JOURNAL

OF THE PENNSYLVANIA ACADEMY OF SCIENCE

Founded on April 18, 1924

ISSN: 1044-6753

December 2011
Volume 85 Number 4

Jane E. Huffman, PhD, MPH
Editor
Department of Biological Sciences
East Stroudsburg University
East Stroudsburg, PA 18301

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Contents

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A Publication of
The Pennsylvania Academy of Science

Journal of the Pennsylvania Academy of Science 85(4): 123–124, 2011

COMMENTARY: MENTORS WHO HAVE INFLUENCED THE CAREER OF BERNARD FRIED FROM 1951–1963

BERNARD FRIED

Department of Biology, Lafayette College, Easton, PA 18042

Jane Huffman asked me to write a brief review of my career in invertebrate zoology and parasitology for publication in the *Journal of the Pennsylvania Academy of Science (JPAS)*. I thought I would reflect on some of my mentors who influenced my academic career during the 1951–1963 period. From 1951 to 1954, I was an undergraduate at the University Heights campus of New York University (NYU) in the Bronx, New York. A great influence on my career during those undergraduate years was the distinguished American parasitologist, Dr. Horace W. Stunkard. He taught a one year general biology course at NYU that I was fortunate enough to have taken. In that course, he covered in great depth the vast scope of biology as we knew it in those pre-DNA days. He also gave lectures on occasions that I attended on overviews of parasitology in general and helminthology in particular. His course and those outside lectures had a considerable influence in my choice of parasitology as a discipline. Following graduation from NYU with my BA degree, I had many opportunities to read some of his excellent publications. Of particular importance on my career were his papers on avian and turtle blood flukes, his studies on the evolution and systematics of flatworms and his work on the role of parasitology during World War II. He was a prolific writer having published more than 300 papers and monographs during his long and distinguished career.

A funded teaching assistantship and the lure of a strong program in invertebrate biology influenced my pursuit of a Master of Science (MS) program at the University of New Hampshire (UNH), Durham in the fall of 1954. That program gave me the initial incentive to pursue a career in teaching and research at the college level. Of considerable influence on my studies at UNH was Dr. Wilbur L. Bullock. Bullock taught parasitology and histology-microtechnique. He was the first mentor to introduce me to living parasites including larval and adult stages of trematodes and acanthocephalans. His lectures and labs were excellent and under his tutelage I became well trained in wildlife and medical parasitology. He was a skilled microtomist and histochemist who taught me how to prepare and stain sections for light microscopical histological and histochemical studies. His lessons in microtomy proved very helpful to me since I used many histological and histochemical procedures in my research during my career. An examination of his publication record indicates that he published about 30 papers while at UNH, with most of these studies on the life history, biology, histology and histochemistry of various acanthocephalans. He also published work in medical parasitology, including studies on human parasites in New Hampshire. It was Bullock who influenced me to continue in parasitology after I received my MS in zoology from UNH in 1956.

I then opted to go for the Ph.D. at the University of Connecticut (UConn), Storrs, under the tutelage of Dr. Lawrence R. Penner (LRP). I had developed an interest in marine avian schistosomes and schistosomiasis while at UNH and while there learned there was an expert in that area (LRP) at nearby Storrs. Penner accepted me into his program with a graduate teaching fellowship at UConn. I worked on the Ph.D. at Storrs from 1956 and 1961 and was awarded that coveted degree in 1961 based on my studies on marine acquired avian philophthalmiasis. My dissertation covered in vivo, in vitro and in ovo studies on the cultivation of a new eye fluke, *Philophthalmus hegeneri*, although my doctoral work also involved other aspects of the biology of *P. hegneri* and its marine snail vector *Batillaria minima*. I should note that my initial attempts to do work on marine acquired avian schistosomiasis at Storrs met with numerous obstacles and I abandoned the schistosome research about midway through my doctoral program.

My work with Penner both at Storrs and at the Archbold Biological Station in Lake Placid, Florida opened up new vistas of independent exploration for me. Penner let me explore many research topics on my own. I examined numerous invertebrate and vertebrate hosts for parasites and learned how to do host necropsies, examine snails for larval trematodes and to prepare slides of parasites for inclusion in permanent collections. I made thousands of slides under Penner's supervision and learned how to identify and catalog helminths. This experience was enhanced when I took his course in helminthology in Storrs, CT in 1957. In that course we had to collect parasites from hosts that we necropsied and to stain and identify them for a slide collection for LRP. It was a labor intense and exhausting experience that kept me busy for hundreds of hours. It was a type of learning experience that one never forgets. Your distinguished editor, Dr. Jane Huffman, also took this course in the 70s during her MS thesis program under Dr. Penner's supervision and can probably comment on her experiences with him. Penner was a man of boundless energy, took great joy in teaching parasitology to his undergraduate and graduate students and did significant work in wildlife and medical parasitology during his career. His work encompassed more than just helminthology and

his expertise was also demonstrated by his productivity in fields such as entomology, protozoology, bacteriology and public health. As far as I can determine, his publication record in refereed journals was about 50. He has influenced many students who worked with him. I often reflect on my experiences in the Penner lab with awe and wonder. He was an exceptional and fulgent personality.

While at Storrs, I was influenced by the developmental biologist Dr. Hugh Clark. Clark studied the physiology and biochemistry of chick embryos with particular interest in nitrogen excretion and metabolism. While in his lab I learned basic cell and tissue culture techniques and was introduced to elegant chick embryo procedures, particularly chick choriallantoic implantation techniques. His training provided me with the impetus to develop in vitro, in ovo, and ectopic cultivation studies first with philophthalmids (eye flukes) and later with other trematodes. I do not think I would have had success in my numerous studies on the in vitro and in ovo cultivation of trematodes without the initial help of Hugh Clark.

While at UConn, my career was influenced by the biochemist Dr. C. Albert Kind. Kind taught a one-year elegant and sophisticated course in general biochemistry. The lectures and labs in that course were very well organized and the experience I gained therein influenced my later work in the broad area of helminth biochemistry. Kind was a well-trained lipid and sterol biochemist. He taught me the laborious pre-chromatographic techniques then used for the analysis of lipids and sterols. Under his guidance, I did work on lipids and sterols of certain marine snails from Florida with and without parasites. The work never materialized for a variety of reasons but I gained valuable experience in lipid chemistry. I have since used newer chromatographic techniques to analyze lipids and sterols in gastropods with and without larval trematode infections. Most of this biochemical work has since been done at Lafayette College in collaboration with my distinguished analytical chemist colleague Dr. Joseph Sherma.

My desire for additional training in the physiology and biochemistry of parasites in 1961 led me to the lab of Dr. Chauney G. Goodchild at Emory University in Atlanta, GA under the sponsorship of an NIH postdoctoral fellowship. I was there from 1961–1963 and developed some expertise in physiological, biochemical and immunological parasitology in the Goodchild lab. Goodchild was an impressive figure, i.e., tall, handsome, distinguished looking, and always immaculately dressed. He was a commanding presence in the Department of Biology at Emory. He was Department Head in those days, did a lot of travel relative to "grantsmanship" and guest lectures at other institutions. He had numerous graduate and postgraduate students in his program. In his lab, I also developed considerable expertise on various aspects of the biology of turtle blood flukes (spirorchids) and the small intermediate planorbid snail *Menetus dilatatus* that served as a vector of *Spirorchis* sp. in Southeastern, USA. My work with him on the spirorchids led to two quick publications which we completed and submitted within two weeks of finishing the research. I was amazed at his rapid insistence on publishing the work as soon as possible. The Goodchild model of completing a study and then getting it ready for publication soon thereafter has remained with me as my mode of operation. The desire to publish research findings soon after completion of the work is something he instilled in me and a legacy I have left with many former students and colleagues. Goodchild published about 100 papers in many diverse areas of parasitology and invertebrate biology during his distinguished career.

This review has examined the mentors who have influenced my career. All of these men loved their work and had a great tenacity to excel in their particular research areas. The zeal and enthusiasm they had for their work has rubbed off on me.

ACKNOWLEDGMENT

I thank Ms. Amanda Balaban of Lafayette College for her assistance in preparing this manuscript.

Dr. Bernard Fried is the Kreider Professor Emeritus of Biology at Lafayette College, Easton, PA 18042, USA. friedb@lafayette.edu He has written and or edited more than 500 scholarly works including original research articles, reviews, book chapters, and books. He is a member of numerous biological and chemical societies and serves as an editor and or referee for numerous biological and chemical journals. His areas of expertise include Parasitology, Malacology and the Biological Applications of Chromatography. He has a Ph.D in Zoology (Parasitology) from the University of Connecticut, Storrs granted in 1961. From 1961 to 1963 he was an NIH Postdoctoral fellow in Parasitology at Emory University in Atlanta, GA. From 1963 until his retirement in 2000 he as Professor of Biology at Lafayette College. Although retired, he continues to maintain an active research program at Lafayette College with students and colleagues from around the world.

Dr. Fried was the president of the Pennsylvania Academy of Science from 1972–1974.

Journal of the Pennsylvania Academy of Science 85(4): 125–131, 2011

A FABRICATED RESISTIVITY APPARATUS TO EXPLORE BURIED STRUCTURES ON THE BENCH AND IN THE FIELD[1]

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ABSTRACT

In the following study, an electrical resistivity apparatus was created and implemented in the laboratory as well as tested in the field, to accurately identify the location of a buried building foundation. The primary goal of the study was to test the accuracy of the apparatus which costs a fraction of the price of available electrical resistivity instrument. The apparatus consists of four electrodes set in a linear spread. An electrical current, produced by a standard 12-volt battery, is injected into earth material through the outer electrodes and the potential voltage is measured across the inner electrodes using a multimeter. The recorded potential and current values can then be used to calculate apparent resistivity of any given material. The study implemented Wenner array which consists of four electrodes placed equally distant from each other and also allows for higher accuracy and greater resolution when investigating lateral variations of resistivity in near-surface formations.

The device successfully produced consistent results on the bench level revealing the location of small bricks buried under soil material. In the field, multiple transects were conducted in the summer of 2010 on Susquehanna University Campus where the foundation of Gustavus Adolphus Hall is located. The apparatus consistently provided accurate results when compared to a commercially produced electrical resistivity device, Iris SYSCAL R1+, and successfully revealed the location of the foundation.
[J PA Acad Sci 85(4): 125–131, 2011]

INTRODUCTION

Geophysical prospection is useful for many applications, from investigating composition, stratigraphy, or exploring objects beneath the ground surface. These methods are currently well established and are routinely and successfully used in the detection and mapping of concealed subsurface archaeological structures (Papadopoulos 2009). Electrical resistivity (ER) surveying is one of many non-invasive methods techniques used in geophysical investigation including ground-penetrating radar, seismic refraction, electromagnetic surveying, and many others. (Bonomo 2010, Conyers 2004, Leucci 2006, Michelsen 2008, Victoria 2011). In ER surveying, direct current or low-frequency alternating current is applied at the ground surface, and the electrical potential is measured between two points (Herman 2001). Potential changes due to differences in conductive properties of subsurface materials. In this study, an electrical resistivity apparatus was created using simple components. This study set its aim on two goals; investigate the buried foundation of Gustavus Adolphus (GA) Hall; once located on Susquehanna University Campus, and test the accuracy of the electrical resistivity apparatus. Electrical resistivity techniques are widely applied in archaeological prospection as the rapidly collected data yields to more precise images of subsoil (Cardarelli 2009). Furthermore, area surveying is now the dominant method as it produces accurate analysis of surface models (Gaffney 2008). Proof that the apparatus could produce results as accurate as an ER device would be beneficial because the apparatus costs a fraction of the price of a commercially produced device, making it ideal for small studies and general use in a geophysics laboratory setting. More importantly, this is an affordable tool to both instructors and students to experiment and apply theoretical knowledge in lab and in field practice.

GA burned down in 1964, but some of the foundation walls are believed to still exist beneath the ground-surface. GA was a relatively large building measuring approximate-

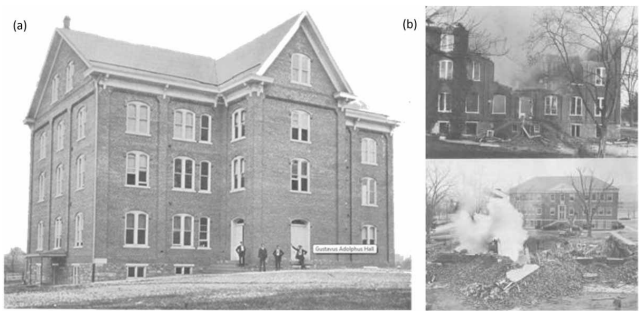

Figure 1. Undated photograph of Gustavus Adolphus Hall as it stood in the middle of campus (a) and during the fire in 1964 (b)

[1]Submitted for publication 30 April 2011; accepted 1 December 2011.

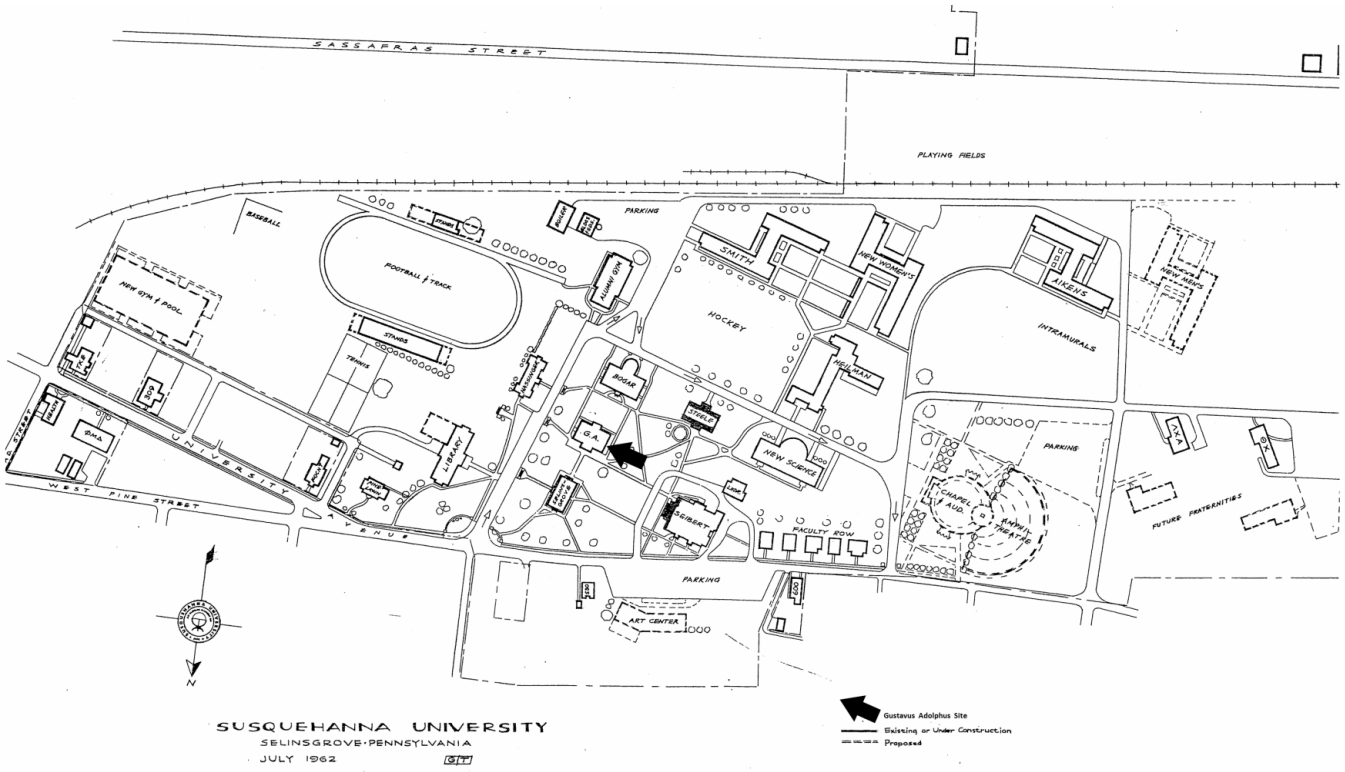

Figure 2. Susquehanna University campus map, July 1962.

ly 16. 5 meters wide by 25 meters long with a 4 meter by 7 meter rectangular structure merging in the front of the building (Figure 1). Figure 1b shows one of the last photographs taken of GA before the fire in 1964, recovered from the Susquehanna University campus library archives. Figure 2 below shows a campus map of Susquehanna University in July of 1962. This campus map was also collected from Susquehanna University campus library archives.

Electrical resistivity surveying methods usually rely on the use of four electrodes placed in a linear array. There is a large variety of established collinear configurations. Most common among these are the *Wenner array*, *Schlumberger array*, the *Dipole-dipole array*, and the *Wenner/Lee array*. Each has specific advantages and disadvantages. This study implements the use of the *Wenner array* which calls for four electrodes equidistant by a spacing denoted *a*. There are several advantages of using this method. First, the mathematical theory is simple and straight-forward. Second, the *Wenner array* is highly sensitive to lateral variations and good for shallow investigations, making it ideal for this study (Herman 2001, Griffiths 1994). ER surveying is conducted by shifting the entire four electrode array linearly across the desired region. The displacement over which the array is shifted is denoted as *x*. As a general rule *a* should always be greater than *x* in order to accurately map all subsurface features. Figure 3 displays the *Wenner array* configuration.

Electrical resistivity is first understood as resistance (R) to an electrical current of an ideal cylinder of length L and cross-sectional area A of a uniform composition. Resistivity

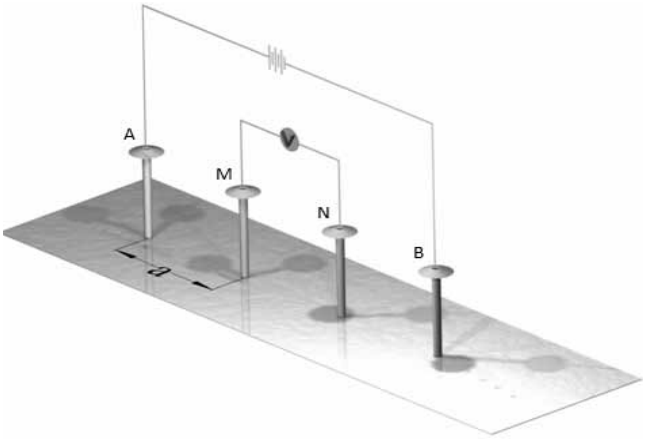

Figure 3. *Wenner array* configuration. Each electrode A, M, N, and B are equidistant a from one another.

(ρ) appears as a material-specific constant in the expression for total resistance of the cylinder.

$$R = \rho \frac{L}{A}$$

The total resistance R may be obtained through **Ohm's Law** given as:

$$R = \frac{V}{t}$$

where *V* is the voltage potential between the ends of the

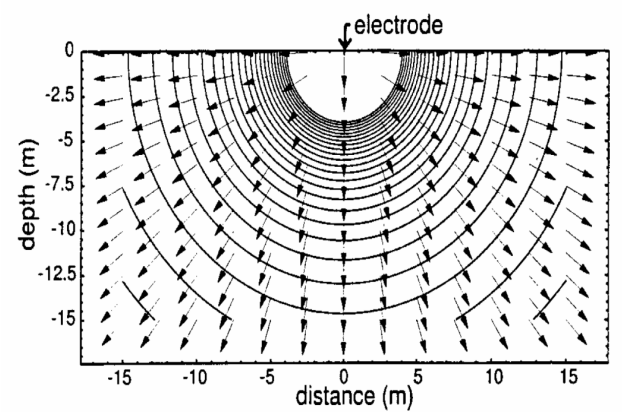

Figure 4. Current flow from a single electrode in a homogenous medium. Arrows represent the direction of flow while the solid lines represent equipotential surfaces (Herman 2001).

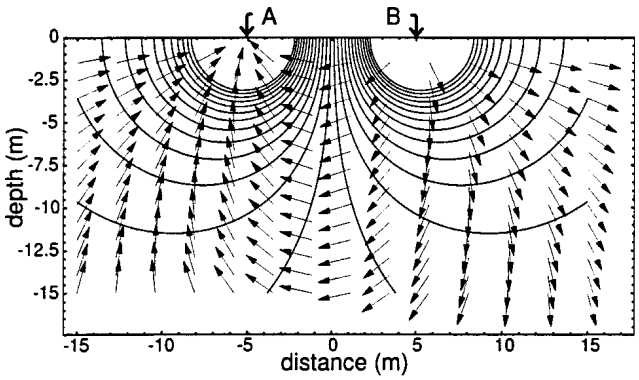

Figure 5. Current flow and equipotential surfaces between two electrodes in a homogeneous formation (Herman 2001).

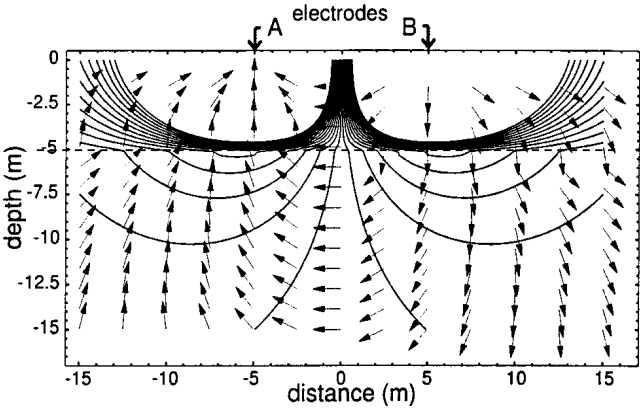

Figure 6. Current flow and equipotential surfaces between two electrodes in a heterogeneous formation. Illustrates a situation where the upper region of the subsurface is of a higher resistivity than the lower region(Herman 2001).

cylinder and i is the total current flowing through the cylinder. It is, however, more difficult to determine the resistivity of material that is geometrically more complex than a cylinder. Current applied, from a single electrode, to the ground of a uniform composition will flow radially away from the electrode. Figure 4 portrays the flow in two-dimension.

A more realistic situation in an ER surveying, involves two electrical electrodes where current flows from one electrode (source) to the other (sink). In this situation, the flow of current can vary depending on if the medium is a homogeneous or a heterogeneous formation. These two situations are depicted in Figure 5 and Figure 6.

The spacing of the electrodes directly affects the depth at which the electricity will penetrate. This is called the effective depth. If the spacing is very large, the current will travel deeper within the subsurface and therefore, the resistance the current encounters will be affected. This is irrelevant for a homogenous formation, such as Figure 5 depicts, but in a heterogeneous formation, as shown by Figure 6, the effective depth becomes important when investigating a feature deep within the subsurface.

When a current is applied to a heterogeneous region, the measured resistivity is not of any particular formation within the subsurface, but rather an apparent resistivity of the entire region for which the electricity travels. This apparent resistivity ρ_a can be expressed by the following simplified formula when using *Wenner array*:

$$\rho_a = \frac{2\pi a V_{MN}}{i}$$

where a is the spacing between each electrode, V_{MN} is the electrical potential difference between electrodes M and N (Figure 3), measured in volts, and i is the current intensity measured in amps.

In this study, electrical resistivity methods were primarily aimed for archaeological purposes. Geophysical surveys in geology typically focus on vertical changes in lithology. Archaeo-geophysical surveys generally concentrate on extreme lateral changes near surface in order to locate and define features (Kvamme 2003). When these lateral changes can be attributed to certain aspects of an archaeological site high-definition maps and images of buried remains can be produced (Conyers 2004).

METHOD AND MATERIALS

The concept behind electrical resistivity studies in geophysics may begin with the vector form of Ohm's law:

$$J = \sigma E = \frac{1}{\rho} E = -\frac{1}{\rho} \nabla V \qquad (1)$$

Where J is the current density vector measured in units of A/m^2, σ is the conductivity measured in $(ohm \cdot m)^{-1}$, E is the electric field vector measured in units of volts/m, ρ is the resistivity measured in $(ohm \cdot m)$ and V is the electric potential in volts. The physical interpretation of current density is that each component of J gives the amount of current flowing through each square meter of a two-dimensional surface perpendicular to the direction of flow.

Figure 5 depicts a homogeneous subsurface with one source and one sink electrode. The electrodes may be treated as point sources or sinks of symmetric current flow in the half plane below the surface. The total current *i* flows away or toward each electrode across a half sphere with an area equal to $1/2(4\pi r^2)$. Ohm's law for one electrode then has a simple form:

$$V(r) = \frac{i}{\frac{1}{2}(4\pi r^2)} = -\frac{1}{\rho}\frac{dV}{dr} \tag{2}$$

For a constant ρ, equation (2) is integrated and yields:

$$V(r) = \frac{\rho i}{2\pi r} \tag{3}$$

for a potential at a distance *r* from the electrode. With the distances between the electrodes (Figure 3), and $V = 0$ infinitely far from the current source, the potentials at M and N are given by:

$$V_M = \frac{\rho I}{2\pi}\left(\frac{1}{AM} - \frac{1}{MB}\right) \tag{4}$$

and

$$V_N = \frac{\rho I}{2\pi}\left(\frac{1}{AN} - \frac{1}{NB}\right) \tag{5}$$

The total potential difference between M and N is thus:

$$V_{MN} = V_M - V_N = \frac{\rho I}{2\pi}\left[\left(\frac{1}{AM} - \frac{1}{MB}\right) - \left(\frac{1}{AN} - \frac{1}{NB}\right)\right] \tag{6}$$

This expression can be rearranged to yield:

$$\rho = \frac{V_{MN}}{I} K \tag{7}$$

where:

$$K = 2\pi\left[\left(\frac{1}{AM} - \frac{1}{MB}\right) - \left(\frac{1}{AN} - \frac{1}{NB}\right)\right] \tag{8}$$

where K is the "geometric factor" which will acquire a particular value for any given electrode spacing. For the *Wenner array*, all distances between the electrodes are equal to a constant value *a*, therefore the geometric factor for *Wenner* array takes the form $K = 2\pi a$. Thus, the apparent resistivity for the *Wenner array* is

$$\rho_a = \left(\frac{V_{MN}}{I}\right) K = \left(\frac{V_{MN}}{I}\right) 2\pi a \tag{9}$$

One of the primary goals of this study was to test the accuracy of the ER apparatus. The apparatus used consists of four electrodes each, to be placed in the ground in a linear array and two EXTECH 540 multimeters, one to measure the current intensity between the outer electrodes and the other to measure the voltage between the inner electrodes. The two multimeters employ a wireless data transmission directly to a computer via a built-in radio frequency transmitter. Power was supplied by a 12 volt deep-cycle battery

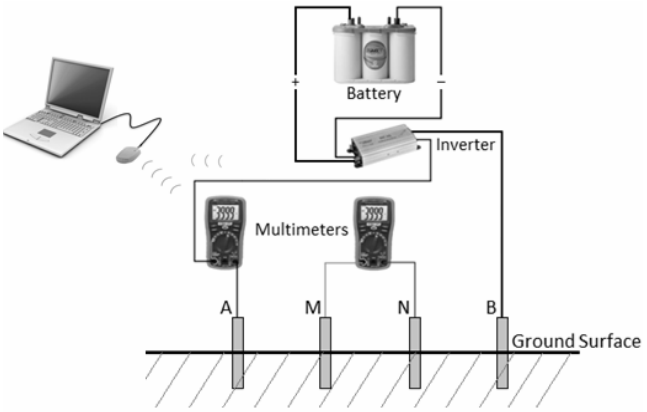

Figure 7. Apparatus setup showing battery(top), followed by the DC to AC inverter and two multimeters, seen at the bottom. Data transfer to a computer is possible via built-in radio frequency transmitter.

connected to an APS600-12 Pure Sine Power Inverter to convert the Direct Current (DC) to an Alternating Current (AC). This was done to prevent macroscopic polarization which can cause inconsistent results. All the connecting electrical wires were 18 gauge. The deep-cycle battery helps to prevent a significant loss of charge while taking the measurements however in theory any standard battery could be used. All of these components can be purchased for a little less than $1, 000, again making the apparatus ideal for small studies and regular use in a laboratory setting. Figure 7 displays the setup of the apparatus.

Similar electrical resistivity apparatus were used in other studies such as in (Herman 2001), (Avants et. al 1999). Others have used designs more closely related to a Terrameter such as in (Olowofela and Jolaosho 2005). All designs provide identical results, however, the design implemented in this study, is more simple and easier to assemble. This is ideal when having to transport the apparatus to the field which at times requires quick disassembly and reassembly.

RESULTS AND DISCUSSION

Several tests were conducted on the bench model and in the field. Apparent resistivity mapping techniques implemented in this study are similar to that of (Klasner 1981). On the bench level, bricks ($2'' \times 2'' \times 8''$) were buried in a sandy soil leaving approximately two inches of material between the top of the brick and the surface of the soil. The electrodes used on the model were standard 2. 5″ stainless steel nails. For each test conducted on the bench model, the displacement increment *x* was taken as 2 in and the electrode spacing *a* as 1 in. Figure 8a shows the bench model where the apparatus was implemented. It was noticed that inaccurate results were often produced by small movement of electrodes during their placement in the soil which introduced void space causing higher resistant medium. To help prevent this, electrodes were secured into a thin wood block

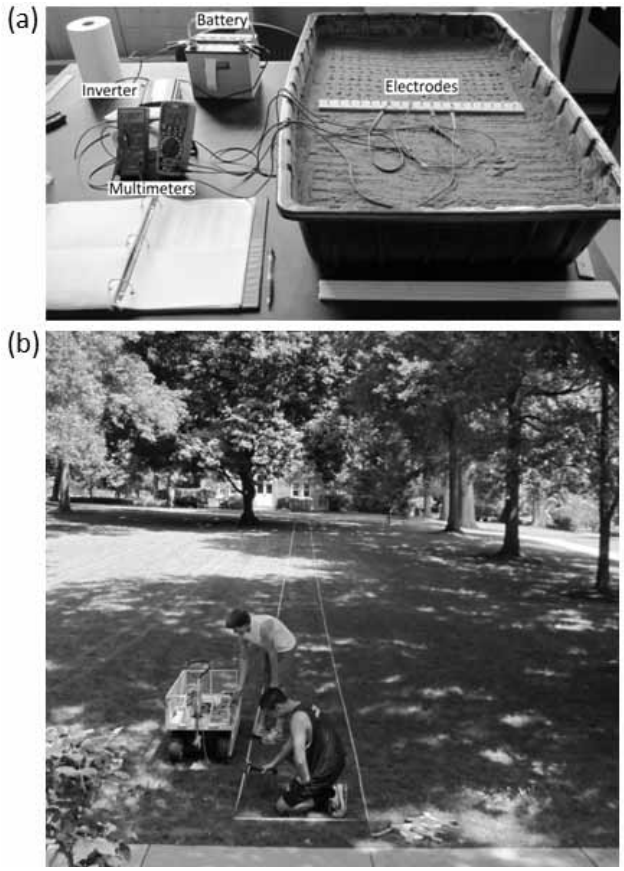

Figure 8. Experimental setup of apparatus on the bench model and in the field.

and placed on the soil only one time. Because wood is also a highly resistant material surface current travel between the electrodes was not an issue.

