two (32.8%) ticks, including *A. americanum*, *A. maculatum*, *D. variabilis*, and *I. scapularis*, tested positive for *Rickettsia* spp., however, none were confirmed as the pathogenic *R. rickettsii*. The researchers propose that the lack of pathogens detected in their study could be due to the low number of ticks collected or because black bears in that geographic region may not be important hosts of those pathogens. Although low tick abundance was a concern in the Florida and Georgia study, their 65 *I. scapularis* represented more than double the amount of *I. scapularis* (29) in the present New Jersey study, in which *B. burgdorferi* and *A. phagocytophilum* were both detected. The absence of *B. burgdorferi* and *A. phagocytophilum* in ticks in Florida and Georgia, both of which were detected in ticks in New Jersey, is likely due to geographic location differences, as these pathogens are more prevalent in the northeastern United States than they are in the southeastern United States (CDC, 2013a; CDC, 2015b). Another study conducted in the southern United States tested ticks removed from black bears in Louisiana for tick-borne pathogens using PCR (Leydet and Liang, 2013). They removed a combination of 86 *A. maculatum*, *D. variabilis*, *I. scapularis*, and *A. americanum* from 17 black bears, in that order of abundance. *Borrelia burgdorferi* was detected in 2 (13.3%) *I. scapularis*, *Rickettsia parkeri* in *A maculatum* (66%), *D. variabilis* (28%), and *I. scapularis* (11%), and no *A. phagocytophilum* or *B. microti* in any of the 86 ticks tested. The tick species distribution and the lack of *B. microti* and *A. phagocytophilum* contrasting what was found in New Jersey is likely due to the difference in geographic location.

Zolnik et al. (2015) found that black bear blood from New Jersey was qPCR positive for *A. phagocytophilum* and *B. microti*. Of 65 blood samples tested by qPCR, 32.3% were positive for *A. phagocytophilum*, 9.2% were positive for *B. microti*, and like the present study, none were positive for *B. burgdorferi*, which the authors also state was not unexpected. In another *U. americanus* study in New Jersey, *Babesia* spp. were detected in 84 of 201 (41.8%) blood samples and were confirmed by sequencing (Shaw et al., 2015). Bove (2012) evaluated the molecular prevalence of *A. phagocytophilum*, *F. tularensis*, *Babesia* spp. and *R. rickettsii* in blood samples from 227 New Jersey black bears from 2010-2011 and in 220 *I. scapularis* and 414 *D. variabilis* removed from the bears. *Francisella tularensis* and *R. rickettsii* were not detected in any blood samples. *Anaplasma phagocytophilum* was detected in 2 (0.01%) of the blood samples and 101 (44.4%) of the blood samples were positive for *Babesia* spp. A comparable result to the present study was that *R. rickettsii* was detected in 5 (5.2%) of the *D. variabilis* pools. *Babesia* spp. were detected in 52

(94.5%) of the *I. scapularis* pools. *Francisella tularensis* and *A. phagocytophilum* were not detected in any of the tick pools screened.

The presence of pathogens in adult ticks feeding on bears but absence from bear blood suggests that the tick acquired the pathogen from some other host, likely a small mammal, as larvae or nymphs and transferred to adults transstadially. In the present study, *Bartonella* spp. were detected in ticks attached to black bears, but not in the blood, although future studies may consider the presence of this pathogen in the tick population associated with bears as information on this relationship is scarce. Although Zonik et al. (2015) and Bove (2012) reported *A. phagocytophilum* in New Jersey black bear blood and the present study did not, this could be explained by the small sample size in this study (14) compared to the larger sample sizes tested by Zolnik et al. (65) and Bove (227). While *Babesia* spp. in New Jersey black bear blood were detected in this study and by others (Bove, 2012; Shaw et al., 2015; Zolnik et al., 2015), it does not indicate reservoir competence and further research exploring this relationship should be conducted. For example, Yabsley and Shock (2013) did not mention black bears in their review of the current knowledge on the ecology of *Babesia* spp. among their rodent reservoir and tick hosts with an emphasis on the role of wildlife as reservoirs. The instance of co-infection in ticks in the study is also consistent with similar research of pathogens in ticks of the Northeast. Aliota et al. (2014) found co-infection with 2 pathogens in 13.3% of ticks collected in New York State and Adelson et al. (2004) found 0.9%-8.4% co-infection rates in ticks collected in northern New Jersey. Both of these studies also found instances of tri-infections in ticks, though this was not observed in this study. The low prevalence of tick-borne pathogens detected in ticks removed from black bears and absence of most tick-borne pathogens from the bear blood suggests that black bears in northwestern New Jersey may not be important hosts of these tick-borne pathogens; however, American dog tick abundance on bears suggests that black bears are important for completion of the tick life cycle, which supports the conclusions of other black bear studies in New Jersey (Bove, 2012; Zolnik et al., 2015).

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