The apparatus was also implemented in the field. GA foundation was investigated by using a Wenner Array configuration with an electrode spacing (a) of one meter and a displacement increment (x) of 50 centimeters. The apparatus was moved along the site using a small cart (Figure 8b).

Test 1 was conducted on a bench with a single transect over a buried brick. Figure 9a displays apparent resistivity versus distance in the x-direction. The brick can be easily identified. Figure 9b displays a theoretical apparent resistivity curve as an electrode array is moved across a similar contact. The array is moved from a soil of lower resistivity, across a contact of higher resistivity, and returns to the lower resistivity soil. When comparing Test 1 with the theoretical curve (Figure 9b) direct similarities can be drawn.

The a-spacing to object width ratio seen in the theoretical can be compared to the a-spacing to object width ratio in the test. In other words, the object labeled "reef" in Figure 9b is approximately 20 ft (Van No strand 1966). The a-spacing used in the theoretical model is 30 ft. The brick width and the a-spacing used in this test were both 2 in. Thus, it can be hypothesized that when the a-spacing is similar to the width of the contact, the trend seen in Figure 9 may be seen. Information like this could prove vital when trying to identify a feature that may have a similar resistivity to surrounding material. For the scope of this study, this preliminary test showed that the apparatus was performing properly and was capable of producing feasible results.

For Test 2, a total of 29 transects were performed to cover the entire model. Each transect consisted of 30 data points. Results were placed in a 3-D surface plot using Matlab software (Kattan 2010). Figure 10a displays the results from this test while Figure 10b shows the arrangement of bricks within the bench model.

The location of the bricks can clearly be identified from the apparent resistivity profile. Higher peaks of resistivity values toward the edges and in certain spots of the model can be explained by the existence of fissures caused by the dryness and cracking of the soil or movement of the electrodes. This hinders electrical current to travel in the medium and therefore increases resistivity.

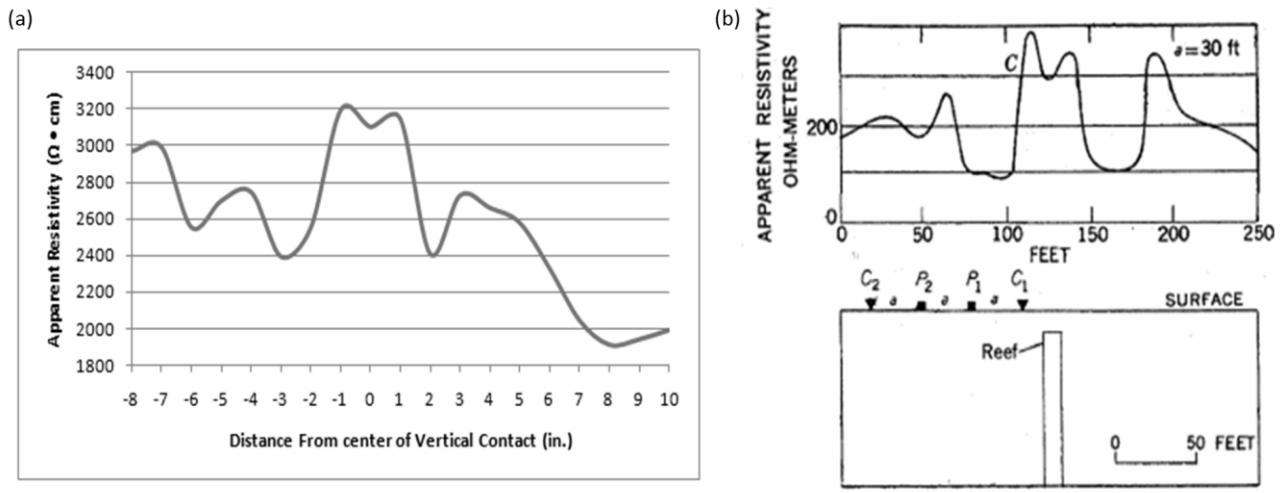

Figure 9. (a) Results from Test 1, ρ_a versus x. (b) Theoretical curve for the contact shown (reef) (Van No strand 1966).

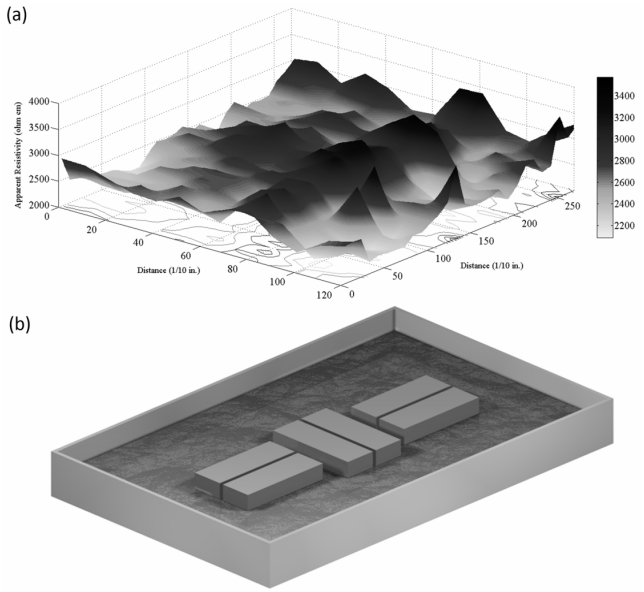

Figure 10. Apparent resistivity 2-D surface plot for bench level test 2. Areas of lighter shade indicated regions of higher apparent resistivity while areas of darker shade indicate lower apparent resistivity. Dotted lines indicate the approximate location of buried bricks.

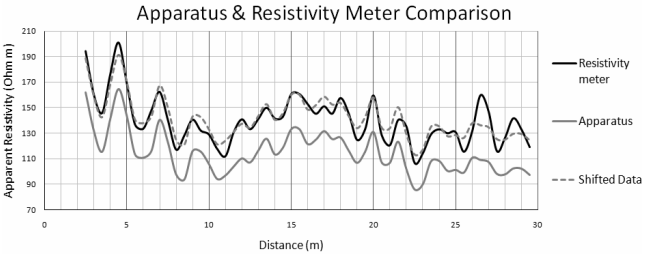

Figure 11. A Single transect comparison of ER apparatus and Iris SYSCAL R1+. Dotted line indicates the apparatus results shifted by the average delta between the ER device results and the ER apparatus results. Transect preformed on Susquehanna University Campus, Selinsgrove, PA.

The final test, Test 3, was conducted before the apparatus was to be applied in the field. In this test the apparatus was directly compared to the Iris SYSCAL R1+ ER device. A single transect was performed using both the apparatus and the ER device. Results from this test can be seen in Figure 11.

Figure 11 conclusively shows that the apparatus was capable of reproducing similar results to that of a commercial device. It is believed that a small difference in the frequency output of the ER device and the APS600-12 Pure Sine Power DC to AC Inverter used in the apparatus caused the apparatus to record slightly lower apparent resistivity values than the ER device. In addition, this slight difference between the two systems can be ignored since ER surveying relies more on the trend of apparent resistivity to reveal features rather than the direct apparent resistivity values. For ease of viewing the ER apparatus results were shifted by the average delta between the ER device results and the ER apparatus results.

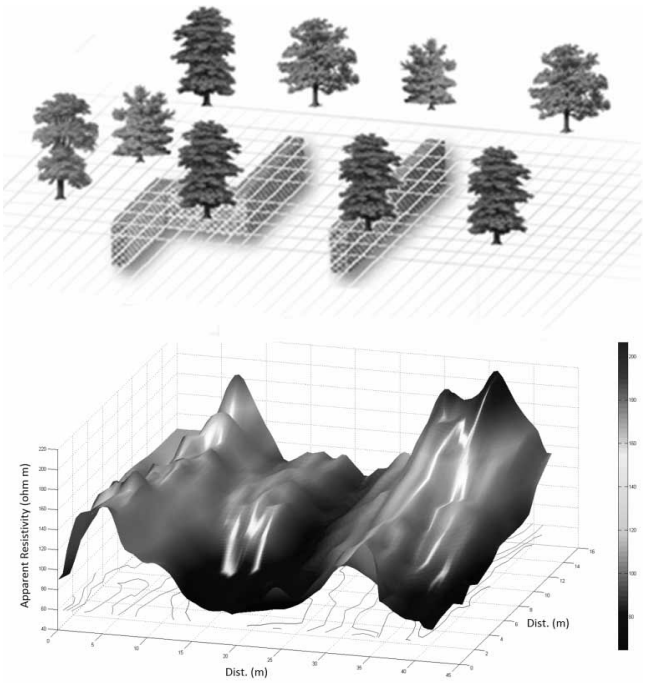

Figure 12. Apparent resistivity data in 3-D surface plot collected using ER apparatus at GA plot, Susquehanna University, Selinsgrove, PA (bottom). Location and arrangement of trees and approximate location of buried foundation in respect to apparent resistivity data.

The final objective of the study was to implement the apparatus in the field at the GA site. A total of seventeen 42-meter transects were performed using an *a*-spacing of one meter and a *x*-displacement of 50 centimeters. Figure 12 shows a number of features; the results from the entire survey plotted as a 3-D apparent resistivity surface using Matlab, the arrangement and location of trees in the GA site, the approximate location of the buried foundation based on the results from the survey, the campus map of July 1962, using GIS information, and a basic sketch of the building from the time of construction recovered from archives at Susquehanna University campus library. The illustrated walls in Figure 12 are understandably exaggerated to better visualize the foundation of the building and how it matches the resistivity contour plot (Figure 12).

Abnormal peaks in apparent resistivity seen in Figure 12 are due to the root systems of the trees in the area which are approximately 50 years old. The trees were found to be planted on the boundary limits of GA outer walls. The apparent resistivity data collected from the GA site matched well with GA dimensions collected from aerial photos, campus maps, and GIS information. An important feature to note is the "L-shape" feature seen in the apparent resistivity data and depicted in the illustration of the walls in Figure 12. If we refer back to the photo of GA and the campus map, Figures 1 & 2, it can be seen that the front of the building had a rectangular extension protruding from it. It is believed that this "L-shape" feature corresponds to this rectangular portion in the front of the building. It can be seen however that the entire building was not revealed by the investigation.

The apparatus succeeded in all levels of the study. It accurately revealed the GA foundation and gave similar values to that of SYSCAL R1+. This is evidence that an apparatus similar to the one used in this study could be relied on for consistent and accurate results in the laboratory and low budget applications.

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Journal of the Pennsylvania Academy of Science 85(4): 132–139, 2011

RELATIONSHIP BETWEEN EXOTIC INVASIVE SHRUBS AND AMERICAN WOODCOCK (SCOLOPAX MINOR) NEST SUCCESS AND HABITAT SELECTION[1]

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ABSTRACT

Habitat loss is thought to be a cause of woodcock population declines, however little is known about the impact of exotic invasive vegetation on woodcock nest site selection and nesting success. In March and April of 2009 and 2010, we examined nest success and nesting habitat selection in relation to the abundance of exotic invasive vegetation at 13 nests in southeastern Pennsylvania. We used logistic regression and Akaike's Information Criterion (AIC) to determine the best models for nest success and habitat use. Woodcock avoided exotic invasive vegetation when selecting nest sites. Nest success and habitat use decreased significantly with an increase in percentage of exotic invasive woody vegetation. Models containing percentage of exotic invasive woody vegetation were highly supported for nest success and habitat selection. We recommend that managers attempt to control and remove exotic invasive vegetation to promote increased woodcock nesting success and habitat use.

[J PA Acad Sci 85(4): 132–139, 2011]

INTRODUCTION

American woodcock populations have experienced declines from 1968 to present (Sauer and Bortner 1991, Cooper and Parker 2010). These declines are thought to be linked to the degradation and decline of early successional habitats (Owen et al. 1977, Dwyer et al. 1983, Straw et al. 1994, Dessecker and McAuley 2001). Woodcock population trends in Pennsylvania are comparable to other eastern populations (Palmer 2008) and it may be reflective of the regional trend. Additionally, the importance of habitat components like singing grounds, feeding, nesting, and diurnal cover to woodcock survival has been studied in Pennsylvania (Liscinsky 1972, Kinsley and Storm 1988). Liscinsky (1972) reported that when one or more of these habitat components was missing, woodcock use declined rapidly. These studies documented the importance of native plant species like alder (*Alnus* spp.), crabapple (*Malus* spp.), hawthorn (*Crataegus* spp.) and dogwood (*Cornus* spp.) and recommended management on a shorter rotation that will allow habitat to remain in a young stage that is suitable for American woodcock (Liscinsky 1972, Gutzwiller 1980, Kinsley and Storm 1988). Previous research also concluded that woodcock had low selectivity for nesting site habitat, and utilized many different cover types (Mendall and Aldous 1943, Sheldon 1967, Liscinsky 1972, Coon et al. 1982). Adequate soil moisture is recognized as an important characteristic of woodcock feeding areas and is considered a limiting factor for woodcock habitat use, although previous research also concluded that drier, upland sites are typically used for nesting (Liscinsky 1972, Gregg and Hale 1977, Coon et al. 1982).

Exotic invasive vegetation (hereafter invasive vegetation or invasives) was not as prevalent during the time of previous studies as it is now, and little is known about the effect of invasive vegetation on woodcock nesting success and habitat use. Dense thickets of multiflora rose (*Rosa multiflora*), autumn olive (*Elaeagnus umbellata*), and tatarian honeysuckle (*Lonicera tatarica*) prevent native shrub and forb establishment and may be detrimental to nesting native birds (Luken and Thieret 1996, Hutchinson and Vankat 1997). These invasive shrubs have become common in southeastern Pennsylvania and are now listed as a serious threat because they out-compete native vegetation in the region (PADCNR 2009).

The Pennsylvania Game Commission's Wildlife Action Plan (2008) identifies the management of early successional habitat as a high priority because of the decline in its quantity and quality. In Pennsylvania, the amount of forested acreage has not drastically declined in recent years (McWilliams 2007), but rather the forests have aged to a point that deters use by woodcock (Kinsley and Storm 1988). Moreover, the invasion of exotic shrubs is thought to influence native forest quality by reducing sunlight and soil nutrients available to native shrubs (Bratton 1982, Wilcove et al. 1998, Zavaleta 2000, Sala et al. 2000).

Woodcock have been listed as a species of greatest conservation concern in the Pennsylvania Wildlife Action Plan (PGC 2008), because they require early seral habitats (Men-

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dall and Aldous 1943, Sepik et al. 1981). Given American woodcock population decline and encroachment of invasive vegetation, our objective was to determine whether invasive vegetation affects American woodcock nest success and nesting site selection. We hypothesize that American woodcock would show no preference for native vegetation over invasive and that nesting success would not differ between nest sites that contained native or invasive vegetation.

MATERIALS AND METHODS

Study site.

Our study was conducted at Swatara State Park near Suedberg in Lebanon and Schuylkill counties, in southeastern Pennsylvania (Fig. 1). The park is roughly 14 km^2 and lies in the Ridge and Valley Province (Bailey 1995). Dominant cover types include invasive shrublands, red maple (*Acer rubrum*) – elm (*Ulmus americana*) palustrine flood plains, red oak (*Quercus rubra*) – mixed hardwood forests, and northern hardwood forests (Fike 1999).

Nest success.

Singing ground surveys were conducted in early March of 2009 and 2010 by walking roads and hiking trails at dusk and listening for singing males. Singing grounds were identified using a Global Positioning System (GPS; Garmin, Olathe, KS).

Mist nets were strung across singing grounds to capture female woodcock who were visiting displaying males (Sheldon 1967) and trained pointing dogs (Ammann 1981) were used to locate nests in proximity to occupied singing grounds. For all woodcock captured, we recorded date, time, location of capture, capture method, bill length (mm), mass (g), and widths of outer 3 primary feathers (P10, P9, P8) measured 2 cm from the feather tip. Bill length, sum of P10 + P9 + P8 width measurements, and body mass were used to determine sex (Martin 1964).

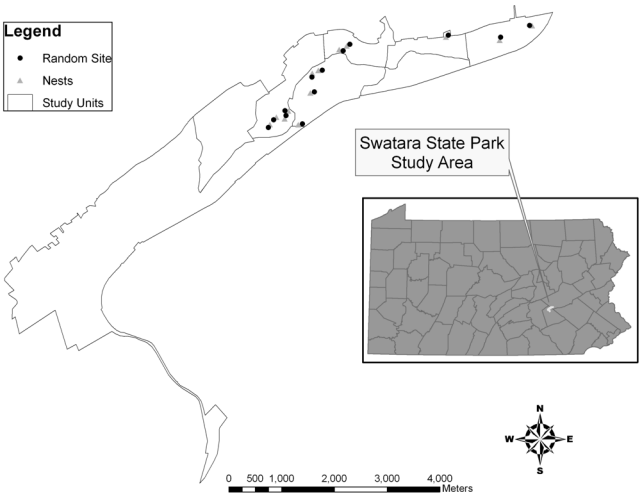

Figure 1. Map of Swatara State Park including nest and random sites and an insert map of Pennsylvania showing location of American woodcock study area in Schuylkill & Lebanon Counties, PA, USA during 2009–2010.

All woodcock were fitted with United States Geological Survey leg bands. A 3 g radio transmitter (Model SOPB-2190, Wildlife Materials, Inc., Murphysboro, IL) was attached to all captured females using all-weather animal tag cement and a belly-loop wire harness (McAuley et al. 1993). Tracking of females began after release using a portable receiver and handheld yagi antenna (Wildlife Materials, Inc., Murphysboro, IL) to determine habitat used. When monitoring indicated that a female was in the same 0.5 ha area for 2 consecutive days, we located the bird visually to confirm nesting and recorded the number of eggs. Nests were checked remotely with binoculars every 3 days to verify their fate. If a signal was located in an area away from the nest, we visually determined the fate of the nest. We considered all nests that hatched ≥ 1 egg to be successful.

Habitat composition.

Two days after fledging or immediately following depredation or abandonment, we marked the location of each nest using a GPS, and recorded habitat composition in an 11.3 m radius plot (0.04 ha; James and Shugart 1970, McAuley et al. 1996) around the nest. Woody vegetation composition was measured by counting native and invasive woody vegetation stems ≥ 30.5 cm in height that emerged from the soil in the plot. Woody vegetation under 30.5 cm was omitted due to potential difficulty in identifying species without the presence of leaves. Soil moisture was measured using a RapiTest moisture meter with probe (Scherm et al. 1998; Luster Leaf Products, Inc., Woodstock, IL) that ranged from 0 for dry to 10 for wet. Soil moisture at each site was measured three times within 10 minutes and the average was calculated. Stem density was defined as the total number of native and invasive woody vegetation stems ≥ 30.5 cm tall within the plot divided by the plot area. Vegetation cover was obtained by dividing the number of invasive woody vegetation stems by the total number of woody vegetation stems found in the plot.

Habitat selection.

Habitat conditions between paired use and random locations were assessed for factors in nest site selection. Used sites were defined as nest locations and random sites were determined by randomly selecting a direction by flipping a coin twice (first for north or south and second for east or west) and then walking 70 m from the nest location in the combined direction. This distance was chosen to reflect the average distance that nests were found in relation to an occupied singing ground (Miller 2010).

Data analyses.

An information-theoretic approach (Burnham and Anderson 2002) was used to assess the importance of habitat covariates to woodcock nest success and habitat selection using logistic regression, based on models generated to reflect competing hypotheses. Relative support was then evaluated for these competing models using Aikake's Information Criteria (AIC; Burnham and Anderson 2002). We developed a set of *a-priori* models to predict the effect of habitat variables on nesting success and nesting habitat use (Table 1). A Hosmer and Lemeshow (2000) goodness-of-fit test on the global model (Table 1) was conducted to determine whether it fit the observed values. Akaike's Information Criterion adjusted for small sample size (AIC_c) and weight of evidence (w_i) were used to rank and select the most parsimonious models fit to the data (Burnham and Anderson 2002). We considered models with $\Delta_i \geq 2$ to have substantial support and models with $\Delta_i \geq 4$ to lack support (Burnham and Anderson 2002, Lloyd and Martin 2005). Student's *t*-tests compared the means of normally distributed habitat variables recorded at paired nest and random sites. An Independent Mean test compared variables that were not normally distributed from successful and failed nests. All statistical analyses were performed using SPSS (Version 18, SPSS, Inc. 2009).

Table 1. Model selection results, bias-corrected Akaike's Information Criterion (AIC_c), model weights (w_i), and parameters estimated (K) used to evaluate success of American woodcock nests in Swatara State Park, Schuylkill & Lebanon Counties, PA, USA during 2009–2010.

Model[a]	AIC_c	Δ_i	w_i	K
PER_INV	15.978	0.000	0.616	2
PER_INV + MOISTURE	18.862	2.884	0.146	3
NATIVE	20.210	4.232	0.074	2
STEMDENS	21.138	5.160	0.045	2
MOISTURE	21.248	5.270	0.044	2
INVASIVE + MOISTURE	21.977	5.999	0.031	2
INVASIVE	22.052	6.074	0.030	3
NATIVE + MOISTURE	23.665	7.687	0.013	3
GLOBAL	36.160	20.182	0.001	6

[a]PER_INV= percentage of invasive stems present in 0.04 ha circular area around each nest.
MOISTURE = amount of moisture measured in soil with a RapiTest soil meter; 0 = dry, 10 = wet.
NATIVE = number of native stems ≥ 30.5cm within 0.04 ha circular area round each nest.
STEMDENS = density of woody vegetation ≥ 30.5cm in height /m^2.
INVASIVE = number of invasive species ≥ 30.5cm present within 0.04 ha circular area round each nest.
GLOBAL = contains all measured variables.

Table 2. Akaike-weighted odds ratios of variables occurring in models of nest success in Swatara State Park, Schuylkill & Lebanon Counties, PA, USA during 2009–2010.

				95% Confidence Interval	
Variablea	β	SE	Odds Ratio	Lower	Upper
NATIVE	0.014	0.011	1.015	0.993	1.037
MOISTURE	0.006	0.606	1.006	0.306	3.301
STEMDENS	0.004	0.011	1.004	0.982	1.026
INVASIVE	–0.054	0.036	0.947	0.882	1.017
PER_INV	–3.178	1.568	0.042	0.002	0.900

[a]NATIVE = number of native stems ≥ 30.5cm within the 0.04 ha circular area round each nest.
MOISTURE = amount of moisture measured in soil with a RapiTest soil meter; 0 = dry, 10 = wet.
STEMDENS = density of woody vegetation ≥ 30.5cm in height /m^2.
INVASIVE = number of invasive species ≥ 30.5cm present within 0.04 ha circular area around each nest.
PER_INV = percentage of invasive stems present in 0.04 ha circular area around each nest.

RESULTS

Nest success.

Five nests were located in 2009 and 8 in 2010, for a total of 13. Mean clutch size for all nests was 3.85 (SE = 0.15). Apparent success, which is the proportion of observed nests that are successful, was 69%. Three of the 4 failed nests were lost to predation.

The goodness-of-fit test indicated the global model fit the observed values ($\chi^2_8 = 6.61$, $P = 0.581$). The highest ranking model was the percentage of invasive stems present in the plot (PER_INV; $AIC_c = 15.978$, $w_i = 0.616$; Table 1). The next best model was PER_INV + MOISTURE ($\Delta_i = 2.884$, wi = 0.146; Table 1). We also evaluated the importance of each covariate individually (Burnham and Anderson 2002). Odds ratios are considered meaningful when 95% confidence intervals do not overlap zero. The odds ratios suggested that nesting success decreased with an increase in percentage of invasive woody vegetation. Additionally, for every native stem in the plot, the probability of success increased by 1.02 (Table 2).

Across all nest sites, the percentage of invasive woody stems in the plot averaged 38.1% (SE = 6.94). At successful nest sites, the percentage of invasive woody stems (30.3% ± 8.64; X ± SE) was significantly less than at unsuccessful nest sites (55.8% ± 5.41; $t_1 = 6.74$, $P = 0.021$). Stems of native species did not vary between successful and failed nests ($t_1 = 0.034$, $P = 1.0$; Fig. 2). Multiflora rose, tatarian honeysuckle, and autumn olive were the dominant invasives; they occurred at 85%, 77%, and 51% of our plots respectively, and accounted for 9.9%, 10.9%, and 3.3% of the total stem count. Total stem density varied greatly across nest sites (84.23 ± 16.33; range = 12.0 – 195.0). Soil moisture was rated as dry to slightly moist across nest sites (2.15 ± 0.29).

Habitat selection.

A Hosmer and Lemeshow (2000) goodness-of-fit test indicated that the global model fit the observed values ($\chi^2_7 = 5.01$,

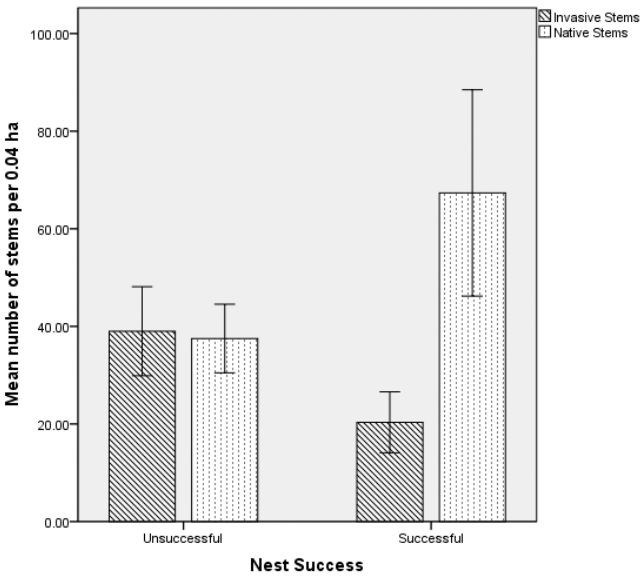

Figure 2. Observed mean values (±1 SE) for invasive and native woody vegetation recorded at American woodcock nest sites in Swatara State Park, Schuylkill & Lebanon Counties, PA, USA during 2009–2010.

Table 3. Model selection results, bias-corrected Akaike's Information Criterion (AIC_c), model weights (w_i), and parameters estimated (K) used to evaluate nest site habitat selection of American woodcock in Swatara State Park, Schuylkill & Lebanon Counties, PA, USA during 2009–2010.

Model[a]	AIC_c	Δ_i	w_i	K
PER_INV	34.770	0.000	0.492	3
INVASIVE	36.926	2.156	0.167	2
PER_INV + MOISTURE	37.301	2.531	0.139	2
NATIVE	38.516	3.746	0.076	2
INVASIVE + MOISTURE	39.440	4.670	0.097	3
STEMDENS	40.472	5.702	0.048	2
MOISTURE	40.492	5.722	0.028	2
GLOBAL	40.896	6.126	0.023	6
NATIVE + MOISTURE	41.003	6.234	0.022	3

[a]PER_INV = percentage of invasive stems present in 0.04 ha circular area around each nest.
INVASIVE = number of invasive species ≥ 30.5cm present within 0.04 ha circular area round each nest.
MOISTURE = amount of moisture measured in soil with a RapiTest soil meter; 0 = dry, 10 = wet.
NATIVE = number of native stems ≥ 30.5cm within 0.04 ha circular area round each nest.
STEMDENS = density of woody vegetation ≥ 30.5cm in height /m^2.
GLOBAL = contains all measured variables.

$P = 0.661$). The highest ranking model described the effect of percent of invasive stems on nest site selection (PER_INV) and was the only one considered substantially supported ($AIC_c = 34.770$, $w_i = 0.492$; Table 3). We also evaluated the importance of each covariate individually (Burnham and Anderson 2002). Odds ratios indicated that habitat used by woodcock for nesting decreased with an increase in percentage of invasive vegetation present (Table 4).

Invasive vegetation comprised 57.1% (SE = 5.86) of the woody vegetation on random plots. Mean soil moisture (2.09 ± 0.21) and stem density (81.2 ± 10.24) did not vary between used and random locations. Total woody vegetation stems ≥ 30.5 cm in height per plot did not vary ($\chi^2_{24} = 0.29$, $P = 0.772$) across nest sites (84.23 ± 16.33) and random sites (78.09 ± 12.98). We did, however, detect a significantly lower percentage of invasive vegetation between nest sites and random sites ($\chi^2_{24} = 2.04$, $P = 0.050$). Native stem density was not different on nest sites and random sites ($\chi^2_{24} = 1.41$, $P = 0.174$; Fig. 3). Multiflora rose and tatarian honeysuckle were present on 100% of the random sites compared to 85% and 77% of nest sites, respectively (Table 5). Additionally, multiflora rose and tatarian honeysuckle comprised 50% of the total stem count on random sites compared to

Table 4. Akaike-weighted odds ratios of variables occurring in models of nest site habitat selection in Swatara State Park, Schuylkill & Lebanon Counties, PA, USA during 2009–2010.

				95% Confidence Interval	
Variablea	β	SE	Odds Ratio	Lower	Upper
NATIVE	0.014	0.011	1.010	0.993	1.037
MOISTURE	0.102	0.375	1.107	0.531	2.310
STEMDENS	0.002	0.008	1.002	0.982	1.018
INVASIVE	–0.035	0.021	0.965	0.882	1.006
PER_INV	–2.015	0.891	0.133	0.002	0.765

[a]NATIVE = number of native stems ≥ 30.5 cm present within 0.04 ha circular area around each nest.
MOISTURE = amount of moisture measured in soil with a RapiTest soil meter; 0 = dry, 10 = wet.
STEMDENS = density of woody vegetation ≥ 30.5 cm in height /m^2.
INVASIVE = number of invasive species ≥ 30.5 cm present within 0.04 ha circular area around each nest.
PER_INV = percentage of invasive stems present in 0.04 ha circular area around each nest.

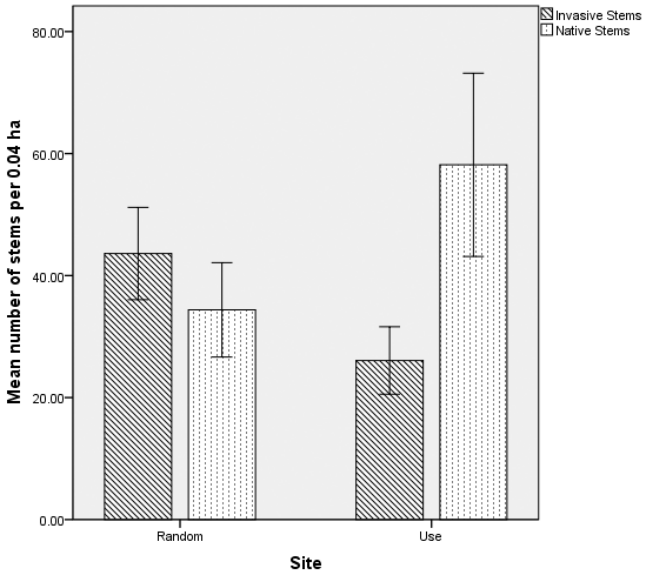

Figure 3. Observed mean values (± 1 SE) for invasive and native woody vegetation recorded at American woodcock nest and randomly selected sites in Swatara State Park, Schuylkill & Lebanon Counties, PA, USA during 2009–2010.

Table 5. Woody vegetation ≥ 30.5-cm in height present within a 0.04 ha plot around nest and randomly selected sites in Swatara State Park, Schuylkill & Lebanon Counties, PA, USA during 2009–2010.

		Nest location		Random location	
Species	Common Name	% occurrence[a]	% total stems[b]	% occurrence[a]	% total stems[b]
Rosa multiflora[c]	Multiflora rose	85.0	9.9	100.0	23.8
Fraxinus americana	White ash	76.9	3.7	76.9	4.3
Lindera benzoin	Northern spicebush	76.9	11.1	46.2	7.9
Lonicera tatarica[c]	Tatarian honeysuckle	76.9	10.9	100.0	25.7
Prunus serotina	Black cherry	69.2	4.2	46.2	2.8
Acer rubrum	Red maple	53.9	2.9	46.2	3.9
Elaeagnus umbellata[c]	Autumn olive	38.5	3.3	53.8	4.7
Cornus amomum	Silky dogwood	46.2	4.3	23.1	0.4
Juglans nigra	Black walnut	46.2	1.0	23.1	0.6
Viburnum prunifolium	Blackhaw	38.5	7.3	23.1	0.5
Rubus allegheniensis	Blackberry	30.8	3.0	30.8	3.1
Rubus idaeus	Raspberry	30.8	1.3	7.7	0.4
Pinus sylvestris[c]	Scotch pine	23.4	0.2	15.4	1.2
Viburnum dentatum	Southern arrowwood	23.1	12.9	7.7	0.1
Liriodendron tulipifera	Tulip poplar	15.4	4.9	15.4	1.1
Smilax rotundifolia	Greenbrier	7.7	7.6	0.0	0.0
Robinia pseudoacacia	Black locust	7.7	5.0	0.0	0.0
Crataegus spp.	Hawthorn	7.7	1.7	7.7	1.4

a % of sites at which ≥ 1 species stem was found.
b % of total count of woody stems ≥ 30.5-cm.
c Invasive vegetation.

20% on nest sites (Table 5). Occurrence of autumn olive was also higher at random sites than at nesting sites (Table 5). White ash was the most prevalent native species and was found consistently on both nest and random points; however, it only comprised a low percentage of the total stem density. Southern arrowwood (*Viburnum dentatum*), northern spicebush (*Lindera benzoin*), greenbrier (*Smilax rotundifolia*), and blackhaw vibrurnum (*Viburnum prunifolium*) accounted for the highest percentage of the total stem count (Table 3).

DISCUSSION

The number of nests we located over the course of this study was comparable to previous studies (Parris 1986, Chambers 1976, Bourgeois 1977, Kinsley and Storm 1988, Harris et al. 2009). We found our apparent nest success rate of 69% to be consistent with the range of rates (50–85%) reported by previous research (Mendall and Aldous 1943, Liscinsky 1972, Whitcomb 1974, Gregg 1984).

Nest success was greater in plots containing a lower percentage of invasive stems and models that included the percentage of invasive woody vegetation within the nest plot ranked highest in our nest success model set. Models that included the percentage of invasive woody vegetation within the plot also ranked high in our habitat selection model set. Nesting habitat selection appeared to be strongly associated with an avoidance of invasive shrubs, particularly multiflora rose and tatarian honeysuckle and we suggest that the factors involved in this avoidance are deserving of further investigation. Soil moisture and stem density did not appear to influence habitat selection however woodcock typically nest on drier sites (Liscinsky 1972, Gregg and Hale 1977, Coon et al. 1982) and we did experience dry weather during the course of the study.

Woodcock are cryptically colored and well adapted to nest on the ground, and their eggs are mottled and blend in with the leaf litter. They are well adapted to visually detect predators while nesting as evidenced by the location of the eyes towards the back of the head allowing woodcock to see in nearly a 360° arc (Mendall and Aldous 1943). Moreover, the low stem density in our study indicated that woodcock appeared to nest in less-dense cover. Tirpak et al. (2006) suggested that ruffed grouse (*Bonasa umbellus*), which also nest on the ground, may not rely on dense understory vegetation to avoid predators due to well-camouflaged plumage. Conversely, invasive woody vegetation can form a dense understory and rapidly degrade native habitat (Underwood et al. 1996, Deering and Vankat 1999, Kaufman and Kaufman 2007). Multiflora rose can form nearly impenetrable thickets which exclude native vegetation (Kaufman and Kaufman 2007), and bush honeysuckles may exhibit allelopathic effects, preventing other shrubs from going in close proximity to it (Williams 1994). Additionally, Rhoads and Block (2000) reported that multiflora rose and tatarian honeysuckle leaf out much earlier than native shrubs in Pennsylvania. If woodcock nesting locations are chosen in areas that are composed of a high percentage of invasive shrubs, the early leaf out and dense growth may prohibit detection of approaching predators. However we do acknowledge that the low number of nests makes our results preliminary. In support of our findings, Borgmann and Rodewald (2004)

reported that nest success was lower for American robin (*Turdus migratorius*) nests located in invasive shrubs and invasive shrubs reduced the nesting success of forest birds.

Previous studies of woodcock habitat preference do not include much information on invasive woody vegetation, most likely because invasives were not as widespread at the time of the studies. Current woodcock habitat management, however, must address exotic invasive woody vegetation effects and we are the first to document this relationship. We believe this is vital if the national objective of returning American woodcock populations to 1970s levels (Kelley and Williamson 2008) is to be successful.

Throughout woodcock range, habitat loss and degradation are a major cause of population decline and creating and maintaining young forested habitat is important to returning woodcock populations to past levels. Such efforts will be difficult where invasive vegetation is present. Treating invasive vegetation is expensive and managers should utilize tools that are most cost-effective. The odds of native woody vegetation competing with or out-competing invasive woody vegetation can greatly increase with proper invasive control methods like prescribed fire. Timing of control methods is critical to effectively combating invasive vegetation (Richburg 2005). By conducting repeated growing season fire treatments, which reduce the plant's vigor, further stress can be placed on the root system. This stress decreases sprout vigor, causes root death, and increases the vulnerability of the plant to competition and disease (Richburg 2005). Because exotic invasive vegetation is extremely resilient, exotic invasive shrub control should occur over long periods of time and will require a tremendous amount of work and effort from biologists, land managers, and the public. The continued conservation of American woodcock and their habitats, however, greatly outweighs the costs of such efforts.

ACKNOWLEDGMENTS

This project was made possible with funding from the Wildlife for Everyone Endowment. We thank the anonymous reviewers for their constructive comments. Special thanks to Mike Kotay for his tremendous help in conducting the research. We are grateful to Erik Blomberg, John Dunn and Ben Jones for their comments on earlier drafts of this manuscript. We thank the Quittapahilla Chapter of the Audubon Society for the valuable help in capturing woodcock. We thank the numerous dog handlers for their help in conducting nest searches, and PA DCNR for granting us permission to conduct the study on their land.

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Journal of the Pennsylvania Academy of Science 85(4): 140–146, 2011

RELATIVE DISEASE SUSCEPTIBILITY OF CULTIVATED VARIETIES OF POTATO TO DIFFERENT ISOLATES OF *PHYTOPHTHORA INFESTANS*[1]

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ABSTRACT

Late blight, caused by the Oomycete pathogen *Phytophthora infestans*, continues to be the most devastating and economically important disease affecting potato crops worldwide. Recent geographical migrations of the pathogen have displaced the 'old' US-1 clonal lineage with 'new', genotypically distinct, lineages. These new genotypes have shown increased pathogenic fitness and diverse environmental requirements. Interactions between two different isolates of the US-8 race and three potato (*Solanum tuberosum*) cultivars were analyzed through *in planta* infection, under controlled laboratory conditions. Disease development over time was rated for each experimental unit by determining incubation period, foliar lesion area and relative number of leaves displaying necrosis. Difference in incubation period between isolates was nearly significant in Russet Burbank, with disease symptoms presenting much later for replicates infected with PSUPotb. No significant difference was seen in incubation period between isolates in cv. Kennebec or cv. Red Norland, with the shortest incubation period occurring for isolate NC092ba in Kennebec. Incubation period did not differ for isolate NC092ba across cultivars. Isolate PSUPotb had a longer incubation period in Russet Burbank than in the other two cultivars. Data suggest, therefore, that differential pathogenicity between the US-8 isolates exists in at least one cultivar. Cultivar susceptibility was also variable. As suggested in previous studies, it was found that cv. Kennebec was least susceptible to either isolate. Confirmation of pathogen presence in infected leaf tissue was successfully obtained using previously developed *P. infestans* specific primers in a standard Polymerase Chain Reaction (PCR) assay.
[J PA Acad Sci 85(4): 140–146, 2011]

INTRODUCTION

Late blight disease, caused by the oomycete *Phytophthora infestans*, devastated potato crops and led to as many as one million deaths in Ireland and a major diaspora from the country during the 1840's. The cause of this widespread potato disease was largely unknown at the time, as the malady occurred well before the germ theory of disease became a universal paradigm (Erwin and Ribeiro 1996). Today, the overall costs to control potato late blight disease by means of fungicide applications and other management strategies, and the expenses incurred due to crop losses exceed six billion dollars annually (Haverkort et al. 2008).

Phytophthora infestans is a member of the Phylum Oomycota within the Kingdom Stramenopila (Alexopoulos et al.1996). Although initially classified within the Kingdom Fungi, recent studies have revealed that Oomycetes are more closely related to brown algae and diatoms than to Fungi (Haas et al. 2009). *Phytophthora infestans* is a heterothallic oomycete that requires two different mating types (A1 and A2) for sexual reproduction (Goodwin 1997). Until 1984, only isolates of the A1 mating type existed outside Mexico, which is considered the center of origin of this pathogen (Goodwin and Drenth 1997). However, for reasons that have been linked to both pathogen mutation and migration of infected potato tubers via international trade, the A2 mating type has become widespread throughout Europe and North America (Goodwin 1997). Since then, there has been an increase in genetic diversity within the population of *P. infestans* (Goodwin 1997).

Clonal lineages (races) of *P. infestans* are generally considered clonal descendents of the same asexually reproducing genotype. They are described based on several different markers including mating type, DNA fingerprint analysis, and allozyme genotype at the glucose-6-phosphate isomerase (*Gpi*) and peptidase (*Pep*) loci (Kato et al. 1997; Goodwin et al. 1995). Prior to the migration of A2, the primary clonal lineage of the pathogen was US-1, which is considered now to be the "old" clonal lineage (Lamour and Kamoun 2009). In the decades since the migration of A2, the old clonal lineages have been replaced with genotypically distinct "new" clonal lineages, such as US-7 and US-8. The presence of new clonal lineages in a particular region may be due to migration, sexual recombination or mutations within an already established clonal lineage (Goodwin et al. 1995). Several key differences between clonal lineages of *P. infestans* have been noted, including pathogenic fitness, fungicide sensitivity and environmental preferences (Kato et al. 1997; Fry and Goodwin 1997). This suggests that con-

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ventional wisdom on the biology of this pathogen may not apply to new, genotypically distinct lineages.

We have investigated the interactions between two *P. infestans* isolates belonging to the US-8 genotype and three different potato (*Solanum tuberosum*) cultivated varieties (referred to as cultivars), namely cv. Kennebec, cv. Red Norland and cv. Russet Burbank. These three cultivars represent the three major skin colors found in commercially marketed potatoes: white, red and russet, respectively. Kennebec was among the first varieties of potato to be bred purely for resistance to potato late blight and became available in the 1940's as a resistant variety (Akeley et al. 1948). It is a white skinned variety originally described as "vigorous, fast-growing, high-yielding and late maturing" (Akeley et al. 1948). At the time, it was found to be superior to varieties that were marketed for their resistance to late blight. The Red Norland cultivar was released in 1957 and described as a red skinned potato with moderate resistance to common scab and high susceptibility to late blight (Johansen et al. 1959). Russet Burbank is a russet-skinned variety that has demonstrated high susceptibility to potato late blight (Douches et al. 1997). Most research on susceptibility of these cultivars was done prior to the 1980's and thus before the "new" clonal lineages of *P. infestans* predominated in the United States. Therefore, while many experiments have examined susceptibility of these cultivars to late blight, few have compared resistance relative to one another when exposed to a pathogen of the US-8 clonal lineage under laboratory conditions.

Field studies in 1996–97 exposed numerous commercial cultivars, including those noted above, to US-8 isolates in an attempt to establish resistance rankings based on the Relative Area Under the Disease Progress Curve (RAUDPC; Jenkins and Jones 2003). Although none proved suitably resistant, of the many varieties being examined in this previous study cv. Kennebec displayed the lowest susceptibility, with cv. Russet Burbank being slightly more susceptible and cv. Red Norland being highly susceptible (Jenkins and Jones 2003). However, preliminary observations in our laboratory suggested that Russet Burbank is among the most susceptible varieties to isolates of the US-8 clonal lineage, with disease progressing rapidly relative to Kennebec and Red Norland, respectively.

Field experiments, while critical in providing general conclusions regarding resistance in commercial varieties, are subject to variations due to environmental conditions, mechanisms of inoculation, and insect and other pathogen presence. Therefore, conducting these experiments under controlled laboratory conditions, with each plant receiving uniform exposure to inoculum, will be critical in confirming the validity of the results obtained in the field. In addition, the use of new strains of the pathogen, recently isolated from potato fields, will serve to assess the general response of these potato cultivars to other members of the US-8 race. This study aims to quantify levels of resistance to two different isolates of the US-8 clonal lineage among the three potato varieties by *in planta* infection under controlled laboratory relative humidity, temperature and photoperiod conditions.

MATERIALS AND METHODS

Living material

Phytophthora infestans *Isolates*

Isolates for this study were chosen based on their ability to consistently cause infection of potato plants under laboratory conditions. Isolates PSUPotb and NC092ba were obtained from the United States Department of Agriculture (USDA) in Beltsville, MD. Each isolate had been confirmed to be of the US-8 clonal lineage and A2 mating type (F. Perez, USDA, Pers. Comm.). Cultures were maintained in the laboratory on Rye A Agar (Caten and Jinks 1968) and were re-isolated onto Nonclarified V8®/Lima bean agar (see below) for sporulation and inoculum preparation (Anonymous, 2001). Nonclarified V8®/Lima bean agar was prepared by autoclaving 80g of frozen baby lima beans in 200ml of distilled water for 10 min at 15psi. Upon cooling, lima beans were strained from liquid and discarded, retaining the liquid. Next, 200ml of V8® juice and 1.4g of $CaCO_3$ (Fisher Scientific, Fair Lawn, NJ) were added along with distilled water to bring mixture to volume. Adjustment of pH to 6.0 was achieved by adding the appropriate amount of KOH. Then, 30g of Difco Agar, granulated (Becton-Dickinson, Sparks, MD) was added and the medium was autoclaved once more for 15 minutes at 15psi (Anonymous, 2001). All cultures were grown in the dark at 18ºC.

Cultivars

Locally grown seed tubers of cvs. Kennebec, Russet Burbank and Red Norland were obtained from the Netherland Bulb Company (Easton, PA) and the Penn State Cooperative Extension division of Lehigh County. Blocks of 2 cm^2 were excised around visibly germinating tuber eyes and planted in 20-cm terracotta pots with Miracle-Gro® Potting Mix. Pots were placed in a growth chamber at 24ºC, with 16-hr light and 8-hr dark photoperiod. Plants were watered every 3 days throughout the duration of the study.

In planta Infection

Inoculum Production

Each isolate of *P. infestans* was transferred from Rye A agar to plates of Nonclarified V8®/Lima bean agar and new cultures were stored at 18ºC in the dark. After two weeks of incubation, plates were flooded with 5ml of ddH_2O. Mycelium was agitated for one minute using a pipette tip to dislodge sporangia from hyphal tips. Liquid was drawn back into the pipette tip, placed in 15ml conical centrifuge tube,

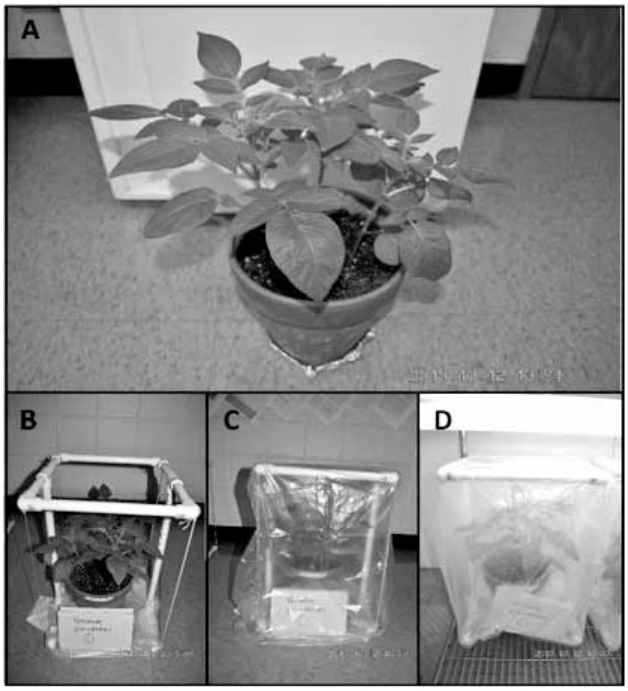

Figure 1. *In planta* infection experiment. cv. Kennebec replicates (A) prior to infection with isolate PSUPotb. Infection chamber, constructed of PVC frame (B), was covered with 0.6mil pre-moistened plastic bag (C). Experimental unit was then placed in growth chamber for the duration of the study (D).

and set on ice. This procedure was repeated with a second aliquot of ddH_2O and both sporangial suspensions were combined and centrifuged for two min at 500 *g*. Following centrifugation, 5ml of supernatant was carefully decanted. Sporangium concentration was determined using a hemocytometer and adjusted to 1.5×10^4 sporangia/ml. This technique was repeated with two more plates until 15ml of concentrated sporangial suspension had been obtained and subsequently incubated at 4ºC for two hours to promote zoosporogenesis. This procedure was completed for each of the two isolates (Becktell et al. 2005; Douches et al. 1997).

Preparation of Plants

At five weeks old, a total of 27 plants (n = 3 per cultivar, for j = 2 isolates plus control, in triplicate) were obtained for this study. Replicate sets of cultivars had been grown together in 20-cm terracotta pots. Pots were placed in individual infection chambers constructed of a PVC frame and covered with transparent plastic bag with 0.6mil thickness to allow adequate light exposure (Figure 1). Infection chambers also served to maintain high relative humidity (close to 100%) in the disease system while preventing cross contamination between experimental conditions.

Plant infection

After a two-hour incubation period, sporangial suspensions were transferred to two hand-held atomizers. One atomizer was filled with an equal volume of distilled water as an appropriate control. To ensure uniform inoculum exposure, each plant was sprayed 13 times, distributed evenly, until every leaf appeared wet. The amount of inoculum received per plant was quantified by obtaining an average volume for each spray (~1.82ml), and then determining the approximate total number of sporangia present in infection. Control plants were sprayed in the same manner with distilled water. The inside of growth chambers was then sprayed with distilled water and sealed to promote high relative humidity and to ensure infection establishment. Infection chambers were kept at 24ºC with a 16-hour photoperiod.

Assessments

One experiment including three replicates for each cultivar was conducted. Disease progression was observed every 24 hours after inoculation (HAI) during a seven-day period. The first dependent variable measured was incubation period (IP), defined as the HAI when first visible necrosis was observed (this measure is sometimes referred to as the latent period). After initial infection had been established in an experimental unit, each infected leaf was photographed daily. Lesion area was calculated using ImageJ computer software by determining the proportionate lesion area per infected leaf (PLA) relative to healthy leaf mass. Number of leaves per plant and number of leaves infected (showing visible signs of necrosis) were also recorded daily. Seven days after initial inoculation, visibly infected leaves were removed from each replicate and flash frozen in liquid nitrogen and stored at –80ºC for future DNA extraction. Plants were autoclaved and discarded two weeks after initial inoculation.

Molecular Confirmation of *P. infestans* Presence

Genomic DNA extraction was performed from visibly infected leaves detached from each replicate plant (168 HAI), as well as uninfected controls. For a positive control, genomic DNA was extracted from mycelium of *P. infestans* isolate NC092ba that had been grown on Pea Broth media at 25ºC. All extractions were performed using GenElute™ Plant Genomic DNA Miniprep Kit according to manufacturer (Sigma-Aldrich, St. Louis, MO) recommendations. To confirm the presence of *P. infestans* DNA in the samples, standard PCR assays were performed in 25µl reaction volumes, each with 1µl genomic DNA. Reactions also contained 17µl ddH_2O, 5µl 5× GoTaq® Buffer (Promega, Madison, WI), 0.5µl 5U/µl GoTaq® DNA Polymerase (Promega), 0.5µl 10µM dNTPs (Promega), and 0.5µl each of 10mM *P. infestans* primers PiITS5 and Pinf2. Primer sequences were 5'-GGA AGT AAA AGT CGT AAC AAG-3' and 5'- CTC GCT ACA ATA GCA GCG TC-3', respectively (Trout et al. 1997). Reactions were conducted using Bio-Rad's MyCycler™ (BioRad Laboratories, Hercules, CA). PCR parameters included an initial 2-min 92ºC denaturation phase, followed by 35 cycles of denaturation at 92ºC for 15 sec,

annealing at 57ºC for 15 sec, and 30 sec of extension at 72ºC. Reactions always included appropriate positive and negative controls. Products were electrophoresed on 1.2% agarose gels containing 500µl 1µg/ml Ethidium Bromide in 1× TAE buffer at 60V for 45 minutes. Samples were run along with 1kb DNA Ladder (Promega) as a standard.

Statistical Analysis

Summary statistics analyzed in all experiments included treatment of arithmetic means with standard errors computed using Microsoft Excel (Excel version in Microsoft Office 2007 for Windows). Where suitable, statistical assessment typically included one-way analysis of variance (ANOVA).

RESULTS

In planta infection

Incubation period

Infection was successfully established in all plants (n = 3 for each cultivar) infected with isolate NC092ba and in all plants but one replicate of cv. Kennebec infected with PSUPotb. No disease symptoms appeared in control plants. No significant difference was seen in incubation period between isolates in cv. Kennebec (P = 0.251) or cv. Red Norland (P = 0.374), with the shortest incubation period occurring for isolate NC092ba in Kennebec ($\overline{x}$ = 32 ± 16.0, n = 3) (Figure 2). Difference in incubation period between isolates was nearly significant in Russet Burbank (P = 0.055), with disease symptoms presenting much later for replicates infected with PSUPotb ($\overline{x}$ = 88 ± 8.0, n = 3) than with NC092ba ($\overline{x}$ = 40 ± 16.0, n = 3). Incubation period did not differ for isolate NC092ba across cultivars (P = 0.702).

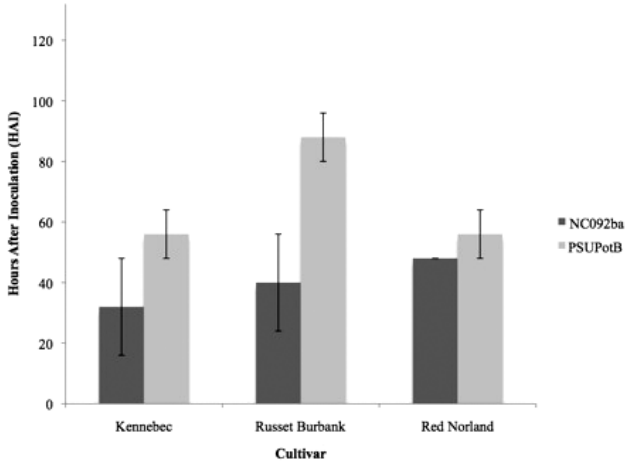

Figure 2. *In planta* incubation period. Hours after inoculation (HAI) when first visible signs of necrosis appear. Black bars represent isolate NC092ba and grey bars represent isolate PSUPotb. Error bars represent the standard error of the mean time of visible infection for each experimental unit (n = 3).

Isolate PSUPotb had a longer incubation period in Russet Burbank than in the other two cultivars (P = 0.05).

Disease Area

Daily photographs were taken of each leaf showing visible necrosis, for each plant. Using ImageJ computer software, daily proportionate area of necrotic tissue for each leaf was manually delineated in pixels and compared to total leaf size in pixels. This measure gives a proportionate lesion area per infected leaf (PLA). Although data were obtained and analyzed for all time points, it was necessary to choose a single time point to examine disease progression. The time point 96 HAI was chosen as a conservative estimate for each condition, when highest number of leaves appeared to be infected, but prior to excessive leaf loss due to disease (Figure 3). Infected leaves were pooled together from all replicates (n = 3), for each condition, to obtain each PLA value (Figure 4).

There was no difference in PLA between isolates NC092ba ($\overline{x}$ = 0.061 ± 0.022, n = 5) and PSUPotB ($\overline{x}$ = 0.054 ± 0.038, n = 5) in cv. Kennebec (Figure 4). There also appeared to be no difference between NC092ba ($\overline{x}$ = 0.390 ± 0.376, n = 34) and PSUPotb ($\overline{x}$ = 0.376 ± 0.060, n = 36) in cv. Russet Burbank. In cv. Red Norland, PLA was much greater for isolate NC092ba ($\overline{x}$ = 0.722 ± 0.054, n = 47), than for isolate PSUPotb ($\overline{x}$ = 0.269 ± 0.057, n = 27). At 96 HAI, cv. Kennebec had a significantly lower PLA for each isolate than Russet Burbank or Red Norland (Figure 4). Russet Burbank appeared to have intermediate PLA when compared to Kennebec and Red Norland. Interestingly, PLA in Red Norland was not different from Russet Burbank for isolate

Figure 3. Foliage of each experimental unit. Photographs were taken at 96 hours after infection. Inset: photograph of an infected leaf from each plant at the given time point.

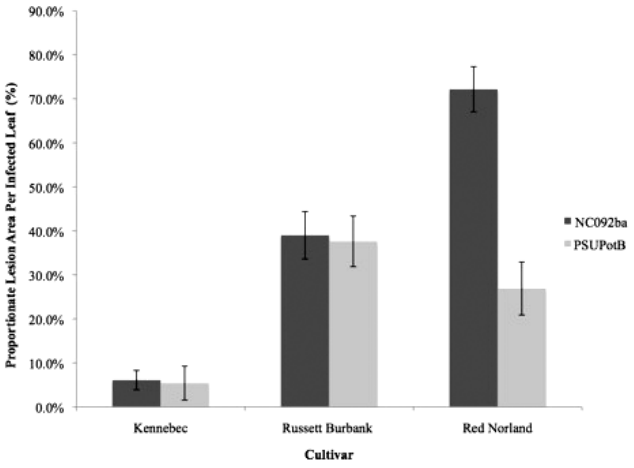

Figure 4. Mean proportionate lesion area (PLA) showing necrosis for all infected leaves at 96 hours after inoculation (HAI). Black bars represent isolate NC092ba and grey bars represent isolate PSUPotb. Error bars represent standard error of the mean disease area for all infected leaves at each condition for each of three trials (n = 5 – 47).

PSUPotb, but was much greater for isolate NC092ba. When isolates were compared between cultivars, PLA for NC092ba was smallest in Kennebec, intermediate in Russet Burbank and greatest in Red Norland. PLA for isolate PSUpotb was also smallest in Kennebec but was intermediate in both Russet Burbank and Red Norland (Figure 4).

Molecular Confirmation

Infection was successfully confirmed in all infected replicates via Polymerase Chain Reaction (PCR) assay. The expected ~600-bp fragment was visualized on 1.2% agarose gel electrophoresis in all samples from infected leaf tissue and the positive control. No product was visible in noninfected negative controls (Figure 5).

DISCUSSION

We have investigated the interactions of three different potato cultivars, developed in the 1900's, with two geographically different isolates of *P. infestans* belonging to the clonal lineage US-8. Results of our study revealed interesting trends in the disease cycle of the three cultivated varieties of potato, specifically in regards to the time elapsed between the moment of inoculation and when the first visible signs of necrosis appear. In addition, significant differences were observed in the mean proportionate lesion area (PLA) for all infected leaves 96 hours after inoculation. Data suggest that variable susceptibility to *P. infestans* exists between cultivars, with each cultivar developing some level of disease (Figure 2); thus no complete resistance was observed. Interestingly, there was also an isolate effect on disease development (cf. cultivar Russet Burbank, Figure 2, and cultivar Red Norland, Figure 4). This is surprising, as asexually derived members of the same clonal lineage are generally believed to be genotypically identical descendents of the same ancestor (Kato et al. 1997).

When infected with isolate NC092ba, cv. Kennebec was least susceptible, cv. Russet Burbank was moderately susceptible and cv. Red Norland was most susceptible (Figure 4). This was determined by comparing the amount of necrotic tissue that had been integrated into each infected leaf at 96 hours after inoculation (HAI). These findings corroborate field studies in which several commercially important cultivated varieties were exposed to US-8 isolates (Jenkins and Jones 2003). From these investigations, it was concluded that Russet Burbank was moderately susceptible while Kennebec was much less susceptible (Jenkins and Jones 2003). In our study, however, a similar trend was not apparent when cultivars were infected with isolate PSUPotb. While cv. Kennebec was shown to be least susceptible, there was no difference in susceptibility between cv. Russet Burbank and cv. Red Norland (Figure 4).

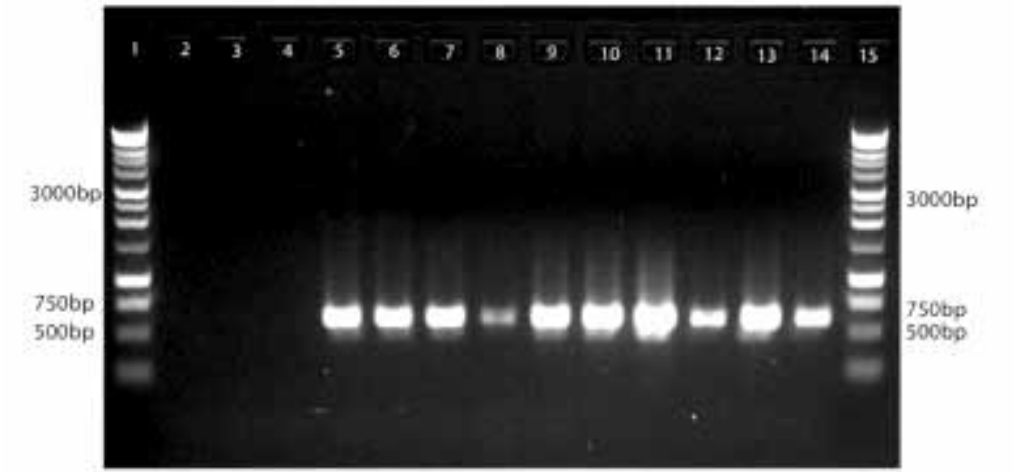

Figure 5. Detection of *P. infestans* in infected tissue. Expected ~600- bp PCR products visualized on 1.2% agarose gel electrophoresis. 1 kb DNA Marker (Lanes 1 and 15). Negative control DNA from uninfected foliage from Kennebec (Lane 2), Russet Burbank (Lane 3), and Red Norland (Lane 4). Positive control DNA extracted from mycelia of *P. infestans* isolate NC092ba (Lane 5). *In planta* infection: Leaves infected with *P. infestans* isolate NC092ba for cv. Kennebec Replicates 1-3 (Lanes 6, 7 & 8), cv. Russet Burbank Replicates 1–3 (Lanes 9, 10 & 11) and cv. Red Norland Replicates 1-3 (Lanes 12, 13 & 14).

Potatoes of the same cultivated variety used for this study had been vegetatively propagated, and therefore, are considered genetically identical. Because of this, variable disease response within cultivars with respect to different isolates [as seen, for example, with the PLA of Red Norland (Figure 4)], must be the result of variable pathogenic fitness between isolates. There are a number of reasons why this variability may exist within isolates of the same clonal lineage. One may be that because these strains were isolated from two distinct geographic regions, during different years, pathogens have undergone slight evolutionary divergence, with changes affecting their pathogenicity. There are several steps that must be accomplished for a pathogen to successfully infect a host. These include adhesion to host, penetration, down-regulation of plant immune response, acquisition of nutrients and colonization, which eventually leads to necrosis and plant death (Lamour and Kamoun 2009). Any changes in the genes that regulate these functions may alter the pathogenicity of the isolate. Because *P. infestans* is polycyclic, meaning the pathogen goes through several generations per growing season, it is conceivable that mutations could accumulate in these genes over time (Campbell 1998). As a result, genetic adaptation could occur based on local abiotic factors and the selection pressure exerted by potential resistance genes present in the host. Furthermore, it is possible that pathogenicity differences are not a product of different genes but rather a product of variable expression of homologous genes.

Previous studies examining pathogenic variability within clonal lineages have shown that asexually reproducing lineages can still diverge rapidly and overcome local compatible resistance (R) genes (Goodwin et al. 1995). It has also been shown that pathogenic divergence increases with time and, therefore, higher divergence is seen in older lineages (Goodwin et al. 1995). The US-8 clonal lineage, which is under examination in this study, was first detected in the United States in 1992. The long time elapsed between the first detection of this lineage and the collection of these new isolates might be sufficient for pathogenic divergence to occur within this clonal lineage. This is also evidence supporting the notion that, while classification of the pathogen within clonal lineages is an important tool, it is not necessarily indicative of pathogenic fitness.

For *in planta* infection, PLA was used as the primary means for assessment of disease severity. This is considered a good standard because assessments were frequent and because, in general, lesion area is a good measure of disease integration over time (Mizubuti and Fry 1998). Although it would have been possible to apply a semi-quantitative rating scale to assess disease severity, we chose not to do so, because these rating scales are difficult to analyze for a variable population size, such as that seen in plant infections with different leaf numbers between replicates (Dorrance and Inglis 1997).

The second dependent variable measured in this study was incubation period, or the amount of time that lapses between inoculation and the appearance of visible signs of disease (Agrios 2005). This is an important fitness component in the disease cycle of different isolates of *P. infestans* because the faster a pathogen can establish infection, the greater the number of reproductive cycles it can initiate during the infection process. In the *in planta* infection, incubation period was only variable in one cultivar, Russet Burbank, after inoculation with isolate PSUPotb. In these replicates, first disease symptoms were not visible until over three days after inoculation (Figure 2). However, when compared with the PLA measurements at 96 HAI, incubation period length had no apparent effect on overall disease progression throughout the course of the infection. Detached leaf assay infection experiments show a similar trend of long incubation period in Russet Burbank inoculated with PSUPotb at 18°C and 24°C (Andersen and Ospina-Giraldo. Unpublished). This suggests that these two methods of laboratory disease assessment may have similar predictive power in regards to the disease development. The extended amount of time that isolate PSUPotb requires before symptoms appear suggests that a unique isolate-host interaction exists whereby it takes the pathogen longer to overcome the plant's innate defenses. Once infection is visible, however, disease progression does not appear to be affected.

We have investigated the degree of resistance of potato cultivars against *P. infestans* infection using foliar indicators. However, it is important to note that foliar response to *P. infestans* does not necessarily correlate to tuber response to this pathogen (Inglis et al. 1996). For example, in a quantitative trait loci (QTL) study, it was shown that markers for foliar resistance were not associated with tuber resistance to blight; conversely, most markers associated with tuber resistance were not associated with foliar blight resistance (Mayton et al. 2009). There are two general types of resistance, vertical (qualitative) and horizontal (quantitative). Vertical resistance, said to involve one or very few resistance (R) genes, targets a specific pathogen race, and in potato, it has been usually introduced into cultivated varieties from wild *Solanum* species. This type of resistance has not proven efficient because individual R genes can be quickly overcome by new lineages of *P. infestans.* In contrast, horizontal resistance relies on multiple genes, has a broad spectrum, and appears to be more stable in the field (Agrios 2005). In recent years, plant breeders have focused less on race-specific resistance and have put more interest into understanding quantitative resistance. While this more general resistance proves promising for the development of durable resistance, relationships between foliar and tuber resistance remain complex (Mayton et al. 2009). Understanding foliar resistance remains important, however, because foliage remains the primary source of inoculum in the field. A significant step towards understanding the factors leading to late blight disease development has been the recent sequencing of the *S. tuberosum* (Unpublished) and *P. infestans* (Hass et al. 2009) genomes. The latter, at ~240Mb, is larger and more complex than previously sequenced parasitic oomycetes. Additional field studies focusing on *P. infestans* pathogenicity and *S. tuberosum* resistance will be greatly

assisted by an in-depth analysis of both genomes, particularly of those gene pathways that have been identified as critical for this interaction to occur.

In conclusion, these investigations have revealed a difference in pathogenicity between two isolates of the same clonal lineage (race). It is critical for disease forecasting and management to understand that these differences may exist within clonal lineages, as this will allow the implementation of appropriate cultural practices and chemical treatments, based on local host/pathogen populations and environmental conditions.

ACKNOWLEDGEMENTS

We would like to thank Dr. Nancy Waters for her contributions to experimental design and data analysis for this project and Mr. Philip Auerbach for his technical assistance. We would also like to thank Mr. Bob Leiby, the Lehigh County Extension Director for the Penn State College of Agricultural Sciences Cooperative Extension Division, for providing seed tubers for this study, and Ms. Frances Perez of the United States Department of Agriculture Agricultural Research Service (USDA-ARS) for providing the *P. infestans* isolates used in our investigations. This project was funded by Lafayette College.

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Journal of the Pennsylvania Academy of Science 85(4): 147–150, 2011

ADDITIONAL EVIDENCE FOR BAROTRAUMA AS A CAUSE OF BAT MORTALITY AT WIND FARMS[1]

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ABSTRACT

The discovery of extensive bat mortality at wind farms has puzzled biologists, especially since many carcasses found under turbines exhibit no obvious injuries. A recent study proposed barotrauma – damage to body tissues caused by changes in pressure – as a possible explanation. We tested this hypothesis by examining 146 carcasses of hoary bats (*Lasiurus cinereus*), eastern red bats (*Lasiurus borealis*), and silver-haired bats (*Lasionycteris noctivagans*) collected during mortality surveys at a wind farm in eastern Pennsylvania. All carcasses were examined for both external and internal injuries. To assess barotrauma, we looked for blood in the nose and mouth, hemorrhaging in the lungs, and pooled blood in the thoracic and abdominal cavities. Of 146 carcasses examined internally, 54 (37%) had signs of barotrauma but no external injuries, 48 (33%) had external injuries but no signs of barotrauma, and 44 (30%) had both signs of barotrauma and considerable external injuries. A chi-square test found no association between the age or sex of bats and the incidence of barotrauma. These results support barotrauma as a contributing cause of bat mortality at wind farms.
[J PA Acad Sci 85(4): 147–150, 2011]

INTRODUCTION

As our society seeks alternatives to fossil fuels, wind energy is often proposed as a substitute, and Pennsylvania alone has a goal of producing 4000 MW of electricity via wind power by 2020 (Pennsylvania Department of Community and Economic Development 2008). Although wind energy does provide an alternative to fossil fuels, there are growing concerns that wind farms have negative impacts on bird and bat populations (Arnett et al. 2008; Kunz et al. 2007; Tuttle 2004). One recent review of bat mortality surveys has estimated that up to 69.9 bats are killed per turbine per year, although location and turbine type play major roles in the extent of mortality (Arnett et al. 2008).

Approximately 75% of bat mortalities at wind farms appear to be migratory bats, particularly hoary bats (*Lasiurus cinereus*), eastern red bats (*Lasiurus borealis*), and silver-haired bats (*Lasionycteris noctivagans*; Arnett et al. 2008; Kunz et al. 2007). Most wind turbines in the northeastern U.S. are placed along ridges where the wind is strongest and most consistent. Fiedler et al. (2007) suggested that migrating bats may move along these ridges, contributing to the high mortality in these species.

The extent of bat mortality at wind farms is puzzling, and Cryan and Barclay (2009) reviewed hypotheses that have been proposed to explain it. Bats are able to catch very tiny insects in midair, so their inability to avoid large turbine blades is unexpected. Another baffling aspect of this mortality is that many of the dead bats exhibit no outward signs of injury. They often have no broken bones and no open wounds, and many carcasses appear in perfect condition.

These observations led Baerwald et al. (2008) to evaluate barotrauma as a cause of death for many of the bats found dead at wind farms. Barotrauma is defined as tissue injury caused by changes in pressure that lead to the compression or expansion of gases contained in the body (Beers 2003). In relation to atmospheric pressure, spinning wind turbine blades have areas of lower pressure along their entire lengths, with the greatest pressure gradient around the tip of the blade. The actual pressure required to kill a bat is unknown, but Baerwald et al. (2008) reported that blade-tip vortices have pressure drops in the range of 5-10 kPa, and noted that pressures within this range are known to cause death in larger mammals. For example, in rabbits the blood gas barrier of the lung sustained significant damage at a pulmonary capillary transmural pressure of 5.13 kPa (Birks et al. 1994). Baerwald et al. (2008) also noted that these pressure differences may explain why relatively little bird mortality has been reported from eastern wind farms: birds have a completely different structure to their respiratory anatomy (Liem et al. 2001), which may make them less susceptible to changes in barometric pressure.

To test the barotrauma hypothesis, Baerwald et al. (2008) examined 188 bats collected at a wind farm near Calgary,

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Alberta. Of the 188, 87 (46%) had no obvious external injuries. A subset of 75 fresh bat carcasses was necropsied in the field and assessed for both external and internal injuries. External traumatic injuries included broken wings, broken vertebrae and ribs, and muscular contusions. Internal injuries included blood in the nose and mouth, abdominal hernias, burst stomachs, intramuscular bruising and hemorrhaging, contused lungs, hemorrhaging in the lungs, detached heart and lungs, and hemorrhaging in the thoracic and abdominal cavities; presence of these injuries in a bat was interpreted as indicating barotrauma, in accord with the previously stated definition. Of the 75 necropsied bats, 69 (92%) showed hemorrhaging in the thoracic and abdominal cavities, and 43 of these (57% of the total) had no external injuries. Only 6 bats (8%) had external injuries with no signs of internal hemorrhaging. Baerwald et al. (2008) also examined tissue samples from 17 carcasses and looked for damage to the lungs, including pulmonary hemorrhage and fluid-filled alveoli. The 17 carcasses varied in external condition, but when examined histologically, all had evidence of pulmonary lesions, a sign of barotrauma. Baerwald et al. (2008) concluded that barotrauma contributed to a large proportion of bat deaths at that wind farm. More recently, Grodsky et al. 2011 suggested that barotrauma, and the cause of death for bats at wind turbines in general, is very hard to detect due to the condition of the bats and the various ways injury could occur. Bats found under turbines could have experienced barotrauma, but they could also have hit the blades, the monopoles, or the ground, all which could cause similar types of injuries which would be difficult to distinguish.

We assessed bats collected at the Locust Ridge I Wind Farm in eastern Pennsylvania in a similar analysis for signs of barotrauma. Our objective was to determine whether barotrauma was also a significant cause of mortality for bats at this wind farm.

MATERIALS AND METHODS

We examined bats collected from mortality surveys at the Locust Ridge I Wind Farm, Schuylkill County, Pennsylvania, a commercial wind facility containing 13 Gamesa 2-megawatt turbines (described in Zellner et al. 2008). Bats were collected during daily searches from 1 May to 17 November in 2007 and from 15 April to 17 November in 2008. Details of search procedures are found in Zellner et al. (2008). All bats found during mortality surveys had their overall condition noted at initial collection and were then stored in a freezer at –20º C. We restricted our analyses to 146 bats (57 from 2007 and 89 from 2008) that were minimally decomposed or desiccated, and were therefore in good enough condition to be necropsied for the signs of barotrauma recorded by Baerwald et al. (2008). As part of the analysis, photographs of the internal organs were taken, and at the end of the analysis voucher specimens were prepared for all carcasses. One person (SAB) consistently performed assessments and determined the severity and importance of all internal injuries. Chi-square analysis (SPSS Statistics 17.0, Chicago, IL) was used to look for any association between incidence of barotrauma and the age (juvenile vs. adult) and sex of the bats. Because the bats had been frozen prior to examination, we could not perform histological assessments similar to those conducted by Baerwald et al. (2008).

In addition to the bats in our sample, several other bats were found alive under the turbines and taken to a nearby wildlife rehabilitation center. These bats had external injuries, but survived in captivity for approximately 2 weeks after their original collection. Because they survived in captivity for several weeks, we inferred that these bats were not killed by barotrauma and we used them to represent the condition of internal organs of carcasses unaffected by barotrauma.

Table 1. Presence of external and internal injuries in bat carcasses found at the Locust Ridge I Wind Farm in 2007 and 2008, by species.

Species	External Injuries Only	Internal Injuries Only	Both External and Internal Injuries
Hoary Bat	25	23	11
Eastern Red Bat	7	14	20
Silver-haired Bat	16	17	13
Total	48	54	44

RESULTS

Of the 146 bats that we necropsied, 54 (37%) had minimal or no external injuries, while the others had varying degrees of external trauma. A majority of the necropsied bats (98, 67%) exhibited internal injuries, but 48 bats (33%) had considerable external injuries yet no apparent internal injuries (Table 1). The internal injuries we observed were similar to those recorded by Baerwald et al. (2008): contused/hemorrhaged lungs (n=87), hemorrhaging into the thoracic and/or abdominal cavities (n=78), blood in the nose and mouth (n=16), intramuscular bruising/hemorrhaging (n=24), burst stomachs (n=3), and detached heart (n=1; Table 2). Of the necropsied bats that had no apparent external injuries and still displayed substantial internal injuries (54, 37%), the injuries included contused/hemorrhaged lungs (n=51), hemorrhaging into the thoracic and/or abdominal cavities (n=42), blood in the nose and mouth (n=5), and intramuscular bruising/hemorrhaging (n=12). The types of injuries present (Table 1) were independent of both sex and age (sex: $\chi^2_{(5,\ n=146)} = 9.014$, $p = 0.109$, age: $\chi^2_{(5,\ n=146)} = 8.920$, $p = 0.112$).

DISCUSSION

Our results are generally consistent with those of Baerwald et al. (2008) and support the conclusion that barotrauma is a major cause of bat mortality at wind farms. Many of our bats

Table 2. Types of internal injuries in bat carcasses found at the Locust Ridge I Wind Farm in 2007 and 2008, and their association with external injuries, by species. Percentages are totals for the species with the given level of injury (e.g., 18.2% of hoary bats with external injuries had blood in their nose/mouth).

Species	Blood in nose/mouth		Intramuscular bruising/hemorrhaging		Contused/ hemorrhaged lungs		Hemorrhaging into the thoracic and/or abdominal cavities	
	With External Injuries	Without External Injuries	With External Injuries	Without External Injuries	With External Injuries	Without External Injuries	With External Injuries	Without External Injuries
Hoary Bat	2 (18.2%)	4 (16.7%)	4 (36.4%)	8 (33.3%)	8 (72.7%)	22 (91.7%)	9 (81.8%)	19 (79.2%)
Red Bat	6 (30.0%)	1 (7.1%)	3 (15.0%)	2 (14.3%)	16 (80.0%)	13 (92.8%)	16 (80.0%)	11 (78.6%)
Silver-haired Bat	3 (21.4%)	0 (0%)	5 (35.7%)	2 (11.1%)	12 (85.7%)	16 (88.8%)	11 (78.6%)	12 (66.7%)
Total	11 (25.0%)	5 (9.2%)	12 (27.2%)	12 (22.2%)	36 (81.8%)	51 (94.4%)	36 (81.8%)	42 (77.8%)

had minimal external injuries upon gross external assessment, but when examined internally they had a variety of injuries associated with barotrauma. We also recorded a set of internal injuries very similar to those noted by Baerwald et al., including contused lungs, hemorrhaging in the body cavities, and burst stomachs, although we did not observe any incidences of abdominal hernias or detached lungs.

Overall, we recorded a lower percentage of bats with injuries consistent with barotrauma than Baerwald et al. (2008): 37% of our bats had internal signs of barotrauma with no external injuries, whereas 57% of their bats were in this category. However, Baerwald et al. (2008) interpreted intramuscular bruising and blood in the thoracic and abdominal cavities as positive signs for barotrauma. In contrast, we felt that we could not rule out external physical injury or freezing as the cause of bruising and internal bleeding in our bats, and we considered the cause of death in bats with only these injuries to be inconclusive.

Bats that experienced external trauma may have subsequently suffered internal damage unrelated to pressure changes. For example, getting hit by a turbine blade might cause internal bleeding. Also, bats that experienced changes in barometric pressure could have obtained external injuries after experiencing barotrauma by subsequently hitting either a turbine blade or the ground, and this would make it difficult to discern the cause of death as both external and internal injuries would be present. The order in which the injuries occurred is critical, but may be impossible to ascertain from a carcass. Also, red-tinged fluid present in the thoracic and abdominal cavities could be confused for blood when it is simply an artifact of tissue damage from freezing. The fact that our bats were frozen prevented us from examining lung tissue histologically, which may have provided definitive evidence of barotrauma as the cause of death.

In conclusion, even with our conservative assessment of barotrauma, 54 of 146 bats (37%) showed diagnostic signs of barotrauma and had no external injuries likely to have caused mortality. Barotrauma therefore does seem the likely cause of death for a large percentage of bats killed at the Locust Ridge I Wind Farm. Kunz et al. (2007) questioned whether migratory bats will be able to sustain the year-after-year mortality that is occurring at wind energy facilities, and they expressed particular concern about the eastern red bat (*Lasiurus borealis*), one of the species that is killed in largest numbers at wind farms and a species that already appears to be in decline throughout much of its range.

Arnett et al. (2011) and Baerwald et al. (2009) have demonstrated that small changes in wind-turbine operation can result in major reductions in bat mortality, with only marginal loss in power production. These modifications involve either changing the wind-speed at which the turbine blades begin to turn or altering the blade angles to reduce rotor speed; both techniques result in turbine blades that are nearly motionless in low wind speeds, when mortality is greatest. Bat populations around the world face threats from a variety of anthropogenic and natural causes. Being able to understand, and perhaps minimize, one cause of mortality could have an important impact on the survival of some bat species. Deaths due to barotrauma need to be considered when investing in future bat-safe wind turbine technologies. Bats are dying at wind farms, and understanding the causes of these mortalities may help to minimize future mortality.

ACKNOWLEDGMENTS

We thank the Pennsylvania Game Commission and Iberdrola Renewables for funding, East Stroudsburg University for the use of facilities, Jessica Newbern, Shannon Williams, and Mike Scafini for help with the processing of bat carcasses and the preparation of voucher specimens, and Tom LaDuke, Terry Master, Doug Roscoe, and Jane Huffman for comments on early versions of this manuscript. We also thank three anonymous reviewers for their suggestions to improve the manuscript.

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Journal of the Pennsylvania Academy of Science 85(4): 151–158, 2011

A STUDY OF EPIPHYTIC LICHEN COMMUNITIES IN URBAN AND RURAL ENVIRONMENTS IN SOUTHWESTERN PENNSYLVANIA[1]

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ABSTRACT

Community analyses of epiphytic lichens were used to study the controlling factors of lichen abundance and diversity in urban and rural environments of Pittsburgh in southwestern Pennsylvania. Two urban sites included Schenley and Frick Parks in metropolitan Pittsburgh and two rural sites at Mingo Creek County Park in Washington County and Roaring Runs Natural Area in Westmoreland County. Community composition of lichens was measured at six intensive monitoring plots per site and site-wide species diversity surveys. The lichen diversity value, a statistical estimator of the environmental conditions at a site, was greater at the rural sites (20.8 ± 3.0) compared to the urban sites (11.3 ± 3.5) (± standard error), suggesting a less disturbed lichen community at Mingo and Roaring Runs. In the intensive monitoring plots, species richness was greater at Mingo and Roaring Runs compared to Schenley and Frick, averaging 5.2 ± 0.3, 4.7 ± 0.4, 3.7 ± 0.8 and 2.0 ± 0.4, respectively. The dominant lichens across all sites were *Lepraria lobificans*, an unidentified sterile crustose lichen and *Cladonia ochrochlora*. The dominance of nitrophilous and sulfur dioxide–tolerant lichens at all sites suggests that the lichen community within the larger geographical region is influenced by nitrogen and sulfur dioxide air pollutants. The differences between sampling sites are most likely driven by lichens responding to changes in urbanization, which include humidity and habitat fragmentation.
[J PA Acad Sci 85(4): 151–158, 2011]

INTRODUCTION

The diversity of lichens and their differing levels of sensitivity allow for informative community analyses to monitor pollution gradients over large areas. In urban environments, numerous studies have shown correlations between lichen abundance and air quality (Bennett & Wetmore, 2010; Perlmutter, 2010; Washburn & Culley, 2006). Additional factors that may affect the lichen community in an urban area are habitat alteration and the influence of the "city effect" resulting in less atmospheric moisture and greater temperatures than the surrounding countryside. Brodo (1966) was one of the first studies to succinctly conclude that the "city effect" on epiphytic lichens was the primary influencing factor on lichens in a city, whereas, air pollution was the most influential factor describing lichen diversity over a larger area.

Pittsburgh, located in southwestern Pennsylvania, has a long industrial history. This began with the War of 1812, sparked by the region's rich seam of bituminous coal and three navigable rivers, and continued until the collapse of the steel industry in the 1980s. Pittsburgh's economy has shifted away from industry and significant reductions in sulfur dioxide (SO_2) and nitrogen oxides have occurred in the region due to implementation of the 1990 Clean Air Act Amendments and Acid Rain Program (EPA, 2010). In the past ten years, Allegheny and its neighboring counties of Washington and Westmoreland have been two to three times below the U.S. Environmental Protection Agency's National Ambient Air Quality Standards for SO_2 and nitrogen dioxide (NO_2) but continue to exceed the standards for fine particulate matter ($PM_{2.5}$) and ozone (PADEP, 2006).

Our study examines the lichen abundance and diversity between urban and rural environments to determine the extent of impact to the lichen community due to anthropogenic influences. Community analyses were conducted at two urban parks in the metropolitan area of Pittsburgh in Allegheny County and two rural parks in the neighboring counties of Washington and Westmoreland counties to the south and southeast of the city, respectively. The study is in an area that has experienced a decline from a primarily steel-based industry, with a subsequent shift to service-based industries. Additionally, urban sprawl has increased in the past several decades throughout the region, which has contributed to a population increase and greater land fragmentation providing an opportunity to investigate the role of lichens as bioindicators of urbanization.

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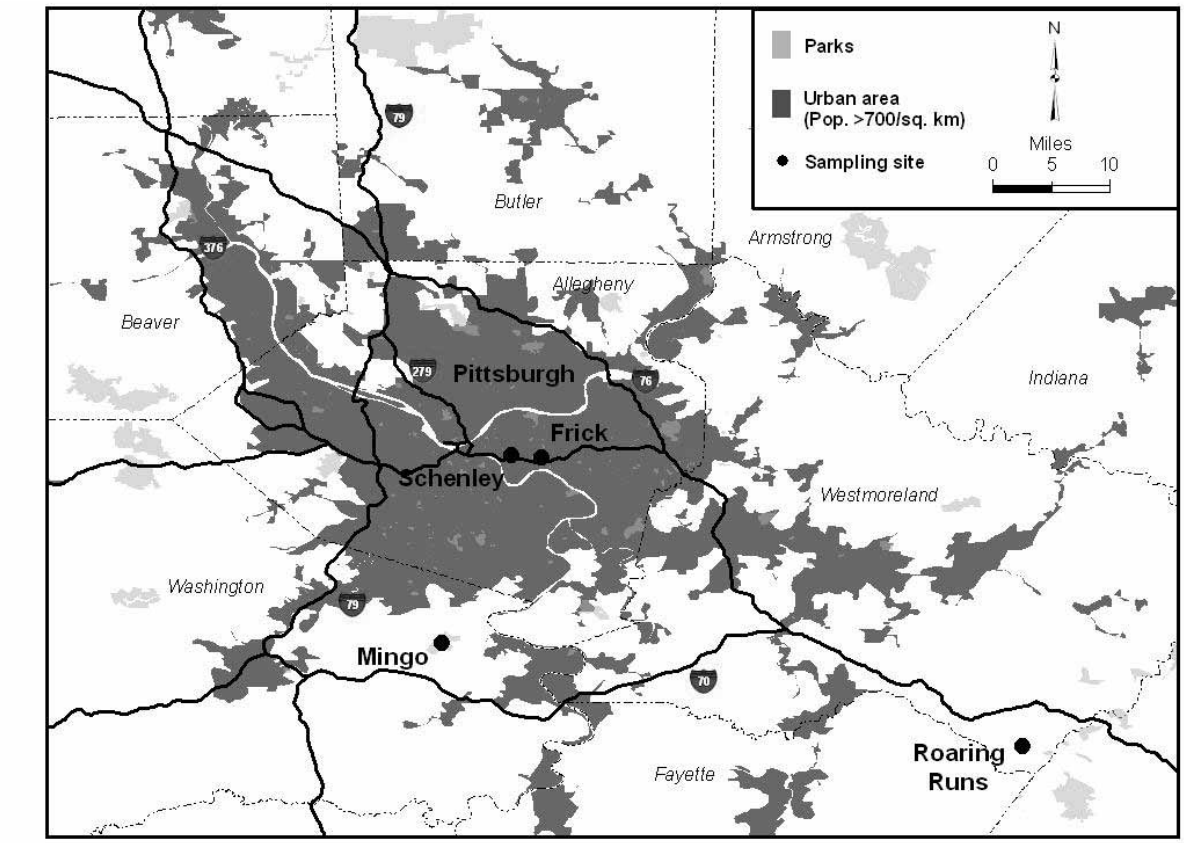

Figure 1. Site map of the locations of sampling sites that include Schenley and Frick in Allegheny County, Mingo in Washington County and Roaring Runs in Westmoreland County in southwestern Pennsylvania.

MATERIALS AND METHODS

Site Description

Lichen communities were sampled at four sites in southwestern Pennsylvania in Allegheny, Washington and Westmoreland Counties (Fig. 1). The study area lies in the Allegheny Plateaus Physiographic Province that is characterized by mixed topography on shale residuum. Winter minimum and summer maximum temperatures for Pittsburgh from 1971–2000 were –30 °C in January and 39.4 °C in July, respectively, and annual mean precipitation and snowfall was 961 and 1024 mm, respectively (NCDC, 2004).

Schenley Park (Schenley) and Frick Park (Frick) are 8 km east of downtown Pittsburgh in Allegheny County. Mingo Creek Park (Mingo) is in rural Washington County approximately 23 km south of Pittsburgh and Roaring Runs Natural Area (Roaring Runs) is part of Forbes State Forest in rural Westmoreland County approximately 61 km southeast of Pittsburgh. All sites are in mesophytic woodlands in the mid–successional stage. The four sites were chosen because of their similarities in forest type, accessibility and relative location to Pittsburgh.

Population and annual traffic densities were used as surrogate variables for urban development and mobile source emissions of NO_2 (Table 1). Population estimates for 2000 were determined from the U.S. Census Bureau for the zip codes in which the sites occur (USCB, 2002). The average population density was determined where multiple zip codes overlap a given site. Schenley and Frick are classified by the U.S. Census Bureau as urban areas, with >386 persons per square kilometer, with Mingo and Roaring Runs being non-urban areas.

Table 1. The surface areas, elevation ranges above sea level, population density estimates for 2000 (USCB 2002) and annual traffic estimates for 2008 (PennDOT 2009) of the sampling sites that include Schenley, Frick, Mingo and Roaring Runs.

Site	Area (km2)	Elevation Range(m)	Population (persons/km2)*	Annual Traffic (106 km/yr.)
Schenley	1.85	232–335	3,944	320
Frick	2.27	238–335	3,944	270
Mingo	10.50	280–378	80	14
Roaring Runs	14.50	561–890	34	10

*Sites in areas with densities >386 are urban and <386 non-urban.

Annual traffic data were obtained from the Pennsylvania Department of Transportation's annual average daily traffic maps for 2008 (PennDOT, 2009). Traffic volume data within a 3 km radius of each site were multiplied by the road lengths and by 365 days per year to determine the annual

vehicle distance traveled within a designated impact area (Washburn & Culley, 2006).

Sampling Design

The epiphytic lichen community of each site was sampled by intensive monitoring plots and species diversity surveys between 2008 and 2010. The location of intensive monitoring plots were chosen based on criteria to minimize differences in lichen communities between sites caused by atmospheric moisture, sunlight exposure and substrate type. At each site, six well-distributed plots in areas of favorable lichen habitat were sampled. Sampling in favorable lichen habitat allowed for plots to be effectively compared and minimized sampling in locations where lichens were absent. Additionally, the plots were located in the interior of the parks, avoiding locations along roadways and in wet environments such as along streams or wetlands. Sampling in the park interior at all sites avoids a complication of within site variability caused by sampling a variety of interior and edge habitats. It also suggests that the intensive monitoring plots will yield more low-light tolerant lichens. A healthy *Quercus rubra* (northern red oak) tree having the most coverage of lichens was chosen as a plot after searching in an area of favorable lichen habitat. *Quercus rubra* was found to support a wide variety and abundance of lichens compared to other species of trees and is endemic to upland, mature woodlands. To further maintain consistency among plots, all plot trees had a diameter at breast height (DBH) exceeding 25 cm and a surrounding canopy cover averaging 80–90%, which was measured using hemispherical photography.

On each plot tree, the percent cover of lichens was estimated using a 20 × 50 cm microplot subdivided with nylon string into 2 × 2 cm squares. The microplot was placed on the tree at eight locations 0.5 and 1.5 m above the ground in the north, east, south and west directions. Additionally, the plot tree served as the center of a 465 m^2 plot in which all living trees with a DBH ≥ 5 cm were surveyed for lichens. The lichens found on these outlying trees were categorized into three groups: crustose, foliose and squamulose. Lichens may be a combination of fruticose and squamulose growth forms, such as *Cladonia sp.*, which have stalks of a fruticose that develop from a squamulose base. Throughout the manuscript the squamulose description will be used to identify all *Cladonia sp.* The percent cover of lichens on a tree up to 3 m above the ground was recorded for each group using the following codes: 0 for <1, 1 for 1–10, 2 for 11–25, 3 for 26–50, 4 for 51–75 and 5 for 76–100%.

At all sites, a species diversity survey was separately conducted from the intensive monitoring plots. Only those lichens attached to fallen stems, tree stumps and tree trunks were collected, with all major habitats within a site surveyed. The amount of time spent searching for lichens in a given site averaged one hour per square kilometer. Lichens were identified in the field whenever possible to avoid collection, or if collected, returned to the laboratory for identification using Lichens of North America by Brodo et al. (2001). The identification of specimens was confirmed by running thin layer chromatography of lichen thalli following methods published by Bungartz (2001) and Orange et al. (2001). Lichen specimens were run in toluene:acetic acid (170:30) and toluene:ethyl acetate:formic acid (139:83:8) solvents and retention (Rf) values were compared to those published in Orange et al. (2001) to identify lichen substances and ultimately, the species. Further confirmation of specimen identification was determined by sending a subset of samples to James C. Lendemer at the New York Botanical Gardens.

Site comparisons of the intensive monitoring plots were analyzed by species abundance, Shannon–Weiner diversity index, relative dominance and frequency and lichen diversity value (LDV). Significance testing of species abundance and percent dominance was completed using one–way Analysis of Variance (ANOVA; Statext v1.2) with a significance level of $\alpha = 0.05$. When ANOVA yielded significant F-values a post hoc Scheffe test (Scheffe test; Statext v1.2) was conducted to determine which mean is significantly different from the others. To determine the Shannon–Weiner diversity index (H), the proportion of species i relative to the total number of species (p_i) is calculated, and then multiplied by the natural logarithm of this proportion ($\ln p_i$). The resulting product is summed across species and multiplied by –1.

Mean percent dominance per species is the mean percent number of grids that a species occurred within the microplots of all six plots, either separately for 0.5 and 1.5 m sampling heights or sampling heights combined. Relative dominance is the proportion of mean percent dominance of a species to the total percent dominance of all species and multiplied by 100. Frequency is the proportion of the number of occurrences of a species in a microplot to the total number of microplots per site. Relative frequency is the proportion of the frequency of a species to the total frequency of all species and multiplied by 100.

The LDV is a statistical estimator of the environmental conditions in a site (Asta et al. 2002). The first step in calculating the LDV of a site (j) is to sum the frequencies of all lichen species found on each tree (i) within the site. Substantial differences in lichen growth may occur on different sides of the trunks, thus, the frequencies have to be summed separately for each aspect (N, E, S, W) to obtain four Sums of Frequencies (SF) at each tree (SF_{iN}, SF_{iE}, SF_{iS}, SF_{iW}). For each aspect, the arithmetic mean of the Sums of Frequencies (MSF) for each site is calculated:

$$MSF_{Ni} = (SF_{1Nj} + SF_{2Nj} + SF_{3Nj} + \ldots + SF_{nNj})/n \quad (1)$$

The LDV of a site is the sum of the MSFs of each aspect:

$$LDV_j = MSF_{Nj} + MSF_{Ej} + MSF_{Sj} + MSF_{Wj} \quad (2)$$

The dominant trees in the plots surrounding the central plot tree of the intensive monitoring plots were determined using importance values, which is sum of relative values for dominance, frequency and density. Relative dominance is

the proportion of basal area for a species to area sampled, which is then divided by the total dominance for all species and multiplied by 100. Relative frequency is the proportion of the number of plots in which a species occurs to the total number of plots sampled which is then divided by the total frequency for all species and multiplied by 100. Relative density is the proportion of the total number of individuals of a species to the area sampled, which is then divided by the total density for all species and multiplied by 100. In the same plots, the statistical significance between sites of percent lichen cover by type (crustose, foliose and squamulose) was determined using one–way ANOVA, followed by a post hoc Scheffe test.

RESULTS

Mean species richness of lichens was significantly greater at Mingo (Scheffe test; $F = 6.37$, $P < 0.01$) and Roaring Runs (Scheffe test; $F = 4.51$, $P = 0.01$) than Frick (Table 2). Although Schenley had a lower mean species richness compared to Mingo and Roaring Runs, it was not significant. The Shannon–Weiner species diversity index for lichens was greatest at Mingo, Roaring Runs and Schenley. Bryophytes (liverworts and mosses), sterile lichen crust, *Cladonia ochrochlora* and *Lepraria lobificans* were present at all sites. The top three dominant epiphytes at each site accounted for >70% relative dominance and >65% relative frequency. Overall, dominance of epiphytes was greater at 0.5 m compared to 1.5 m above the ground, but not significantly. Across all sites, bryophytes and *L. lobificans* were a dominant epiphyte, with relative dominance and frequency averaging 45% and 26% for bryophytes and 20% and 25% for *L. lobificans*, respectively (Table 3). Among the individual sites, sterile crustose lichen was a dominant epiphyte at Frick and Schenley, *C. ochrochlora* at Mingo and *Cladonia caespiticia* at Roaring Runs.

The lichen diversity value (LDV) was greatest at Mingo, followed by Roaring Runs, Schenley and least at Frick (Fig. 2). The average LDV at the rural sites compared to the urban sites was 20.8 ± 3.0 and 11.3 ± 3.5, respectively.

In the extended monitoring plots beyond the central plot tree, the dominant tree species at Schenley were *Q. rubra*, *Fraxinus americana* (white ash) and *Acer saccharum* (sugar maple) with importance values of 119, 31 and 29, respec-

Table 2. Ecological diversity indices by site.
Standard errors are in parenthesis.

Site	Species Richness	Shannon-Weiner Diversity Index
Schenley	3.7 (0.8)	1.4
Frick	2.0 (0.4)	0.3
Mingo	5.2 (0.3)	1.0
Roaring Runs	4.7 (0.4)	1.1

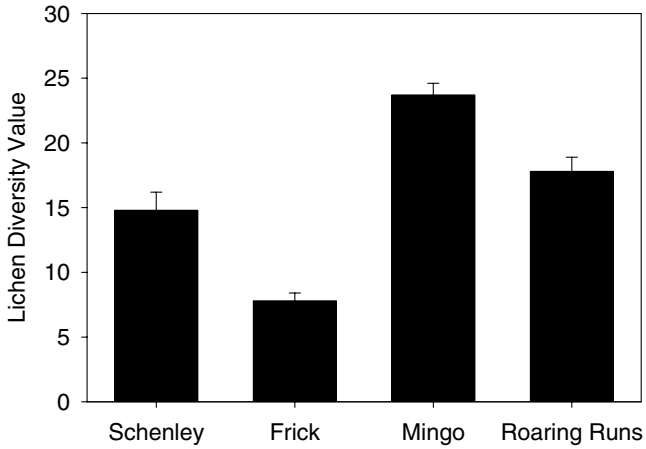

Figure 2. Lichen diversity values for the sampling sites of Schenley, Frick, Mingo and Roaring Runs determined from intensive monitoring plots. Standard error bars are shown.

Table 3. Relative percent dominance and relative frequency of bryophytes and lichens in the intensive monitoring plots at Schenley, Frick, Mingo and Roaring Runs sampling sites.

	Relative % Dominance				Relative % Frequency			
Species	Schenley	Frick	Mingo	Roaring Runs	Schenley	Frick	Mingo	Roaring Runs
Bryophytes	17.9	44.6	48.3	69.1	11.5	25.5	25.1	30.6
Sterile crusts	35.8	2.5	3.5	2.4	27.1	10.6	8.0	10.2
Cladonia caespiticia	-	-	-	7.7	-	-	-	17.8
Cladonia ochrochlora	17.6	0.6	36.1	0.1	17.7	6.4	24.1	1.9
Flavoparmelia caperata	1.9	-	3.2	0.4	12.5	-	12.8	8.9
Hypogymnia physodes	-	-	-	<0.1	-	-	-	1.3
Lepraria lobificans	19.4	51.9	7.3	19.1	14.6	46.8	16.6	19.7
Parmelia sulcata	0.3	-	0.1	<0.1	4.2	-	2.7	1.3
Parmelinopsis minarum	-	-	-	<0.1	-	-	-	0.6
Parmotrema hypotropum	-	-	<0.1	-	-	-	1.1	-
Phaeophyscia rubropulchra	-	-	0.2	-	-	-	2.7	-
Physcia millegrana	7.0	0.4	-	-	12.5	10.6	-	-
Punctelia caseana	-	-	0.1	1.1	-	-	1.6	7.6
Punctelia rudecta	-	-	1.1	-	-	-	5.3	-

tively. The three dominant tree species at Frick and Mingo were *Q. rubra*, *Prunus serotina* (black cherry) and *A. saccharum*, with respective importance values of 68, 43 and 38 at Frick and 76, 33 and 61 at Mingo. At Roaring Runs, the three dominant trees were *Q. rubra*, *Acer rubrum* (red maple) and *A. saccharum*, with importance values of 73, 63 and 37, respectively.

Identifying *Q. rubra* as the central plot tree resulted in its dominance in all plots. Because *Q. rubra* and *A. saccharum* were abundant across all sites, these trees were chosen in this study to compare for lichen cover of crustose, foliose and squamulose lichens. *P. serotina* was abundant at Frick, Mingo and Roaring Runs within our sampling plots, thus, chosen as the third tree. However, *P. serotina* was absent from the sampling plots at Schenley. The number of individual trees of a given species surveyed for lichen cover ranged from 12 to 55 per site, excluding *P. serotina* which were not present in the plots at Schenley.

Crustose lichens, which consisted primarily of sterile crusts and *L. lobificans*, surveyed on *A. saccharum* (Scheffe test; Mingo-Schenley: $F = 3.65$, $P = 0.01$; Mingo-Frick: $F = 14.42$, $P < 0.01$; Mingo-Roaring Runs: $F = 6.33$, $P < 0.01$) and *Prunus serotina* (Scheffe test; Mingo-Frick: $F = 6.39$, $P < 0.01$; Mingo-Roaring Runs: $F = 4.40$, $P = 0.02$) had a significantly greater percent cover on trees at Mingo compared to the other sites by a magnitude of two to four (Fig. 3A). There was no significant difference between sites in crustose cover on *Q. rubra*. In regards to foliose lichen cover, which consisted primarily of *Flavoparmelia caperata*, *Parmelia sulcata*, *Physcia millegrana*, *Punctelia caseana* and *Punctelia rudecta*, there was significantly more than two times the coverage on *A. saccharum* (Scheffe test; Mingo-Schenley: $F = 12.09$, $P < 0.01$; Mingo-Frick: $F = 27.67$, $P < 0.01$; Mingo-Roaring Runs: $F = 13.88$, $P < 0.01$), *P. serotina* (Scheffe test; Mingo-Frick: $F = 26.59$, $P < 0.01$; Mingo-Roaring Runs: $F = 22.45$, $P < 0.01$) and *Q. rubra* (Scheffe test; Mingo-Schenley: $F = 11.84$, $P < 0.01$; Mingo-Frick: $F = 5.24$, $P < 0.01$; Mingo-Roaring Runs: $F = 6.41$, $P < 0.01$) at Mingo compared to the other sites (Fig. 3B). The squamulose lichen coverage, which consisted of *Cladonia sp.,* was greater on all three tree species at Mingo compared to the other sites but only significantly so on *A. saccharum* (Scheffe test; Mingo-Schenley: $F = 38.86$, $P < 0.01$; Mingo-Frick: $F = 18.53$, $P < 0.01$; Mingo-Roaring Runs: $F = 5.01$, $P < 0.01$) and *Q. rubra* (Scheffe test; Mingo-Schenley: $F = 24.79$, $P < 0.01$; Mingo-Frick: $F = 7.71$, $P < 0.01$; Mingo-Roaring Runs: $F = 7.57$, $P < 0.01$) (Fig. 3C). Mingo had significantly more squamulose lichen cover than Frick on *P. serotina* (Scheffe test; $F = 11.41$, $P < 0.01$). On *A. saccharum*, Roaring Runs had significantly more squamulose lichen cover compared to Schenley (Scheffe test; $F = 5.24$, $P < 0.01$) and Frick (Scheffe test; $F = 9.62$, $P < 0.01$). On *Q. rubra*, squamulose lichen cover was significantly greater at Roaring Runs (Scheffe test; $F = 6.80$, $P < 0.01$) and Frick (Scheffe test; $F = 3.05$, $P = 0.03$) compared to Schenley. Overall, the percent cover of squamulose lichens was greatest at the rural sites compared to the urban sites, foliose coverage was only slightly greater at the rural sites if Mingo and Roaring Runs are averaged and there is little difference in crustose coverage between rural and urban sites if Mingo and Roaring Runs are averaged.

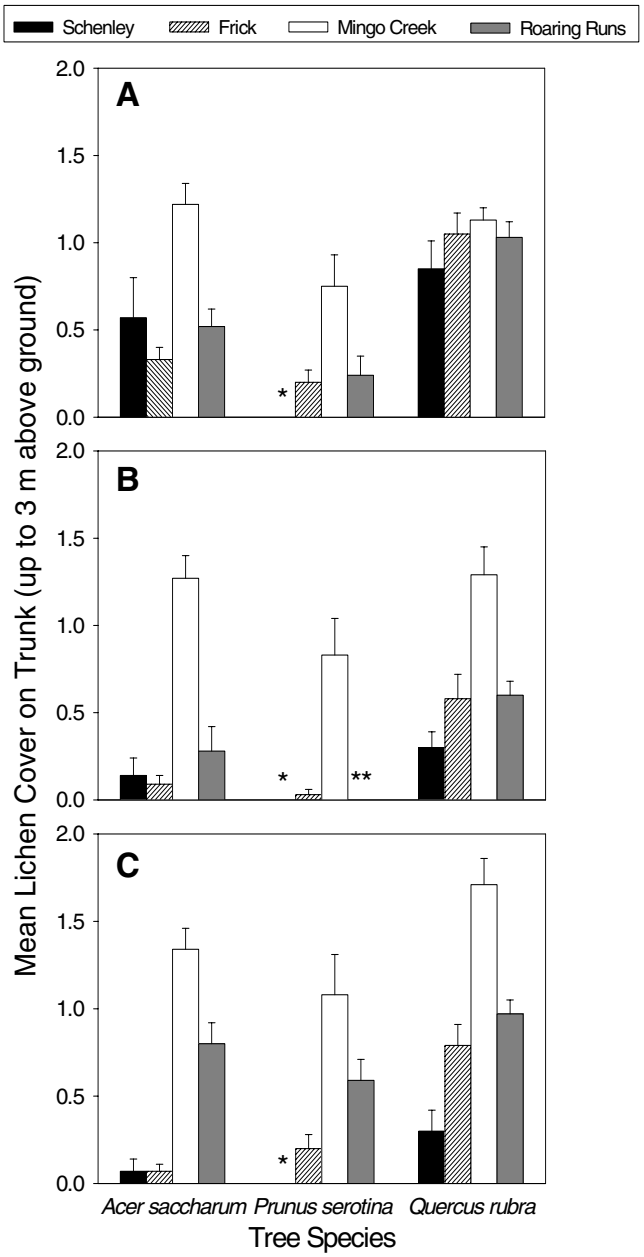

Figure 3. Mean percent lichen cover (0 for <1%, 1 for 1–10% and 2 for 11–25%) of crustose (A), foliose (B) and squamulose (C) lichen forms on *Acer saccharum*, *Prunus serotina* and *Quercus rubra* for the sampling sites of Schenley, Frick, Mingo and Roaring Runs determined from the intensive monitoring plots. Standard error bars are shown. (* no data; ** <1% lichen cover)

In the species diversity survey 46 epiphytic lichens were found throughout all of the sampling sites. The rural sites had more species, with 33 species found at Roaring Runs and 32 at Mingo compared to the urban sites, with 20 species found at Schenley and 19 at Frick (Table 4).

Table 4. Epiphytic lichen species identified during sampling at the intensive monitoring plots and species diversity surveys at Schenley, Frick, Mingo and Roaring Runs sampling sites.

Species	Schenley	Frick	Mingo	Roaring Runs
Allocetraria oakesiana (Tuck.) Randlane & Thell				x
Amandinea polyspora (Willey) Lay & May		x	x	
Amandinea punctata (Hoffm.) Coppins & Scheid.	x			x
Arthonia caesia (Flot.) Korb.		x	x	
Buellia dialyta (Nyl.) Tuck.				x
Candelaria concolor (Dicks.) Stein	x	x	x	
Candelariella efflorescens Harris & Buck	x	x	x	
Cladonia caespiticia (Pers.) Florke		x	x	x
Cladonia cristatella Tuck.	x		x	x
Cladonia macilenta var. bacillaris Hoffm.			x	x
Cladonia ochrochlora Florke	x	x	x	x
Cladonia parasitica (Hoffm.) Hoffm.	x		x	x
Flavoparmelia caperata (L.) Hale	x	x	x	x
Graphis scripta (L.) Ach.				x
Hypogymnia physodes (L.) Nyl.			x	x
Lecania croatica (Zahlbr.) Kotlov	x			
Lecanora saligna (Schaerer) Zahlbr.	x			
Lecanora strobilina (Spreng.) Kieffer		x	x	x
Lecanora symmicta (Ach.) Ach.			x	x
Lecanora thysanophora Harris			x	
Lepraria lobificans Nyl.	x	x	x	x
Lepraria neglecta (Nyl.) Erichsen				x
Lepraria caesioalba (de Lesd.) Laundon				x
Melanelia subaurifera (Nyl.) Essl.		x	x	
Micarea peliocarpa (Anzi) Coppins & Sant.			x	x
Micarea prasina Fr.				x
Myelochroa aurulenta (Tuck.) Elix & Hale		x		
Ochrolechia arborea (Kreyer) Almb.			x	
Parmelia squarrosa Hale			x	x
Parmelia sulcata Taylor	x	x	x	x
Parmelinopsis minarum (Vainio) Elix & Hale	x		x	x
Parmotrema hypotropum (Nyl.) Hale	x	x	x	x
Phaeophyscia adiastola (Essl.) Essl.			x	
Phaeophyscia pusilloides (Zahlbr.) Essl.	x	x	x	x
Phaeophyscia rubropulchra (Degel.) Essl.	x	x	x	x
Physcia millegrana Degel.	x	x	x	x
Physcia stellaris (L.) Nyl.	x			x
Physcia subtilis Degel.		x	x	
Placynthiella dasaea (Stirton) Tonsberg				x
Placynthiella icmalea (Ach.) Coppins & James				x
Punctelia caseana Lendemer & Hodkinson	x	x	x	x
Punctelia rudecta (Ach.) Krog	x	x	x	x
Pyrrhospora varians (Ach.) Harris			x	
Ropalospora chlorantha (Tuck.) Ekman			x	x
Scoliciosporum chlorococcum (Stenh.) Vezda	x			x
Trapeliopsis flexuosa (Fr.) Coppins & James			x	x

DISCUSSION

In the intensive monitoring plots, lichen richness and percent cover of crustose, foliose and squamulose forms were greatest at Mingo and least at Frick. Overall, the percent cover of foliose and squamulose lichens was greatest at the rural sites. Although Schenley had the greatest Shannon-Weiner diversity value from the intensive plots, the species diversity surveys showed an average species count of 33 at the rural sites compared to 20 at the urban sites. During the intensive plot sampling it was also observed that foliose and squamulose lichens had both larger and thicker thalli, suggesting healthier specimens at the rural sites. Additionally, a greater number of *Cladonia sp.* had podetia at the rural sites.

The population and traffic densities surrounding the urban sites are a magnitude of 69 and 25 times greater than at the rural sites, respectively. The greater traffic density in the Pittsburgh area influences local concentrations of NO_2, with an annual average concentration of 2.1×10^4 µg m^{-3} compared to an annual average of 1.4×10^4 µg m^{-3} at air monitoring stations within 33 km of Mingo and Roaring Runs between 1997 and 2006 (PADEP, 2006). Nitrogen

dioxide emitted by road traffic can be an influential pollutant affecting lichen communities in urban environments (Gombert et al., 2003). However, this study cannot distinguish differences in lichen communities between the urban and rural sites due to nitrogen pollution because of the overwhelming dominance of nitrophilous macrolichen species across all sampling sites. The dominant nitrophilous macrolichen species in this study include *Flavoparmelia caperata*, *Parmelia sulcata* and species in the genera *Phaeophyscia* and *Physcia*. Furthermore, most of the dominant macrolichens identified in the study, such as *P. sulcata* and *Phaeophyscia sp.* and *Physcia sp.* are considered to have an intermediate to tolerant sensitivity to SO_2 pollution. The lichens most sensitive to air pollution, such as those with cyanobacteria as their algal component and the noncladoniform fruticose lichens were not found at any sampling sites. However, a historical survey conducted around 1922 in Western Pennsylvania (Mozingo, 1948) and more recent surveys in central and eastern Pennsylvania have yielded these types of lichens (Harris & Lendemer, 2005; Lendemer & Macklin, 2006). The absence of a pollution gradient in our study is presumed the result of a long-term exposure to air pollutants from a steel-based industry. Although air quality has improved in recent years, lichens are slow to colonize and grow.

Brodo's (1966) "city effect" is likely a major factor in describing the differences in the lichen communities between the urban and rural sites. Lichens are indicators of air pollution as well as moisture and temperature. Temperatures between the urban, Pittsburgh area and rural countryside are likely not significant enough to explain the differences in lichen communities. However, we compared the density of streams between sites as a surrogate to humidity and found that stream density was 0.6 km per km^2 and 1.0 km per km^2 at the urban and rural sites, respectively. At Mingo, the mean distance between sampling plots and a body of water was 0.2 km, compared to Schenley, Frick and Roaring Runs which were 0.2, 0.7 and 0.3 km, respectively. The intensive monitoring plots showed that lichen coverage was greatest at Mingo compared to any other site, and we noted throughout our study that the density of lichens was greatest in the bottomlands along streams. If humidity is a factor influencing the lichen community, then the presence of bryophytes should also be more pronounced at the rural sites than the urban sites. Both bryophytes and lichens have relatively similar moisture requirements for metabolic processes. The mean relative dominance and frequency of bryophytes in the rural sites is 59% and 28%, respectively, compared to 32% and 19% in the urban sites, respectively. In a study by Perlmutter (2010), which complements our own study but was conducted around Raleigh, North Carolina, humidity was also found to play a role in lichen community structure. Thus, it appears that humidity may be a factor that influences the abundance and diversity of lichen communities between the urban and rural sites.

An additional factor to consider in its effect on lichen communities in this study is habitat quality. A study by Johansson & Ehrlen (2003) studied the influence of habitat quality on two epiphytic lichens and found that the abundance of the lichens was positively correlated with tree size and that the presence of the lichens was negatively correlated with the isolation of woodlands. The larger and older trees are exposed to colonization for a longer time and may provide more suitable substrate with rough bark to capture dispersing propagules as well as have different bark chemistry than younger trees (Armstrong, 1990; Gustafsson & Eriksson, 1995). The isolated woodlands would imply dispersal constraints, inhibiting genetic diversity among lichens in urban areas and preventing recolonization should the isolated patch become further fragmented through recreational management or environmental degradation (Johansson & Ehrlen, 2003). So while the study shows that urban sites are less suitable for lichens, it is important to recognize that the rural sites are not intact undisturbed habitats. However, Mingo and Roaring Runs consist of older growth forests that would be more suitable for lichen dispersion and propagation than the urban sites. Additionally, the overall park area of the rural sites, as depicted in Table 1, is a magnitude of ten times the urban sites. There is also a greater degree of isolation at the urban sites, being completely surrounded by development, whereas, Mingo and Roaring Runs is surrounded by woodlands or dispersed residential and agricultural lands. Therefore, habitat quality is likely a second factor in defining the differences in the lichen communities between the urban and rural sites.

In conclusion, air pollution defines the lichen community in the larger geographical region of this study as demonstrated by the dominance of nitrophilous and SO^2-tolerant species throughout the sampling sites and the general lack of pollutant-sensitive species. The differences in species richness and diversity between the sites are best defined by Brodo's (1966) "city effect" and habitat quality. Although our study did not directly measure humidity levels at the urban and rural sites, if humidity is lower in urban sites lichens may succumb to increased dessication, resulting in reduced metabolic activity compared to those sites that have greater humidity levels. The more isolated urban parks may inhibit the dispersion and genetic diversity of lichens resulting in a lower diversity, as observed in the species diversity survey.

ACKNOWLEDGMENTS

Funding for this study came from the Pennsylvania Department of Conservation and Natural Resources, Wild Resource Conservation Program (Grant No. WRCP-08296). James C. Lendemer from the New York Botanical Gardens confirmed the identification of lichen specimens and provided feedback on the experimental design. We also would like to thank Heidi Opdyke and Mack Frantz who provided field and laboratory assistance.

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Journal of the Pennsylvania Academy of Science 85(4): 159–164, 2011

SPORTSMEN'S PERCEPTIONS OF COYOTES AND COYOTE ORIGINS IN PENNSYLVANIA[1]

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ABSTRACT

We surveyed central Pennsylvania sportsmen about the origins of the eastern coyote/coywolf (*Canis latrans x lycaon*) in Pennsylvania, the perceived effect coyotes have on white-tailed deer (*Odocoileus virginianus*) population size, and the prevalence of coyote attacks on humans and domestic animals. Though studies suggest that coyote populations expanded into Pennsylvania naturally, a significant proportion of our participants believed that automobile insurers or the Pennsylvania Game Commission introduced coyotes to the state to control deer populations. Participants overestimated the threat posed by coyotes to humans and animals. Policy makers, especially those responsible for animal control and land management, should consider the level of misinformation concerning coyotes when making policy decisions that impact these canids and the people who must live with them.

KEY WORDS: attitudes, *Canis latrans*, *Canis lycaon*, carnivores, conflicts, Pennsylvania, perceptions, sportsmen, surveys.
[J PA Acad Sci 85(4): 159–164, 2011]

INTRODUCTION

Eastern coyotes/coywolves (*Canis latrans x lycaon*; Kays et al. 2009; Way et al. 2010; hereafter coyote) are relatively recent inhabitants of the northeastern United States (Witmer and Hayden 1991; Parker 1995; Hayden 2005). Their recent presence has spurred questions about their geographic and genetic origins (Gompper 2002; Hayden 2005; Hayes 2008), what threats they pose to humans, property, and ecosystems (Witmer and Hayden 1991), and the extent to which their presence is welcome (Stevens et al. 1994; Gompper 2002). The expansion of coyotes into Pennsylvania (coyotes were first recorded in the Commonwealth in 1946; Hilton 1978) have impacted more than predator-prey relations.

Though a second wave of pure coyotes has colonized western Pennsylvania (Kays et al. 2010), the coyotes of central and eastern Pennsylvania are the result of a wave of hybrid coyotes (*Canis latrans*) and eastern wolves (*Canis lycaon*) that mixed in southern Ontario producing what is known as the eastern coyote (Way et al. 2010, Kays et al. 2009). The relatively rapid range expansion into Pennsylvania (Witmer and Hayden 1991) may be contributing factors in the development of conspiracy theories regarding the origin of coyotes within the state of Pennsylvania. Local lore held by sportsmen (here defined as self-identified hunters who have hunted in Pennsylvania during 2008) accuses the Pennsylvania Game Commission (PGC) of breeding wolf-coyote hybrids and then releasing them for the purpose of decreasing the state's deer population (B. Frye, Tribune-Review, personal communication). Another coyote stocking narrative involves automobile insurance companies breeding and strategically releasing coyotes to decrease deer populations and ultimately the gross number of deer-vehicle collisions which would result in fewer claims (B. Frye, Tribune-Review, personal communication). Besides the variation in beliefs to coyote stocking narratives, some sportsmen view coyotes as a challenging game animal and a graceful symbol of returning wilderness, while others view them as unnatural intruders, released by conspiracy to control deer populations (M.J. Lovallo, Pennsylvania Game Commission, personal communication).

Kellert (1985) showed that U.S. hunters' perceptions of predators were more positive than those held by the general public. A review of 37 studies investigating human attitudes toward carnivore reintroductions in the United States, Canada, Scandinavia, Western Europe, and Asia concurred with Kellert's finding (Williams et al. 2002). Recent studies, however, concluded that hunters hold more negative perceptions of carnivores than the general population (Ericsson and Heberlein 2003; Meadow et al. 2005). This is important as support or tolerance of those in direct contact with recov-

[1]Submitted for publication 30 October 2011; accepted 1 December 2011.
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ering carnivores, such as sportsmen, is particularly important because they can directly impact the success or failure of management policy (Ericsson and Heberlein 2003; Berger and Smith 2005; Glikman et al. 2010). For example, Treves and Martin (2011) found that hunters are even less supportive of carnivore conservation and hunting regulations than the general public.

Understanding diverse attitudes toward carnivores and incorporating interdisciplinary research methods is key for measuring the success of wildlife management and for planning future management and conservation policies (Clark et al. 2001; Liu et al. 2001; Treves and Karanth 2003; Treves et al. 2006; Feldman 2007; Glikman et al. 2010). The debate among sportsmen regarding coyotes in Pennsylvania (Witmer and Hayden 1991; Hayden 2005) suggests a need to empirically examine how sportsmen perceive coyotes. Herein, we will address concerns and beliefs surrounding coyotes with the aim of supporting conflict-preventing policy towards large free-ranging carnivores. We hypothesize that sportsmen who subscribe to coyote stocking narratives also believe that coyotes pose an increased risk to humans and domestic animals and view them more negatively.

METHODS

We conducted an in-person survey to assess sportsmen's perceptions of coyotes at a large sportsman's show in central Pennsylvania. In preparing the instrument, the authors spoke with experts on deer management, members of the PGC, and conducted exploratory focus groups with Pennsylvania hunters.

The lead author collected data at The Jaffa Sports Show in central Pennsylvania in one day. The questionnaire contained 25 multiple choice or open ended questions which assessed participant demographic information, basic knowledge about coyotes, perception of coyotes and coyote-deer relationships, and personal experience with the species. Two of the questions were excluded post hoc from analyses due to lack of correlation with perceived risk or subscription to coyote stocking narratives. Though we did not count the number of people who refused to complete the questionnaire, and so cannot measure an actual non-response bias, 3,500 people attended the sportsmen's show and 227 individuals completed the survey. We estimate that we solicited approximately 300 people, for an approximate response rate of ~ 76 %. And only 168 of 227 (74 %) surveys were usable because of incomplete answers or multiple answers on single answer questions.

We used univariate ANOVAs for comparisons of responses. For the purpose of comparison, we grouped several answers for analysis. We grouped answers for how coyotes arrived in Pennsylvania as follows: (a) "stocked by the Pennsylvania Game Commission" and "stocked by insurance companies" were grouped into "Stocked". (b) Coyotes "came on their own through surrounding states" and coyotes "are indigenous to Pennsylvania" were grouped as "Natural." For questions requesting an estimate of "how many coyote attacks to children under 18 occurred in PA in 2008," "how many coyote attacks to any livestock in PA in 2008," "how many coyote attacks to pets occurred in PA in 2008," and "how many coyote attacks to adults occurred in PA in 2008," we grouped the open-ended responses into numerical categories based on the roughly logarithmic bins that were apparent in the data (pets: 0, 1 to 10, 11 to 100, 101 to 1000, and 1001+, children: 0, 1 to 10, 11 to 100, 101+).

RESULTS

Participants were predominately male (84%; Table 1). They reported hunting mostly deer (88%) and turkey (72%), primarily for meat (39%) and sport (40%). One third (33%) reported hunting coyote. Participants who indicated that they hunt "other" species typically hunted small game such as squirrel or rabbit. Less frequently, participants reported hunting larger game out of state, such as caribou. Approximately 10% of participants responded that they do not hunt, so these responses were discarded. Approximately 66% survey participants had some college experience.

Most participants reported having had some contact or experience with coyotes in Pennsylvania during 2008. A majority (67%) had seen fewer than 5 coyotes throughout the year. Of those, 28% did not see a single coyote. While the majority of sportsmen had seen at least one coyote in 2008, very few experienced depredation of either pets or livestock (4% and 6% respectively) and none had been personally attacked by a coyote.

Table 1. Demographic data of respondents (N = 168).
Unless noted, numbers refer to proportion of participants.

Variable	Mean	Median	Standard Deviation	Range
Demographics				
Gender (Male=1)	0.84	1.00	0.37	0.00–1.00
Education (yrs)	13.86	14.00	1.66	10.00–16.00
Hunting activity: "What do you hunt?"				
Don't Hunt	0.10	0.00	0.30	0.00–1.00
Deer	0.89	1.00	0.35	0.00–1.00
Coyote	0.33	0.00	0.47	0.00–1.00
Turkey	0.73	1.00	0.45	0.00–1.00
Grouse	0.39	0.00	0.49	0.00–1.00
Bear	0.39	0.00	0.49	0.00–1.00
Other	0.24	0.00	0.43	0.00–1.00
Reason for hunting: "In 2008, what was the primary reason that you hunted?"				
Didn't Hunt	0.15	0.00	0.36	0.00–1.00
Meat	0.39	0.00	0.49	0.00–1.00
Sport	0.41	0.00	0.49	0.00–1.00
Out in Nature	0.06	0.00	0.24	0.00–1.00

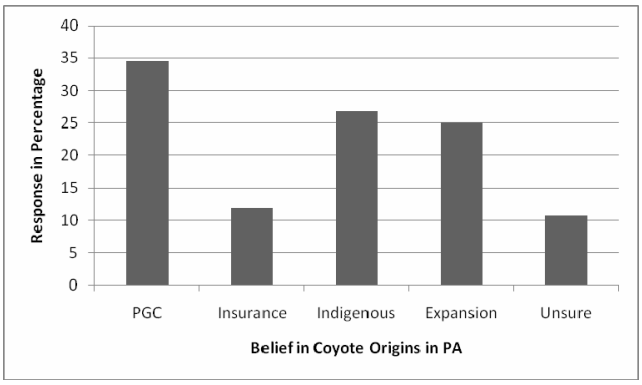

Figure 1: Beliefs about the origins of coyotes by Pennsylvania sportsmen (N=168).

When asked "why do we have coyotes in Pennsylvania?" participants most frequently (34%) reported that the PGC is responsible for stocking Pennsylvania with coyotes (Fig. 1). The natural origin responses, stating that coyotes are indigenous to Pennsylvania (26%) or that they naturally colonized Pennsylvania (25%), were the next most common responses. Finally, the idea that insurance companies stocked coyotes (11%) or that individuals were unsure of the reason that coyotes currently reside in Pennsylvania (10%) had an equal level of responses (Figure 1). Due to the low number of participants who answered "not sure" (11%), this response was dropped from further analysis. Overall, 41% of participants chose at least one "Stocked" response and 49% chose at least one "Natural" response.

Seventy six percent of respondents considered the deer population to be "too small" and 76% reported that they believe hunters kill more deer each year than coyotes do. Analysis by univariate ANOVA revealed participants who believed that coyote were stocked were more likely to believe the deer population to be "too small," whereas participants who believed that the coyote population was not stocked were more likely to believe the deer population to be "neither too small nor too large" ($F(2,162) = 5.14$, $p < 0.05$, $\eta^2 = 0.03$; Stocked: $M = 1.14$, $SD = 0.061$, Not Stocked: M= 1.33, $SD = 0.052$). Analysis by univariate ANOVA revealed that participants who believed the coyote population within Pennsylvania was of natural origin were more likely to believe that coyotes kill more deer than hunters, whereas participants who believed that the coyote population was not natural were more likely to believe that hunters kill more deer, $F(1,156) = 9.98$, $p < .05$, $\eta^2 = 0.06$ (Natural: $M = .278$, $SD = 0.042$, Not Natural: $M = 0.089$, $SD = 0.042$).

Perceived risk of coyote attacks was assessed by asking participants to estimate the number of coyote attacks to humans (by adults and by children under 18), and the number of coyote attacks to pets during 2008 (Table 2).

When responses about perceived number of coyote attacks on humans and pets were compared with perceptions about coyote origins, several trends emerged. As estimated number of attacks on pets increased, the likelihood of believing that coyotes were stocked increased ($F(4,163) = 2.80$, , $\eta^2 = 0.064$, $p < 0.05$) (Table 3).

As the predicted number of attacks on children increased, likelihood of believing that coyotes naturally occur in PA decreased, $F(3,164) = 6.09$, $p < 0.01$, $\eta^2 = 0.10$. Inversely, as predicted number of attacks on children increased, likelihood of believing that coyotes were stocked also increased $F(3,164) = 4.51$, $p < .01$, $\eta^2 = 0.076$. Of the people who predicted that there were no attacks on children, 64% also believed that coyotes naturally occur in PA.

Table 2. Respondent estimated coyote attacks in Pennsylvania in prior year (N = 168).

	Mean	Median	Standard Deviation	Range
Coyote Attacks to Humans				
Children	17.68	1.00	109.12	0.00–1000.00
Adults	13.86	1.00	58.56	0.00–500.00
Coyote Attacks to Animals				
Pets	334.70	20.00	1785.05	0.00–10000.00
Livestock	480.10	50.00	3868.70	0.00–3500.00

Table 3. Respondent estimated number of coyote attacks on pets in prior year among sportsmen who believe coyotes are stocked in Pennsylvania (N = 168).

Attacks on Pets	Mean	Standard Deviation
0	0.29	0.13
1–10	0.29	0.07
11–100	0.45	0.06
101–1000	0.65	0.10
1001+	0.57	0.18

Table 4. Estimated coyote attacks on children in Pennsylvania in prior year (N = 168).

Predicted Attacks on Children	Natural		Stocked	
	Mean	Standard Deviation	Mean	Standard Deviation
0	0.66	0.06	0.27	0.06
1–10	0.43	0.06	0.49	0.06
11–100	0.21	0.11	0.58	0.11
101+	0 .00	0.34	1.00	0.34

DISCUSSION

One common source of contention among sportsmen involves the deer management policy and the number of deer within the state (Frye 2006). While the coyote origins narratives are both attributed to attempts to lower deer population size within Pennsylvania, a majority of participants

believed that hunters kill more deer than coyotes do. This belief, however, was more prominent amongst participants supporting stocked coyote (42 % of surveyed) origin narratives (don't know if narrative is the right word here), which might be interpreted in a couple of ways. First, participants may believe hunters are more efficient than coyotes at managing deer populations and that hunters are the primary, if not premier, deer predator. This is consistent with the idea held by many game management agencies that deer hunting as a management tool cannot be effectively replaced (Southwick 2008). However, the perception that deer populations are too small is held by a majority (76%) of participants, supporting a 1995 survey of Pennsylvania hunters with similar findings (Diefenbach et al. 1997).

The belief that deer populations were too small was more likely to be supported by participants subscribing to stocked coyote origins. Hence this could indicate the belief amongst respondents supporting stocked coyote lore that the number of deer kills by coyotes serves to strain a population already perceived to be too small. Participants supporting natural coyote origins within the state, on the other hand, were more likely to support the belief that coyotes kill more deer than hunters do. While unexpected, this further supports the notion of the natural ecological role of coyotes as deer predators. Also supporting this view of natural ecological processes, participants not subscribing to stocked coyote origins were more likely to believe that the deer population was appropriately sized.

Annual reports of complaints to PGC wildlife conservation officers found concern for deer to be the second highest coyote related complaint within the Commonwealth (Lovallo and Hardisky 2009). This could reflect the findings that bear and coyote are responsible for nearly 70% of neonatal fawn mortality, though there are no indications that this impedes white-tailed deer population growth (Vreeland et al. 2004). Based on our findings, we conclude that although sportsmen subscribing to stocked coyote lore believe hunters kill more deer than coyotes do, their responses indicate that there is little-to-no room in Pennsylvania's ecosystems for coyotes to participate in deer hunting.

In addition to exploring the risk of coyotes to the deer population, we found that many viewed coyotes as threats to domestic animals and human safety. Estimates of that threat, however, tended to be higher than scientifically documented records. Despite the high level of direct experience with coyotes by the majority of our participants, they still tended to overestimate the risk posed by coyotes to pets, livestock, and humans (Lovallo and Hardisky 2009). Recorded coyote-caused pet mortalities in Pennsylvania during 2008 was 36 and has averaged 28 pets annually since 1995 (Lovallo and Hardisky 2009). In our sample of 168 participants, seven individuals reported having lost a pet.

Respondents, on average, estimated 18 attacks by coyotes to children in 2008; however, there has never been a reported incident of a child being attacked by coyotes in the Commonwealth in its long history (PGC 2009). Participants similarly overestimated coyote attacks to adults; as of 2007, there had been one documented coyote attack on an adult (PGC 2009). One concern expressed during survey collection, however, was that there is no central location to report coyote attacks indicating that exact numbers of coyote attacks to domestic animals or humans may be unknown due to under-reporting. Additionally, these responses might also reflect that growing coyote populations have reached a critical mass, possibly indicating that the otherwise covert lives of coyotes are unavoidably colliding with human activities.

A relationship was identified between negative perceptions of coyote impacts on people and pets as well as competition for deer and belief in stocked coyote origins. A positive relationship was found between estimated number of attacks on pets and likelihood of believing that coyotes were stocked, which indicates more negative perceptions of coyotes amongst participants subscribing to stocked coyote narratives. Negative misconceptions related to stocked origins were also linked to overestimations of the danger to humans (both children and adults) from coyotes in Pennsylvania. This relationship between the belief that a government agency or insurance company is stocking coyotes and negative perceptions of coyotes as threatening to humans and human activities is paralleled in the carnivore literature; Wilson (1997: 465) for instance suggested that wolves had become a political symbol in the struggle over "power, property and nature" among rural Americans who are unsatisfied with land use legislation and hold strong feelings of antipathy towards the government. We suggest that coyotes in Pennsylvania have also become a kind of political symbol of the relationship between Pennsylvanians, nature, and understandings of government control amongst this group of sportsmen.

A review of hunters as ecosystem stewards concluded that the activity of hunting, in and of itself, does not foster stewardship or consideration of what is best from an ecological or wildlife management stand-point (Holsman 2000; Treves and Martin 2011). Rather, hunters are often less supportive of ecological management to support biodiversity when it conflicts with game species or property rights (Holsman 2000). In a study of North Dakota and Kentucky residents' participation in hunting, the variable that influenced respondents' support for carnivore management options most was hunting participation: hunters were more likely to agree with management that controlled carnivores rather than protected them (Davenport et al. 2010). A study in three Northern Rocky states and Wisconsin had similar findings which stated hunters do not steward wolves (Treves and Martin 2011).

Coyotes in Pennsylvania and much of the northeastern United States have yet to reach the level of nuisance recorded in western states (Gompper 2002); however, their increasing presence in Pennsylvania, as is evidenced by the increasing number of complaints fielded by the PGC, coincides with an escalation of human-coyote interactions (Witmer and Hayden 1991) and coyote related complaints (Lovallo and Hardisky 2009). The top two complaints fielded by the PGC about coyotes, were complaints expressing

fear of coyotes followed by concern for deer(Lovallo and Hardisky 2009). We expect that as human-coyote interactions increase, negative perceptions of coyotes will also increase, as is the case with wolves in the Great Lakes region (Treves et al. In review). The PGC will continue to receive increasing numbers of complaints about coyotes.

While this study captures sportsmen's attitudes towards and perceptions of coyotes in Pennsylvania, there are some limitations. We surveyed one sportsmen's convention, thus the sample may not be representative of Pennsylvania sportsmen or sportsmen in general. However, we have no reason to believe that our sample was a-typical. While the survey methods provide support for the idea that some sportsmen hold misconceptions about how coyotes arrived in Pennsylvania and their impact on Pennsylvania, replication using more open-ended questions would confirm that sportsmen will independently generate these misconceptions. However, though this study should be replicated with a broader sample and with greater opportunities for free-response from participants, this study does have important implications for exploring the attitudes behind human-wildlife conflict and for the management of Pennsylvania's wildlife.

Management Implications

Participants expressed concern over the fact that the state has no mechanism to systematically report coyote attacks and depredations. While the National Agricultural Statistics Services surveys and estimates coyote depredations of livestock and the PGC records all complaints ad hoc and compiles annual reports regarding coyote attacks (verified or otherwise), there appears to be no single, systematic mechanism in place to report coyote attacks to pets, livestock, and humans. If one does exist, it was not well-known to our respondents. Transparency of the PGC has been called for by hunters in the media (Young 2010), and indeed, transparency has been identified as crucial for the successful co-management of wildlife (Treves et al. 2006). Therefore, we recommend an on-line or telephone-based service be created to document coyote attacks to humans and depredation of pets and livestock. These data would better our understanding of coyotes in Pennsylvania and serve to correct some misconceptions about them. A publicly available outlet may also help to quell misdirected animosity and mistrust toward the PGC.

Mindful wildlife management strategies must take into account the cultural misunderstanding of coyotes in Pennsylvania, especially in policy decisions concerning coyote population thinning operations or when embarking on corrective educational campaigning. Future research could utilize in-depth interviewing techniques to more deeply examine the symbolic quality of coyotes in relation to rural power and politics. We believe that qualitative interviews may reveal that coyotes symbolize government regulation as much as they do a hunter's unabashed first-rights to white-tailed deer.

ACKNOWLEDGMENTS

We thank the organizers of the Jaffa Sports Show and the PA Taxidermy Show, especially T. L. Smith and R. A. Kowalksi. We greatly appreciate the help of B. Frye, M. J. Lovallo, and the many sportsmen who kindly shared their insight about hunting and coyotes in Pennsylvania. We also thank A. Treves and J. Way for their insightful editorial suggestions and J. T. Smith for his input and aid conducting the surveys in addition to Pennsylvania State University's Division of Mathematics and Natural Sciences for funding support for this study.

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Journal of the Pennsylvania Academy of Science 85(4): 165–173, 2011

ASSESSMENT OF THE EFFECT OF TEMPERATURE ON THE LATE BLIGHT DISEASE CYCLE USING A DETACHED LEAF ASSAY[1]

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ABSTRACT

Phytophthora infestans **is a fungal-like organism belonging to the Phylum Oomycota, which is currently classified within the Kingdom Stramenopila. This oomycete is the causal agent of potato late blight, and as such, it is believed to be a major contributor to the Potato Famine, which ravaged Ireland in the 1840's. Current annual losses worldwide due to *P. infestans* gross over $6.8 billion. Interactions between two different isolates of the US-8 race and three potato (*Solanum tuberosum*) cultivars, and in particular, the effect of temperature on the disease cycle, were analyzed using a Detached Leaf Assay (DLA), under controlled laboratory conditions. Incubation period was variable between conditions and, in general, incubation period was longest for each isolate at low temperatures. For isolate NC092ba, mean incubation period was longest at 12°C and did not vary significantly between cultivars. At 18°C, incubation period also did not vary between cultivars but it was shorter than at 12°C. For leaves infected at 24°C with isolate NC092ba, there was a significant difference in incubation period between cultivars, with Russet Burbank being longest. For leaves infected with isolate PSUPotb, incubation period was again longest at 12°C and there was no significant difference between cultivars. At 18°C and 24°C, incubation period was longer for Russet Burbank than for Kennebec or Red Norland. Furthermore, disease progress over time was more severe at higher temperatures for each isolate, across cultivars. Confirmation of pathogen presence in infected leaf tissue was successfully obtained using previously developed *P. infestans* specific primers in a standard Polymerase Chain Reaction (PCR) assay.**
[J PA Acad Sci 85(4): 165–173, 2011]

INTRODUCTION

The plant pathogen *Phytophthora infestans* is most notorious for the widespread devastation of potato crops in Ireland in 1845 and 1846. At that time, it was widely believed that the disease symptoms were a byproduct of the plant itself and a result of poor farming or unfavorable environmental conditions. It is believed that Dr. Charles Montagne was the first to attribute the disease to a fungal pathogen, which he originally named *Botrytis infestans* in 1845 (Matta 2010). It was not until 1876, after over twenty years of careful study of the pathogen's disease cycle that Anton de Bary established that the organism was a member of an entirely undefined genus and renamed the pathogen *Phytophthora infestans* (Erwin and Ribeiro 1996; Matta 2010). Today, the total cost associated with *P. infestans* (including controlling the disease by means of fungicide applications and other management strategies and the loss of approximately 15% of the global production of potato) surpasses $6.8 billion (Haverkort, et al. 2008).

Phytophthora infestans belongs to the Phylum Oomycota, which is included within the Kingdom Stramenopila (Alexopoulos, et al. 1996). Originally, *P. infestans* was grouped with members of the Kingdom Fungi; however, despite several morphological and ecological similarities, many distinct characteristics separate oomycetes from true Fungi (Alexopoulos, et al. 1996). Most notably, zoospore ultrastructure, a diploid thallus, cell wall composition and the production of a sexual spore known as an oospore differentiate the Oomycetes from true Fungi (Alexopoulos, et al. 1996). Recent studies have confirmed that Oomycetes are more closely related to brown algae and diatoms than they are to members of the Kingdom Fungi (Haas, et al. 2009). The genus *Phytophthora* encompasses over sixty different species that give rise to a wide range of symptoms in a number of different plant hosts (Alexopoulos, et al. 1996; Kamoun 2001). Examples of other economically important plant pathogenic species of *Phytophthora* include *P. sojae*, which causes root rot disease in soybeans, and *P. cinnamomi*, which plagues several hosts ranging from eucalyptus forests to avocado and pineapple trees (Erwin and Ribeiro 1996).

Phytophthora infestans can reproduce sexually and asexually. Sexual reproduction, however, requires the presence of two different mating types, A1 and A2 (Goodwin 1997). Fusion of male (antheridium) and female (oogonium) gametes will give rise to the release of diploid structure called oospore (Alexopoulos, et al. 1996). Until the late-1980's, *P. infestans* population diversity was low, with a single clonal lineage, known as US-6, found in Canada and the United States (Tooley, et al. 1985). Outside of Mexico,

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which is considered the pathogen's center of origin, only isolates of the A1 mating type were found (Goodwin and Drenth 1997). In the early 1990's two additional lineages, US-7 and US-8 were also found in the United States and Canada (Goodwin, et al. 1995). It has been suggested that due to both pathogen mutation and migration and international trade of infected potato tubers, the A2 mating type became more prevalent in Europe and North America (Goodwin 1997).

Although sexually produced oospores are resistant to desiccation and other environmental stresses, they have not proven to be a significant contributor to the spread of disease (Drenth, et al. 1995). Most disease outbreaks can be attributed to asexual spores known as sporangia, which are produced on the tips of distinct hyphae known as sporangiophores. Sporangial formation can occur between 9ºC and 22ºC, with 18–22ºC being the optimal range with relative humidity near 100% (Alexopoulos, et al. 1996). Atmospheric conditions such as solar irradiance, temperature and relative humidity are intimately related to the disease cycle of *P. infestans* and are extremely important in the germination of sporangia (Mizubuti, et al. 2000). Previous studies have shown that there are two major mechanisms of sporangial germination, which depend on infection temperature. At low temperatures (optimally below 15ºC, but potentially up to 20ºC) indirect germination, which involves release of motile zoospores from the sporangia, usually occurs (Mizubuti and Fry 1998). Approximately 5–10 zoospores are released per sporangium, each with the ability to penetrate host tissue and cause infection. At higher temperatures (above 20ºC) direct germination, where a germ tube develops directly from the sporangia and infects the host, is favored (Crosier 1934; Maziero, et al. 2009).

In general, the disease cycle favors moderate temperatures and high humidity. Most classical studies of the environmental conditions preferred by this pathogen were done prior to the migration of the A2 mating type, in the early 1980's (Goodwin 1997). But as mentioned above, the genetic diversity within the population of *P. infestans* has increased considerably, and important differences between clonal lineages have been observed. These include pathogenic fitness, fungicide sensitivity and environmental preferences (Fry and Goodwin 1997; Kato, et al. 1997). For example, it has been shown that there are disparities in responses to temperature between "old" and "new" clonal lineages. Notably, sporangial germination in isolates of the "new" lineages, US-8 and US-7, favors much lower temperatures (≤15ºC) than those of the old lineages (Mizubuti and Fry 1998). Therefore, the new, genotypically distinct lineages may be experiencing molecular and physiological changes that challenge the traditional view of *P. infestans* biology.

We have evaluated the response of three popular cultivated varieties (referred to as "cultivars") of potato to inoculation with two geographically distinct isolates belonging to the "new" clonal lineage US-8, at variable temperatures. To assess disease development, we have used a procedure known as detached leaf assay (DLA), which allows assessing disease progression on leaves infected with a controlled amount of inoculum after they have been removed from the plant (Dorrance and Inglis 1997). Earlier studies examining temperature impacts on sporangial germination rates among clonal lineages of *P. infestans* using DLA found that sporangial germination rates of "new" lineages favor low temperatures (Maziero, et al. 2009). We have compared the infection rates observed in DLA with those seen *in planta* infections to determine whether results are consistent between the two means of disease evaluation, and to determine whether temperature significantly affects disease progress in the pathosystem, as previously suggested.

MATERIALS AND METHODS

Living Material

Cultivars

Locally grown seed tubers of cultivars (cvs.) Kennebec, Russet Burbank and Red Norland were obtained from the Netherland Bulb Company (Easton, PA) and the Penn State Cooperative Extension division of Lehigh County. Blocks of approximately 2 cm^2 were excised around visibly germinating tuber eyes and planted in 20-cm terracotta pots with Miracle-Gro® Potting Mix. Pots were placed in growth chamber at 24ºC, with 16-hr light and 8-hr dark photoperiod. Plants were watered every 3 days throughout the duration of the study.

Phytophthora infestans *isolates*

US-8 isolates PSUPotb and NC092ba [A2 mating type (F. Perez, Pers. Comm.)] were obtained from the United States Department of Agriculture (USDA) in Beltsville, MD. Cultures were maintained permanently in the laboratory on Rye A Agar (Caten and Jinks 1968) and were reisolated onto Unclarified V8/Lima bean agar (see below) for sporulation and inoculum preparation (Anonymous 2001). Unclarified V8/Lima bean agar was prepared by autoclaving 80g of frozen baby lima beans in 200ml of distilled water for 10 min at 15psi. Upon cooling, lima beans were strained from liquid and discarded, retaining the liquid. Next, 200ml of V8® juice and 1.4g of $CaCO_3$ (Fisher Scientific, Fair Lawn, NJ) were added along with distilled water to bring mixture to volume. Adjustment of pH to 6.0 was achieved by adding the appropriate amount of KOH. Then, 30g of Difco Agar, granulated (Becton-Dickinson, Sparks, MD) was added and the medium was autoclaved once more for 15 minutes at 15psi (Anonymous 2001). All cultures were grown in the dark at 18ºC.

Inoculum Production

Inoculum was prepared from two-week-old cultures of each isolate (PSUPotb and NC092ba) grown on Unclarified

V8/Lima bean agar and new cultures were stored at 18ºC in the dark. After two weeks of incubation, plates were flooded with 5ml of ddH_2O. Mycelium was agitated for one minute using a pipette tip to dislodge sporangia from hyphal tips. Liquid was drawn back into the pipette tip, placed in 15ml conical centrifuge tube, and set on ice. This procedure was repeated with a second aliquot of ddH_2O and sporangial suspensions were combined and centrifuged for two minutes (500 *g*). Following centrifugation, 5ml of supernatant was carefully decanted. This technique was repeated with two more plates until 15ml of concentrated sporangial suspension had been obtained. This procedure was followed for each of the two isolates (Becktell and Daughtrey 2005; Douches, et al. 1997). Sporangial suspensions were adjusted to $1.5x10^4$ sporangia/ml using a hemocytometer and incubated at 4ºC for two hours to promote zoosporogenesis. Suspensions from each isolate were then transferred to sterile hand-held atomizers and each leaf received three sprays (on average, 1.82ml per spray for an approximate total of 81,900 sporangia/leaf). Control leaves were inoculated in the same manner, using distilled water. All leaves were placed in incubators that had been preset to their respective experimental temperature condition.

Detached Leaf Infection

Three representative temperatures were chosen (12ºC, 18ºC and 24ºC) to examine temperature effect on infection development. To achieve this, leaves were detached at the petiole from five-week-old plants (cvs. Kennebec, Red Norland and Russet Burbank), grown as previously described, and placed adaxially in PyrexÒ containers, atop inverted petri dishes. The bottom of the dish was flooded with water and the container was covered with Saran™ wrap to contain inoculum and promote high relative humidity. Experiment was conducted for each cultivar and each isolate, in triplicate ($n = 3$), at each temperature. Appropriate controls were established for each condition.

Assessment

Infection was assessed for each leaf every 12 hours after inoculation (HAI) during a seven-week period. Incubation period was determined as the time point when first visible sign of infection was present. To assess infection progression, a disease scale with values from 0–6 was used [Modified from Judelson (Pers. Comm.), see Table 1]. Rating scale values were then converted to percentage values (0–100%). To compare disease progression between cultivars, the area under the disease progress curve (AUDPC) was calculated using R Statistical Software (Jeger and Viljanen-Rollinson 2001; Sparks, et al. 2008). AUDPC values represent the amount of disease that has been integrated between assessments and are independent of the disease progress curve shape (Campbell 1998). Values were subsequently assigned ranks. At 168 HAI all leaves were flash frozen in liquid nitrogen and stored at –80ºC for future DNA extraction.

Table 1. Rating scale used to assess disease progression over time in detached leaf infections.

	Detached Leaf Rating Scale
0	No Signs of Infection
1	Few, Small Necrotic Lesions/ <1cm/ <<25% Coverage
2	Larger Necrotic Lesions/ ~25–50% Coverage
3	Larger Necrotic Lesions/ ~50–75% Coverage
4	Larger Necrotic Lesions/ –75–99% Coverage
5	Complete Necrosis
6	Complete Necrosis and Drying

Rating scale modified from Howard Judelson (Pers. Comm.).

Molecular Confirmation of *P. infestans* Presence

Genomic DNA extraction was performed from all detached leaves, from each temperature condition. For a positive control, genomic DNA was extracted from mycelium of *P. infestans* isolate NC092ba that had been grown on Pea Broth media at 25ºC. All extractions were performed using GenElute™ Plant Genomic DNA Miniprep Kit according to manufacturer (Sigma-Aldrich, St. Louis, MO) recommendations. To confirm the presence of *P. infestans* DNA in the samples, standard PCR assays were performed in 25µl reaction volumes, each with 1µl genomic DNA. Reactions also contained 17µl ddH_2O, 5µl 5x GoTaq® Buffer (Promega, Madison, WI), 0.5µl 5U/µl GoTaq® DNA Polymerase (Promega), 0.5µl 10µM dNTPs (Promega), and 0.5µl each of 10µM *P. infestans* primers PiITS5 and Pinf2. Primer sequences were 5'-GGA AGT AAA AGT CGT AAC AAG-3' and 5'- CTC GCT ACA ATA GCA GCG TC-3', respectively (Trout, et al. 1997). Reactions were conducted using Bio-Rad's MyCycler® (BioRad Laboratories, Hercules, CA). PCR parameters included an initial 2-min 92ºC denaturation phase, followed by 35 cycles of denaturation at 92ºC for 15 sec, annealing at 57ºC for 15 sec, and 30 sec of extension at 72ºC. Reactions always included appropriate positive and negative controls. Products were electrophoresed on 1.2% agarose gels containing 500µl 1µg/ml Ethidium Bromide in 1x TAE buffer at 60V for 45 minutes. Samples were run along with 1kb DNA Ladder (Promega) as a standard.

Statistical Analysis

Summary statistics computed in all experiments included treatment of arithmetic means with standard errors computed using Microsoft Excel (Excel version in Microsoft Office 2007 for Windows). Where suitable, statistical assessment also included three-way analysis of variance (ANOVA), conducted using the program JMP version 6.0.3 (SAS Institute, Cary, NC, USA).

RESULTS

Temperature Effects on Disease Cycle

Incubation Period (IP)

Visible infection was successfully established in all replicates for each experimental condition and no control leaves showed any symptoms. IP was significantly variable depending on the conditions (Table 2A) and, in general, it was longest for each isolate at low temperatures (Figure 1). For isolate NC092ba, mean IP was longest at 12°C for cvs. Kennebec ($\overline{x}$ = 100 ± 10.58, n = 3), Russet Burbank ($\overline{x}$ = 116 ± 14.42, n = 3), and Red Norland ($\overline{x}$ = 92 ± 8.00, n = 3) and did not vary significantly between them (p=0.373). IP also did not vary between cvs. Kennebec ($\overline{x}$ = 64 ± 8.00, n = 3), Russet Burbank ($\overline{x}$ = 56 ± 8.00, n = 3) and Red Norland ($\overline{x}$ = 64 ± 8.00, n = 3) at 18°C for isolate NC092ba (p = 0.729). IP was shorter at 18°C for all cultivars (Kennebec p = 0.053, Russet Burbank p=0.022, and Red Norland p = 0.069) than at 12°C (Figure 1A). For leaves infected at 24°C, with isolate NC092ba, there was a significant difference in IP (p = 0.001) between cvs. Kennebec ($\overline{x}$ = 60 ± 0.00, n = 3), Russet Burbank ($\overline{x}$ = 80 ± 4.00, n = 3) and Red Norland ($\overline{x}$ = 60 ± 0.00, n = 3) with Russet Burbank being longest.

For leaves infected with isolate PSUPotb, there was a similar effect between cultivars, within temperatures (Figure 1B). IP was again longest at 12°C and there was no significant difference (p = 0.236) between cvs. Kennebec ($\overline{x}$ = 84 ± 6.93, n = 3), Russet Burbank ($\overline{x}$ = 88 ± 4.00, n = 3) and Red Norland ($\overline{x}$ = 72 ± 6.93, n = 3) at this temperature. At 18°C and 24°C, IP was longer for Russet Burbank ($\overline{x}$ = 64 ± 4.00, n = 3; $\overline{x}$ = 60 ± 0.00, n = 3) than for Kennebec ($\overline{x}$ = 48 ± 0.00, n = 3; $\overline{x}$ = 36 ± 0.00, n = 3) or Red Norland ($\overline{x}$ = 48 ± 0.00, n = 3; $\overline{x}$ = 36 ± 0.00, n = 3) (Figure 1B).

Across temperatures, IP for isolate NC092ba on cvs. Kennebec and Red Norland was longest at 12°C (Figure 1A) and did not vary between higher temperatures (18°C and 24°C). Interestingly, IP for Russet Burbank was longest at 12°C but significantly shorter at 18°C than at 24°C (Figure 1A). For cvs. Kennebec and Red Norland infected with isolate PSUPotb, IP decreased nearly linearly from low temperature

Table 2. Three-way ANOVA tests. (A). General linear model: Effect of Isolate, Cultivar, and Temperature on the incubation period (IP). (B). General linear model: Effect of Isolate, Cultivar, and Temperature on the Area under the disease progress curve (AUDPC).

(A). IP

Source	DF	Sum of Squares	F Ratio	Prob > F
Isolate	1	4,117	35.1	< 0.001
Cultivar	2	2,208	9.4	0.001
Temperature	2	14,563	62.1	< 0.001
Isolate*Cultivar	2	156	0.7	0.522
Isolate*Temperature	2	324	1.4	0.265
Cultivar*Temperature	4	960	2.1	0.109
Isolate*Cultivar*Temperature	4	686	1.5	0.234

(B). AUDPC

Source	DF	Sum of Squares	F Ratio	Prob > F
Isolate	1	278,125	399.9	< 0.001
Cultivar	2	41,416	29.8	< 0.001
Temperature	2	691,246	497.0	< 0.001
Isolate*Cultivar	2	3,923	2.8	0.073
Isolate*Temperature	2	102,723	73.9	< 0.001
Cultivar*Temperature	4	31,287	11.2	< 0.001
Isolate*Cultivar*Temperature	4	32,366	11.6	< 0.001

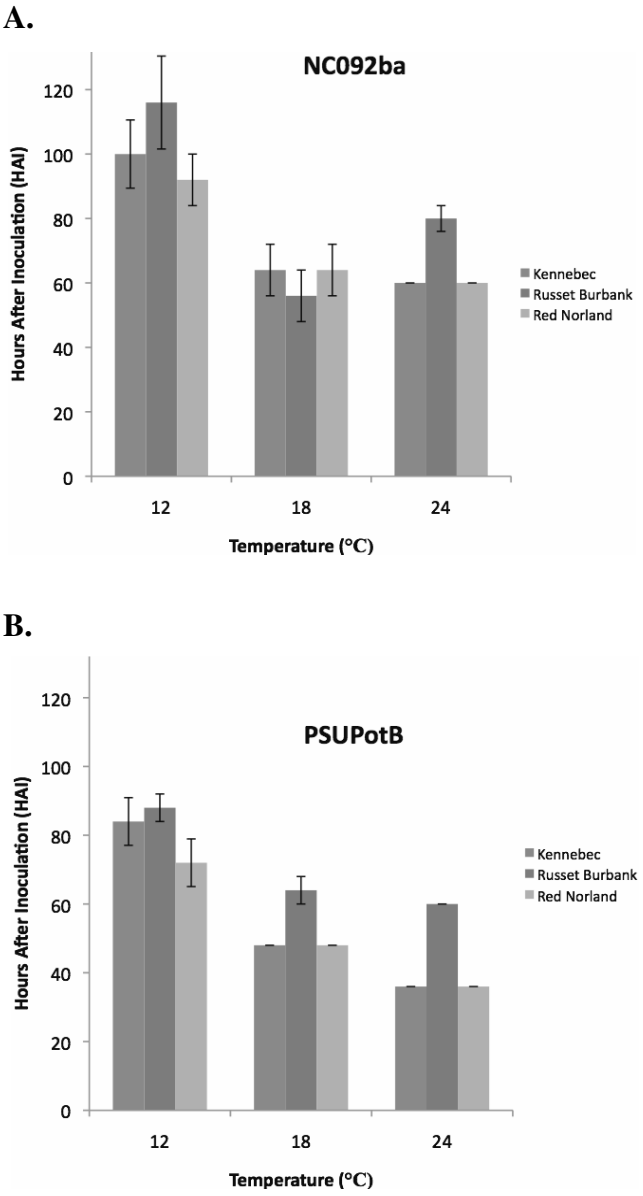

Figure 1. Effect of temperature on incubation period. Incubation period for detached leaves of each of three cultivars, infected with isolate NC092ba (A) and isolate PSUpotb (B). Error bars represent the standard error of the mean time when first sign of infection was visible (n = 3).

Table 3. Test of significance of differences among cultivars, temperatures, and between isolates. (A). Group means and significance of differences for IP. (B). Group means and significance of differences for AUDPC. Significance of differences was tested with Tukey HSD (Honestly Significant Difference) for cultivars and temperatures, and with Student's t-test for isolates. Means within a column that do not share same letter are significantly different at α=0.05.

(A). IP

Cultivar	Mean		Temperature	Mean		Isolate	Mean	
Russet Burbank	77.3	a	12	92.0	a	NC092ba	76.9	a
Kennebec	65.3	b	18	57.3	b	PSUpotb	59.6	b
Red Norland	62.0	b	24	55.3	b			

(B). AUDPC

Cultivar	Mean		Temperature	Mean		Isolate	Mean	
Russet Burbank	4502.0	b	12	3218.0	b	NC092ba	3818.7	b
Kennebec	4926.0	b	18	4220.0	b	PSUpotb	6168.0	a
Red Norland	5552.0	a	24	7542.0	a			

to high temperature. Although IP was also highest for Russet Burbank at 12°C infected with PSUPotb, the effect was not different between higher temperatures (Figure 1B).

Overall, IP was longest for Russet Burbank than for Kennebec or Red Norland; IP length also varied depending on the temperature, being shorter at 12°C than at 18°C or 24°C. In addition, there was a significant difference in the IP when plants were inoculated with NC092ba or PSUpotb ($p \leq 0.05$; Table 3A).

Disease Progress

Disease progress for each replicate leaf was observed every 12 HAI and assigned a value from the Disease Rating Scale (Table 1). In general, disease progress over time was more severe at higher temperatures for each isolate, across cultivars (Figure 2). Temperature also appeared to have a differential effect on disease progress between isolates ($p < 0.001$, Table 2B), especially at 24°C (Figure 2). AUDPC values were calculated to determine the amount of disease integrated between each time point for each condition by converting disease rating to a 1–100 scale (Figure 3). AUDPC values were then assigned ranks to estimate the effect of temperature on the relative susceptibility of each cultivar to each isolate (Table 4).

The highest AUDPC values were assigned to all three cultivars infected with isolate PSUPotb at 24°C while the lowest values were assigned to cultivars infected with isolate NC092ba at 12°C (Figure 3). At 12°C, AUDPC ranks were lower for all cultivars infected with isolate NC092ba than with PSUPotb. At 18°C, temperature effect on isolate was more variable, with AUDPC ranks being lower for Kennebec and Red Norland infected with NC092ba than infected with PSUPot. Interestingly, the opposite was true in Russet Burbank with the AUDPC ranking being higher for leaves infected with isolate NC092ba than isolate PSUPotb (Figure 3). At 24°C, rankings were higher for all cultivars infected with PSUPotb than for those infected with NC092ba.

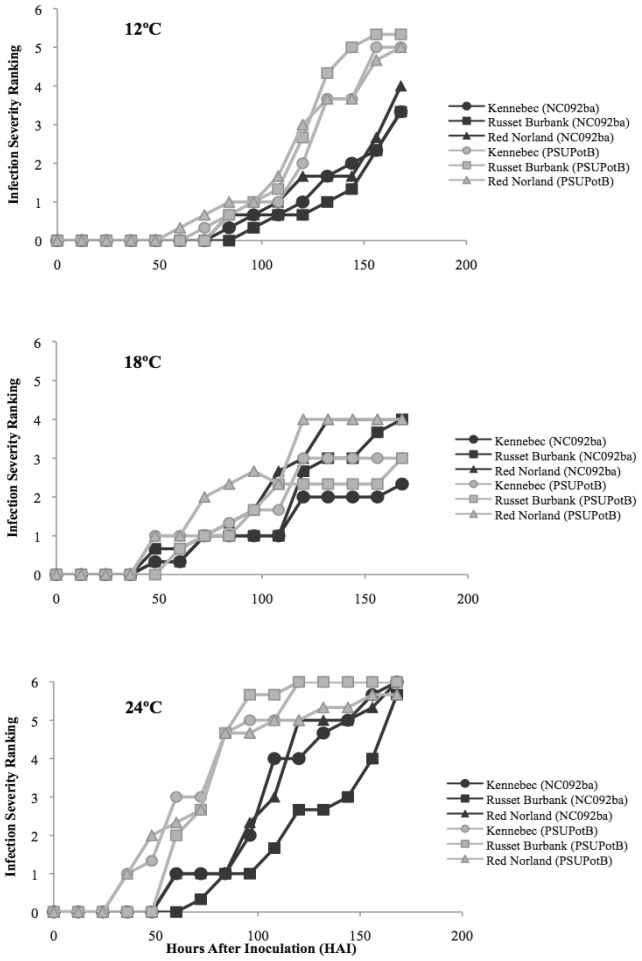

Figure 2. Mean disease development in detached leaves over time (HAI) using detached-leaf rating scale (Table 1) for all temperature conditions (n = 3).

Temperature generally had a positive effect on AUDPC across cultivars, within isolates. In cvs. Kennebec and Red Norland, AUDPC ranking increased with temperature within each isolate (Figure 3). In Russet Burbank, this trend was

Table 4. Area Under The Disease Progress Curve (AUDPC) values (A) and assigned AUDPC rankings (B).

(A). AUDPC

Cultivar	12°C		18°C		24°C	
	NC092ba	PSUpotb	NC092ba	PSUPotb	NC092ba	PSUPotb
Kennebec	2292	3966	2778	4248	6276	9996
Russet Burbank	1608	4590	3942	3516	3840	9516
Red Norland	2406	4446	4890	5946	6336	9288

(B). AUDPC RANK

Cultivar	12°C		18°C		24°C	
	NC092ba	PSUpotb	NC092ba	PSUPotb	NC092ba	PSUPotb
Kennebec	2	8	4	9	14	18
Russet Burbank	1	11	7	5	6	17
Red Norland	3	10	12	13	15	16

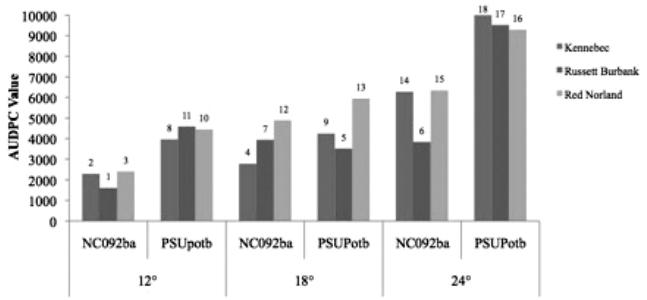

Figure 3. Area Under the Disease Progress Curve (AUDPC) values for detached leaves. Assigned AUDPC ranks (Table 4) are placed above bars for each condition. NC092ba and PSUPotb represent the two isolates of *P. infestans* under investigation along with temperature (°C).

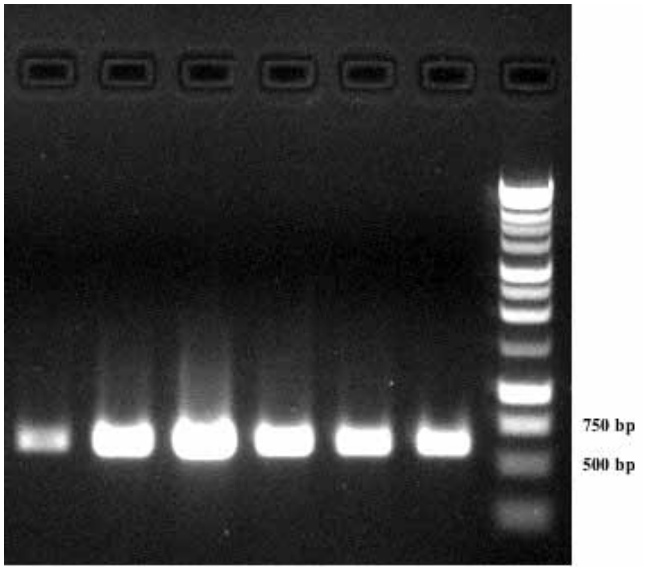

Figure 4. Detection of *P. infestans* in infected tissue. Expected ~600- bp PCR products visualized on 1.2% agarose gel electrophoresis. Detached leaf infection at 24ºC: leaves infected with isolate NC092ba for Kennebec (Lane 1), Russet Burbank (Lane 2) and Red Norland (Lane 3). Leaves infected with isolate PSUPotb for Kennebec (Lane 4), Russet Burbank (Lane 5) and Red Norland (Lane 6). Lane 7: 1-kb DNA marker.

not evident. Within isolate NC092ba, its highest AUDPC ranking was present at 18°C, with 12°C having the lowest rank. When cv. Russet Burbank was infected with PSUPotb, the lowest AUDPC rank was present at 18°C, with 24°C having the highest rank.

In general, these results were confirmed when a 3-way ANOVA was performed, using the original scores, not the converted ones. The model, including factors 'Cultivar', 'Temperature', and 'Isolate', was highly significant for each factor ($p < 0.001$, Tables 2B and 3B). Cultivar Red Norland showed the largest AUDPC value, statistically different from those observed in Russet Burbank and Kennebec (Table 3B). In addition, the mean AUDPC value at 24°C for all cultivars was also statistically significant, when compared with the values obtained at 12°C and 18°C. Finally, there was a significant difference in the effect of the two isolates on the AUDPC (Table 3B). Most factor interactions were also highly significant ($p < 0.001$, Table 2B).

Molecular Confirmation

Infection was successfully confirmed in all infected replicates via Polymerase Chain Reaction (PCR) assay. The expected ~600-bp fragment was visualized on 1.2% agarose gel electrophoresis in all samples from infected leaf tissue and the positive control (Figure 8). No product was visible in uninfected negative controls (Figure 4).

DISCUSSION

Results of this study indicate interesting trends in the disease cycle of three cultivated varieties of potato when exposed to two *P. infestans* isolates of the US-8 clonal lineage. Data suggest that variable susceptibility to *P. infestans* exists between cultivars, with this variation being more

noticeable when cultivars are challenged with different isolates (Figure 2). Each cultivar exhibited some, but not full, level of resistance. Interestingly, there was also an isolate effect on disease development (Tables 2 and 3). This is surprising, as asexually derived members of the same clonal lineage are generally believed to be genotypically identical descendents of the same ancestor (Kato, et al. 1997). Hence, changes in pathogenic fitness must have occurred that have led to variability in the plant response to infection with a specific isolate. Successful infection requires the completion of multiple steps including surface recognition, penetration, modulation of the host resistance response (if any), and colonization. These steps depend on the intertwined function of a myriad of genes and pathways; any changes affecting these genes or pathways could exert a significant effect on pathogenicity (Lamour and Kamoun 2009). Because these isolates were obtained from two different geographic areas, during different years, local adaptations may have developed and the pathogens may have undergone a slight evolutionary divergence, with these changes affecting their pathogenicity on specific cultivars.

As mentioned previously, temperature plays a critical role in the late blight disease cycle. Three representative temperatures, 12ºC, 18ºC and 24ºC, were chosen based on their biological relevance. Previous studies have shown that *P. infestans* favors a moderate temperature range with no disease developing below 7ºC or above 27ºC (Maziero, et al. 2009). The first dependent variable measured in this study was incubation period, or the amount of time that lapses between inoculation and visible signs of disease (Agrios 2005). In general, incubation period was longest for each isolate at low temperatures, across cultivars (Figure 1). Variation in incubation period between temperatures may be a function of different sporangial germination mechanisms utilized by the pathogen at different temperatures. Previous studies suggest that at low temperatures (optimally 12ºC) indirect germination is favored while at high temperatures (above 20ºC) direct germination is favored (Maziero, et al. 2009; Mizubuti and Fry 1998). Results of this study suggest a longer incubation period is needed at lower temperatures, which may be accounted for by the added time needed for zoosporogenesis and subsequent pathogen establishment. Interestingly, the highest variation in incubation period between isolates occurred at 18ºC (Figure 1). In the experiments we are reporting here, isolate NC092ba appeared to require a longer period of time before infection symptoms become visible (Figure 1B), and there was a significant difference in the IP and AUDPC values when plants were inoculated with NC092ba or PSUPotb (Table 3). Such a response within this pathosystem would be indicative of the distinctiveness of the interactions between each potato cultivar and each *P. infestans* isolate.

The detached leaf assay was used to assess temperature effect on the pathogen cycle because it is a practical and commonly used technique in the assessment of foliar susceptibility to *P. infestans* (Dorrance and Inglis 1997). It is also a convenient method as it requires little space and provides replicates of a pre-determined population size. There are, however, several drawbacks to this method of susceptibility assessment. For example, a detached leaf may not be representative of the same immune defenses present in an attached leaf. Furthermore, previous studies have shown that a high number of replicates may be necessary to draw sufficient conclusions about partial resistance in different cultivated varieties (Dorrance and Inglis 1997). Nevertheless, this method of assessment is still an acceptable and convenient approach to compare susceptibility while keeping other environmental conditions, as well as replicate number, uniform.

A semi-quantitative disease scale was used to assess disease severity between experimental conditions (Table 1) in detached leaf infections, over time. To account for inherent subjectivity in this type of assessment, frequency of assessments was increased to every 12 hours. In general, disease progress over time was most severe at the high temperature (24ºC) for each isolate, across cultivars (Figure 2). There also appeared to be a difference between isolates, especially at 12ºC and 24ºC, with disease progress being more severe in cultivars inoculated with PSUPotb (Figure 2 and Tables 2 and 3). To determine how much disease was integrated between each assessment, area under the disease progress curve (AUDPC) values were assigned. AUDPC values correspond to the amount of disease that has been integrated between two consecutive time points and each value is independent of the disease progress curve shape (Campbell 1998). This allowed for relative comparison between each condition. AUDPC rankings show that disease severity was highest in all cultivars infected with isolate PSUPotb at 24ºC and lowest in all cultivars infected with isolate NC092ba at 12ºC (Figure 3). Three-way ANOVA tests conducted using the original scores confirmed that all factors had a significant effect on the AUDPC value ($p < 0.001$) (Tables 2B and 3B), and that the interaction of most of these factors also had a significant effect on the AUDPC value (Table 2B).

A clear pattern emerges in AUDPC values at high (24ºC) and low (12ºC) temperatures, with values being significantly higher in leaves inoculated with isolate PSUPotb than isolate NC092ba (Table 3B). This pattern is absent at the intermediate temperature (18ºC). At 18ºC AUDPC values are variable both between and within cultivars (Figure 3). This could be due to a number of reasons relating to either host defenses or pathogen fitness. Although it is impossible to determine which elements are at play without an extensive look at the molecular pathways involved, it can be surmised that both, host defenses and pathogen fitness, have important roles. As stated previously, the two primary methods of sporangial germination in *P. infestans* are optimal at low and high temperatures (Mizubuti and Fry 1998). Very little information exists about germination rates at intermediate temperatures. It is possible that the pathogen has developed optimal pathogenic capabilities at low and high temperatures rather than at intermediate ones, at which innate structural and biochemical defenses of the plant are likely at optimal levels.

Overall, this study provided a successful evaluation of the interactions between two isolates of the US-8 clonal lineage and three different cultivated varieties of potato in three different temperature conditions. Recognizing the distinctive cultivar responses elicited by diverse isolates is essential for disease forecasting and management. In particular, knowing the differential effects of temperature on the disease cycle will be important when combating the pathogen. As the global demand for potatoes, a nutritious and inexpensive staple food crop, continues to grow so does the threat that late blight poses on crop security. It, therefore, remains critical that research continue toward fully understanding this disease cycle.

ACKNOWLEDGEMENTS

We would like to thank Dr. Nancy Waters for her contributions to experimental design and data analysis for this project and Mr. Philip Auerbach for his technical assistance. We would also like to thank Mr. Bob Leiby, the Lehigh County Extension Director for the Penn State College of Agricultural Sciences Cooperative Extension Division, for providing seed tubers for this study, and Ms. Frances Perez of the United States Department of Agriculture Agricultural Research Service (USDA-ARS) for providing the *P. infestans* isolates used in our investigations. This project was funded by Lafayette College.

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Journal of the Pennsylvania Academy of Science 85(4): 174–182, 2011

2012 DARBAKER PRIZE:

MORPHOMETRIC ANALYSIS OF NUCLEI, LUMENS, AND SHAPE OF LACTIFEROUS DUCTS IN CANCEROUS BREAST TISSUE[1]

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ABSTRACT

As the most common form of breast cancer in women, ductal carcinoma accounts for 70–80% of breast cancer cases. Pathologists often use the Nottingham grading system to assess the abnormality and aggressiveness of invading cancerous cells in ductal carcinoma. A grade of one to three is assigned using a point system based upon variation in nuclear appearance, tubule formation, and mitotic count, but these features are generally not quantified. We collected morphometric data on nuclei and ducts to attempt to quantify nuclear variation and tubule formation. Measurements of over 2000 nuclei and nearly 100 ducts were made using slides of normal breast tissue and the three grades of ductal carcinoma from anonymous patients at Pocono Medical Center. A ratio of luminal area to ductal area in cancerous tissue within each grade was also calculated, and shapes of duct outlines were examined. ANOVA revealed significant differences in nuclear diameter among grades: both diameter and variation in diameter increased with grade. Variability and irregularity of nuclear and duct shape also increased with grade. Lumen/duct area ratios decreased with severity of invasion and approached zero in grade three. Although preliminary, these morphometric data suggest that it may be possible to develop more objective and quantifiable criteria for grade designations.
[J PA Acad Sci 85(4): 174–182, 2011]

INTRODUCTION

Breast cancer is one of the most common types of cancer in women (U.S. Cancer Statistics Working Group, 2010), and is associated with a wide range of manifestations, prognoses, and patient outcomes. A normally functioning human mammary gland resembles a cluster of grapes. Milk glands are composed of clusters of 15 to 20 lobules, which are organized into lobes of the breast (Fechner and Mills, 1990). These lobules drain into the lactiferous ducts, or milk ducts, which converge into several collecting ducts. The collecting ducts are continuous with the lactiferous sinuses, which then exit through the nipple (Fechner and Mills, 1990; Kierszenbaum, 2007; McKinnell et al., 1998; Simon and Zieve, 2009). The walls of the ducts are composed of a single adluminal layer of cuboidal cells and an abluminal layer of myoepithelial cells. The outer, discontinuous layer of myoepithelial cells helps express the secretions of the cuboidal cells through the ducts to the nipple under the stimulation of oxytocin (Kierszenbaum, 2007; McKinnell et al., 1998). When cancer is present, these ducts are scarce. Adipose is abundant in the breast tissue and a network of capillaries surrounds the ducts and lobes along with fibrous connective tissue, such as collagen and elastin (Kierszenbaum, 2007; McKinnell et al., 1998; Simon and Zieve, 2009).

Breast cancer exhibits a wide array of manifestations and can range from relatively benign to an incessant and deadly malignancy. Most breast tumors (75–80%) are classified as invasive ductal carcinoma (Carter, 1990; Fulford et al., 2006). Ductal carcinoma is a cancer of the epithelial cells of the lactiferous ducts. Ductal carcinoma in situ (DCIS) occurs within the duct; the cells have not broken out into the stroma through the duct walls. Infiltrating, or invasive, ductal carcinoma breaks through the duct walls and invades the surrounding tissue (Carter, 1990; McKinnell et al., 1998).

Several histological grading systems have been developed to determine the severity of a ductal carcinoma, and to establish a prognosis and an appropriate treatment plan. The Nottingham grading system, a modification of the Scarff-Bloom-Richardson grading system, considers three factors and uses a 9-point scale to grade each cancer (Bloom and Richardson, 1957; Carter, 1990; Cardiff et al., 2001; Cardiff, 2011; Dalton et al., 1994; Dalton et al., 2000; Halls, 2011; Meyer et al., 2009; Pinder et al., 1998; Thomas, 2011). The three factors are nuclear pleomorphism, tubule formation, and mitotic count per 10 high-power fields (Carter, 1990; Cardiff et al., 2001; Cardiff, 2011; Dalton et al., 1994; Dalton et al., 2000; Elston and Ellis, 1991; Halls, 2011; Meyer et al., 2009; Thomas, 2011).

Nuclear pleomorphism considers the size and shape of the nuclei in the cells comprising the duct wall. Their similarity and the conspicuousness of their nucleoli are observed (Carter, 1990; Cardiff et al., 2001; Cardiff, 2011; Dalton et

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al., 1994; Dalton et al., 2000; Halls, 2011; Meyer et al., 2009; Thomas, 2011). Prominent nucleoli are indicative of increased mitotic activity within the cell (Montanaro et al., 2008). Therefore, one would expect to see enlarged and more abundant nucleoli with increasing grades of ductal carcinoma. Small uniform nuclei with inconspicuous nucleoli score one point, larger nuclei with small nucleoli and some irregularity score two points, and highly pleomorphic nuclei that are variable in size and shape and have prominent nucleoli score three points (Carter, 1990; Cardiff et al., 2001; Cardiff, 2011; Dalton et al., 1994; Dalton et al., 2000; Halls, 2011; Meyer et al., 2009; Thomas, 2011).

Tubule formation assesses the proportion of cancerous tissue that contains ducts with apparent lumens, observed over the entire tumor on low to medium power (Thomas, 2011). Circular tubules with visible lumens are indicative of healthy breast tissue. Tubule formation visible in more than 75% of the cancerous tissue would be assigned one point, tubule formation in 10–75% of the tissue would be assigned 2 points, and tissue with little or no tubule formation (i.e., solid ducts) would be assigned three points (Carter, 1990; Cardiff et al., 2001; Cardiff, 2011; Dalton et al., 1994; Dalton et al., 2000; Halls, 2011; Meyer et al., 2009; Thomas, 2011).

Lastly, the abundance of mitotic figures per 10 high-power (400X) fields is considered, because abundant mitotic figures are indicative of high rates of cell division. In normal breast tissue, mitotic figures are seen not at all or only very rarely. In cancerous tissue, ten consecutive fields are chosen at the periphery of the mass, in areas where mitoses are evident and confounding abnormalities are absent (Medri et al., 2003; Thomas, 2011). Since the area of a high-power field can vary among microscopes (from .40 to .69mm), proper assessment of mitotic counts requires calibrating the field area to standard mitotic frequency scores (Pathology Reporting of Breast Disease, 2005). For example, at a field diameter of .59mm, tissues with fewer than 10 mitotic figures per 10 high-power fields would be assigned one point, tissues with between 10 and 19 mitotic figures would be assigned two points, and tissues with 20 or more mitotic figures would be given three points (Cardiff, 2011). At a field diameter of .50 mm, however, point values of one, two, and three would correspond to mitotic counts of less than or equal to 7, between 8 and 14, and greater than or equal to 15 mitotic figures per high-power field, respectively (Pathology Reporting of Breast Disease, 2005).

Point values derived from consideration of all three factors are added up into a total score, with grade one cancers falling between 3 and 5 points, grade two cancers between 6 and 7 points, and grade three cancers between 8 and 9 points (Carter, 1990; Cardiff et al., 2001; Cardiff, 2011; Dalton et al., 1994; Dalton et al., 2000; Halls, 2011; Thomas, 2011).

Detecting cancers in the early stages of development with more accuracy and immediacy is needed for better chance of survival (Elston and Ellis, 1991; Erukhimovitch et al., 2002; Pinder et al., 1998). Predicting an accurate prognosis, even with recent improvements to grading systems, is not always easy or without ambiguity (Burke and Henson, 1997; Dalton et al. 2000; Pienta and Coffey, 1991). Ductal carcinoma exhibits a wide range of behaviors. Some women with more aggressive cases live for many years with little treatment, while others with early detection die quickly of metastasis even with combative treatment (Bloom and Richardson, 1957). More objective ways of defining grading systems to increase reproducibility among pathologists is an active area of exploration (Dalton et al., 1994; Dalton et al., 2000; Meyer, 2009; Pienta and Coffey, 1991; Thomas, 2011). Problems arise when a certain score deems that a patient be treated one way when a score of just one point lower or higher in another patient results in an entirely different approach to treatment. This problem arises from the imprecise distinction between grades (Dalton et al., 2000), particularly between a grade one and grade two carcinoma. More objective criteria could reduce grading ambiguity and result in improved prognosis and treatment. This is especially the case with respect to nuclear grading, where evaluation is somewhat subjective, leading to inconsistent reproducibility (Pienta and Coffey, 1991; Meyer et al., 2009).

Histological data obtained from image analysis, such as measurements of nuclei and ducts, could decrease subjectivity in grading and lead to greater agreement among pathologists and consistency in prognoses (Cardiff et al., 2001; Cardiff, 2011; Millis et al., 2004; Pienta and Coffey, 1991; Thomas, 2011). We therefore used image analysis to collect morphometric data on the nuclei and ducts of a preliminary set of 11 ductal carcinoma cases in an attempt to quantify two of the Nottingham grading factors: nuclear variation and tubule formation. With quantifiable data, computer-based morphometric image analysis could increase the objectivity of the grading technique. If appropriate sample sizes are available, these data could possibly be used to supplement the Nottingham grading system and promote greater reproducibility in the scoring process.

MATERIALS AND METHODS

Tissues from eleven confirmed cases (individuals) were examined in the Pocono Medical Center (PMC) Laboratory in East Stroudsburg, Pennsylvania, from January 2010 to May 2010. All tissues examined were from anonymous patients with no associated information; patient consent was not an issue and the research was approved by East Stroudsburg University's Institutional Review Board as exempted research. The Nottingham grade of each case and slide was confirmed by PMC pathologists. Two of the eleven cases were examples of normal breast tissue and were used for comparison. Four of the eleven cases were classified as grade one ductal carcinoma, three cases were grade two, and two cases were grade three. An Olympus BX40 microscope with a Leica DFC 290 digital camera accompanied by Leica Application Suite V3 imaging software, housed in the PMC Pathology Department, was used to capture images of hundreds of slides of breast tis-

sue at 200X or 400X. All slides were stained with hematoxylin and eosin (H&E) and the digital images were analyzed by taking measurements on the nuclei and ducts of cancerous and non-cancerous breast tissue. Captured images were also examined in the Biology Department at East Stroudsburg University, where Nikon NIS Elements D V3 imaging software was used to calculate the areas of lactiferous ducts and lumens. In addition, these images were used to make counts of nucleoli and examine the shape of lactiferous ducts.

Maximum Diameter of Nuclei.–The maximum diameters (in µm) of over 2000 nuclei across the 11 cases were measured at 400X. Each case consisted of multiple slides, and multiple images were taken from each. Maximum diameters were measured with the Leica imaging software by zooming in on the images of the nuclei and dragging the mouse across the maximum diameter of each nucleus (Figure 1A). The measurements were then recorded in Microsoft Excel 2010, and IBM SSPS Statistics 18 software was used to perform a nested ANOVA on the data to test for significant differences in the nuclear diameters among grades and cases. A non-parametric Kruskal-Wallis test was also performed after heterogeneity of variances was indicated.

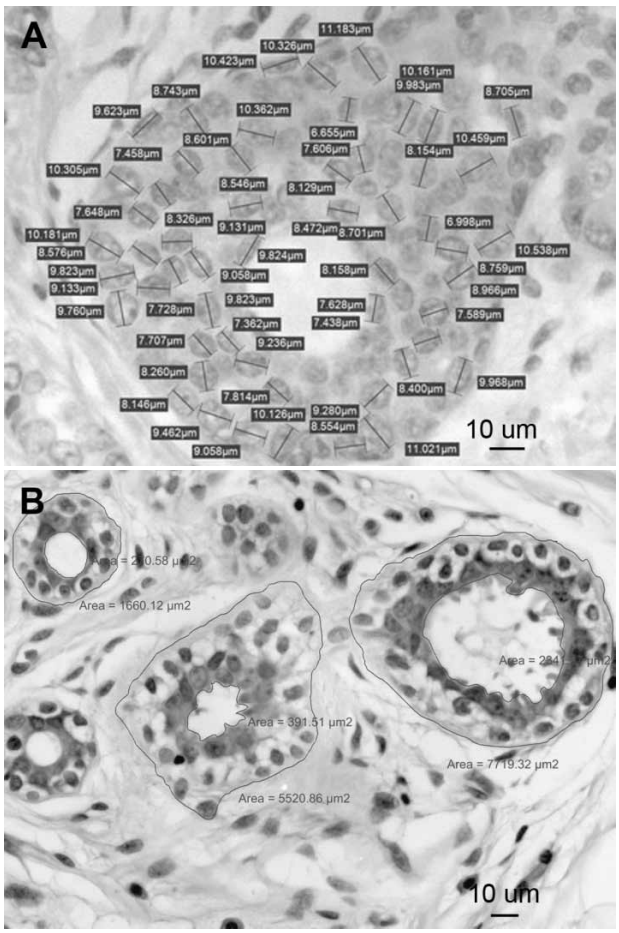

Figure 1. Measurements taken on (A) examples of cell nuclei of grade two invasive ductal carcinoma at 400X and (B) ducts and lumens in areas of normal breast tissue at 200X, stained with H&E.

Prominent Nucleoli.–Nucleoli visible in the images were counted for all cases and grades. The number of nuclei noted to have visible nucleoli was recorded. For each image, the number of nuclei with prominent nucleoli was divided by the total number of measured nuclei to obtain a percentage. Microsoft Excel 2010 and IBM SSPS Statistics 18 software were used to perform a Chi-square test for association to compare the proportion of nucleoli across grades.

Lumen to Duct Areas.–Ductal and luminal areas (μm^2) were measured at 200X in 79 ducts spanning ten of the eleven cases. An additional 15 ducts of grade three cancers were examined but due to the lack of tubule formation lumens could not be measured. Perimeters were indicated (Figure 1B) using the NIS Elements imaging software around each duct and each lumen. Luminal areas were then divided into the ductal areas. All lumen-to-duct area ratios were recorded into a spreadsheet, and IBM SSPS Statistics 18 was used to perform a nested ANOVA and a Kruskal-Wallis test to look for significant differences between the duct ratios across grades.

RESULTS

Maximum Diameter of Nuclei.–Nuclei of ductal carcinoma cells show an increase in size and variability with increasing grade (Figure 2). Maximum nuclear diameter showed a significant increase with grade ($p = .016$), and the standard deviation also increased with grade (Table 1, Figure 3) when cases were pooled within grade. The maximum diameters and standard deviations of grades one and two were much more similar to each other than either was to grade three. A 7.3% increase in size from grade one to grade two was calculated as compared to a 28.5% increase in size from grade two to grade three. Significant differences were

Table 1. Maximum diameter of nuclei among grades and individual cases in samples of normal and cancerous breast tissue.

Grade	Case	N	Mean (µm)	St Dev
Normal	1	290	8.32	0.94
	2	34	8.11	1.06
	Total	**324**	**8.30**	**0.95**
One	3	84	11.22	1.62
	8	315	8.86	1.30
	9	136	7.79	1.29
	12	100	10.73	1.48
	Total	**635**	**9.24**	**1.80**
Two	5	200	9.28	1.45
	6	185	9.85	1.11
	10	80	11.63	2.32
	Total	**465**	**9.91**	**1.73**
Three	7	144	13.82	2.85
	11	471	12.41	2.09
	Total	**615**	**12.73**	**2.36**

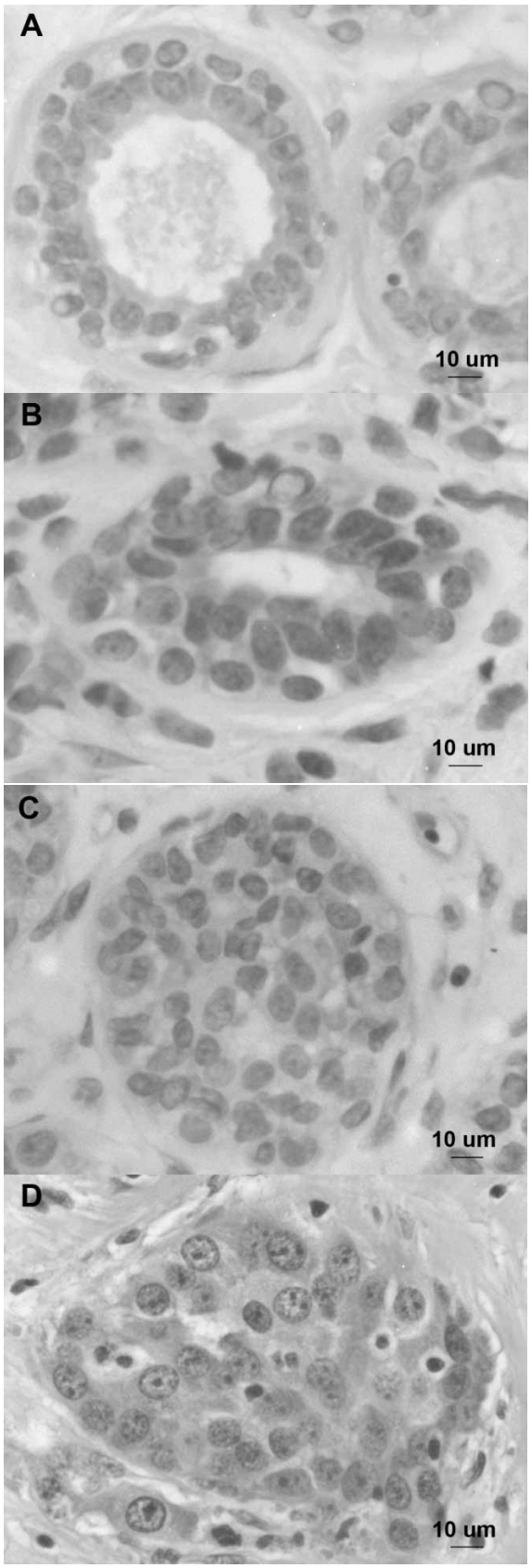

Figure 2. Difference in appearance of nuclei among examples of normal tissue and three grades of ductal carcinoma at 400X and stained with H&E. (A) a duct from normal breast tissue with small, uniform nuclei; (B) grade one ductal nuclei; (C) grade two ductal nuclei; and (D) grade three ductal nuclei.

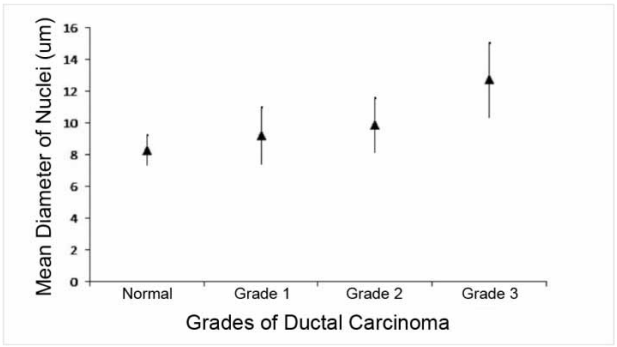

Figure 3. Mean maximum diameter of nuclei in each grade of ductal carcinoma. Triangles represent means for normal (n=324), grade one (n=635), grade two (n=465), and grade three (n=615). Bars represent one standard deviation. Means for individual cases within each grade are displayed in Table 1.

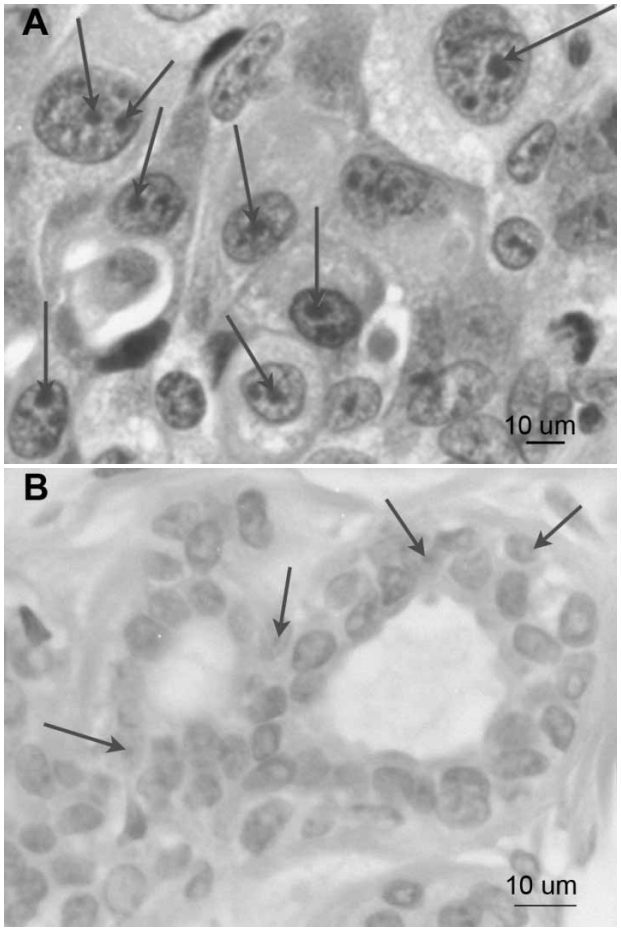

Figure 4. Examples of invasive ductal carcinoma exhibiting (A) nuclei of grade three with abundant prominent nucleoli, and (B) nuclei of grade one showing few visible nucleoli, at 400X and stained with H&E.

also detected between cases. Instead of certain cases from different grades clustering together according to their predetermined grade, almost all of the cases showed significant differences among each other. Even though an upward trend appeared when the grades were compared, individual cases did not reflect the same trend. The significant differences

between the individual cases reflect heterogeneity in the samples partly due to increased pleomorphism and irregularity of nuclei. Therefore, due to heterogeneity of the variances, a Kruskal-Wallis test was performed among the grades of the ductal carcinoma cases to supplement the nested ANOVA results. The Kruskal-Wallis test also revealed significant differences among grades at $p < .001$.

Prominent Nucleoli.–The presence of prominent nucleoli among the measured nuclei significantly increased with grade at $p < .001$ (Figure 4). Of the 324 nuclei measured in the normal breast tissue cases, only 39 (12%) of the nuclei had visible nucleoli. In the grade one cases, 163 of the 663 total nuclei (24%) had visible nucleoli. In the grade two cases, 153 of the 465 total nuclei (33%) had visible nucleoli. Finally, in grade three cases, a remarkable 493 of the 615 measured nuclei (80%) exhibited visible nucleoli (Figure 5).

Lumen to Duct Area.–Upon visual inspection of the four grades, luminal areas decreased while duct wall thicknesses increased as cancerous tissue grade increased (Figure 6). Smaller lumens were seen, if at all, as the grade increased and as the cells penetrated the duct walls into the stroma. Therefore, there is an apparent decrease in lumen/duct ratio (Table 2, Figure 7). Although the calculated lumen/duct ratios (as well as their standard deviations) do decrease from grade one to two, no significant difference was detected in the measured lumen/duct area ratios between these two grades when the nested ANOVA and Kruskal-Wallis tests were performed ($p = 0.837$). The fact that lumens were completely obliterated in most grade three ducts rendered lumen

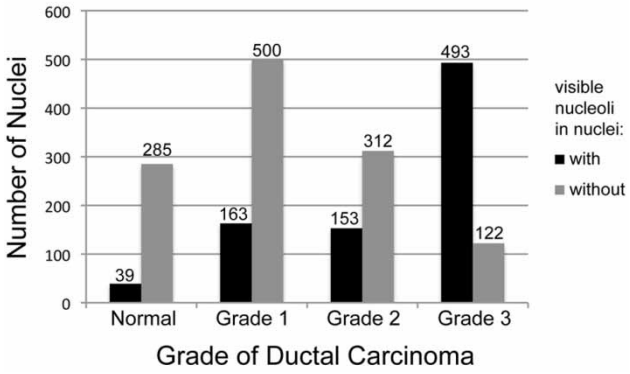

Figure 5. Prominence of nucleoli in different grades of ductal carcinoma: normal (n=324), grade one (n=663), grade two (n=465), and grade three (n=615). Black columns on the left depict numbers of nuclei with visible nucleoli, and gray columns on the right depict numbers of nuclei without visible nucleoli.

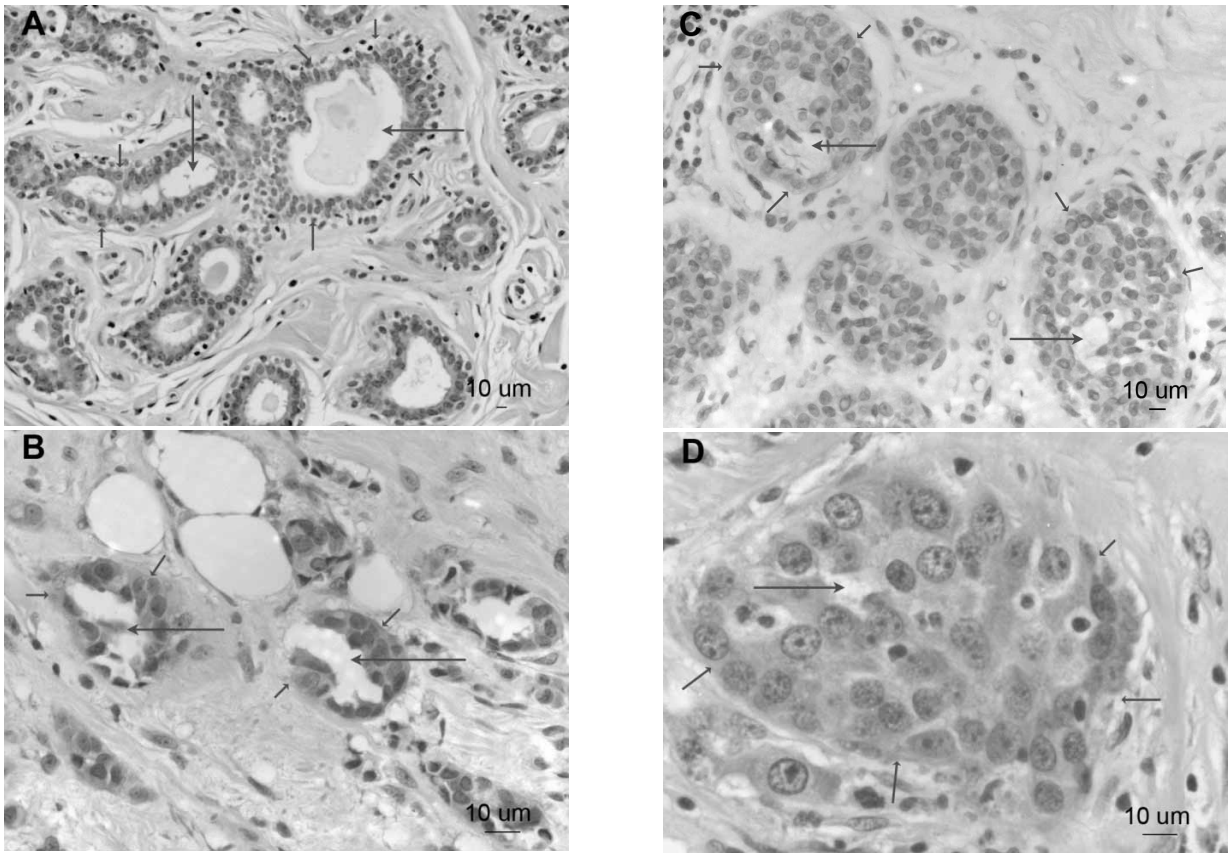

Figure 6. Differences in tubule formation among normal breast tissue and grades of ductal carcinoma at 200X (A, C) and 400X (B, D) and stained with H&E. (A) Normal breast tissue with circular well defined ducts and large lumens, (B) tissue showing a grade one ductal carcinoma, (C) tissue showing a grade two ductal carcinoma with increasingly obliterated lumens, and (D) tissue showing a grade three ductal carcinoma with a barely visible lumen. Small arrows represent boundaries of ducts; large arrows indicate lumens.

Table 2. Lumen area to duct area ratios among grades of ductal carcinoma.

Grade	N	Lumen Area/Duct Area Mean	St Dev
0	44	**0.142**	0.096
1	17	**0.145**	0.116
2	17	**0.120**	0.070
3	16	**0.002**	0.006

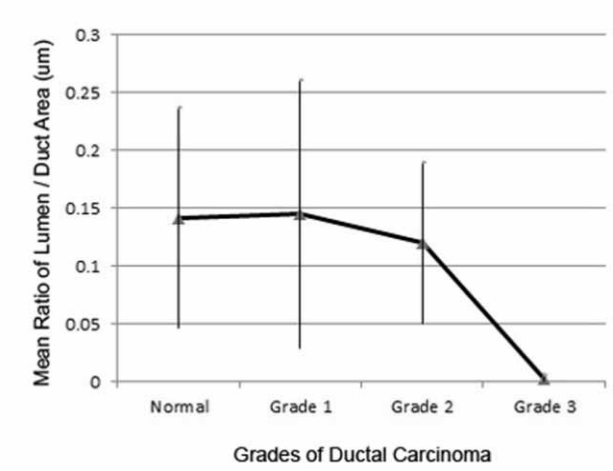

Figure 7. Lumen to duct area ratios among grades of ductal carcinoma. Triangles represent means and bars represent standard deviations, and values are displayed in Table 2.

measurement impossible except for one case in which a single tiny lumen was visible; therefore the mean ratio for grade three ducts is extremely low. When grade three ducts are included in the analysis there is a significant grade effect ($p = .001$) among all grades.

DISCUSSION

Morphometric data from nuclei revealed a significant increase in nuclear size among the three grades of ductal carcinoma. In addition, the standard deviation increased with grade, reflecting the greater variability among the sizes of nuclei as the cancer cells divide without restraint. The different mean nuclear diameters provide a method to quantify differences among each grade. However, it is important to bear in mind that the increase in nuclear variability with grade (reflected in increasing standard deviations) calls for consideration of all the factors used in the grading system to determine the grade.

For example, PMC pathologists confirmed case three to be a grade one carcinoma, but the statistical tests on nuclear diameter exposed it as an outlier. Case three had notably larger nuclei, but they were not irregular and they were all fairly uniform. The nucleoli were inconspicuous and only few were visible. The mean nuclear diameter for all grade one cases was 9.24µm whereas the mean nuclear diameter in case three was 11.22µm. The standard deviation, however, which was only 1.62µm, was only slightly higher than

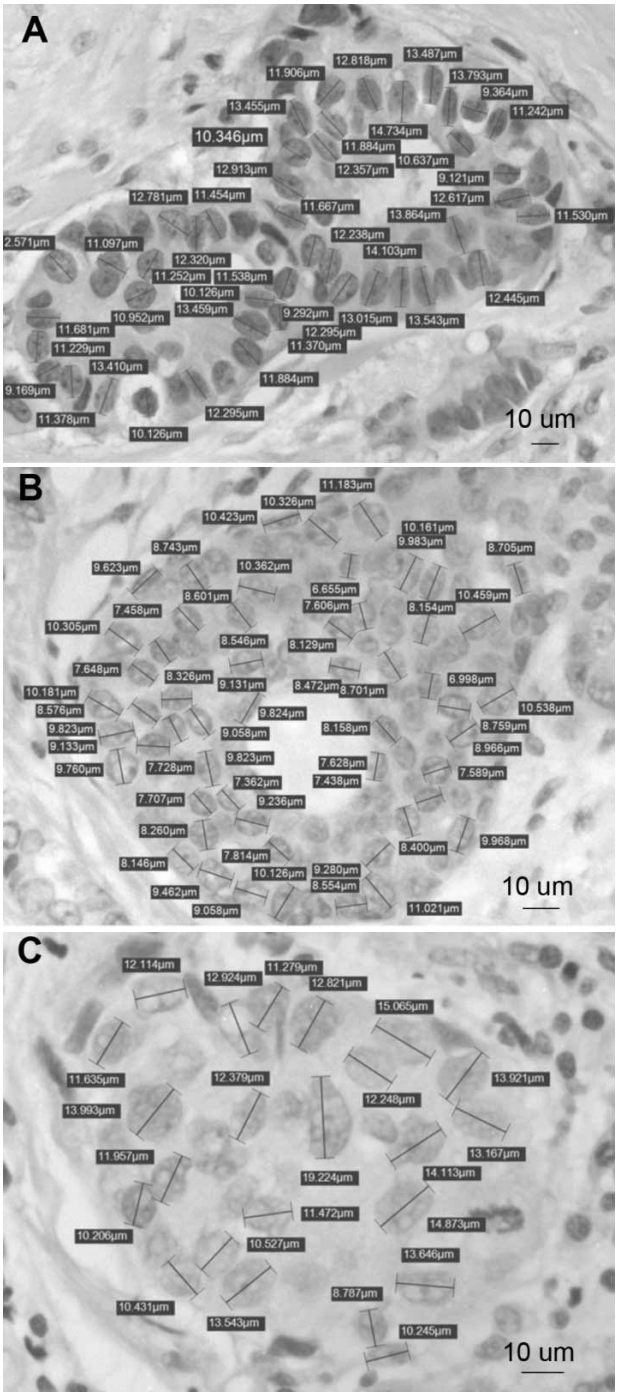

Figure 8. Variation in shape and size of nuclei across grades of ductal carcinoma at 400X and stained with H&E. (A) grade one, (B) grade two, (C) grade three.

the other grade one cases. The standard deviation is meaningful in that it provides an objective way to assess the variability between nuclei. The nuclei may be large in diameter, but their standard deviation can gauge how different they are from each other. A higher standard deviation is indicative of more pleomorphic nuclei, a characteristic that could help define them as part of a low or high-grade tumor. When looking at the other aspects involved in grading case three,

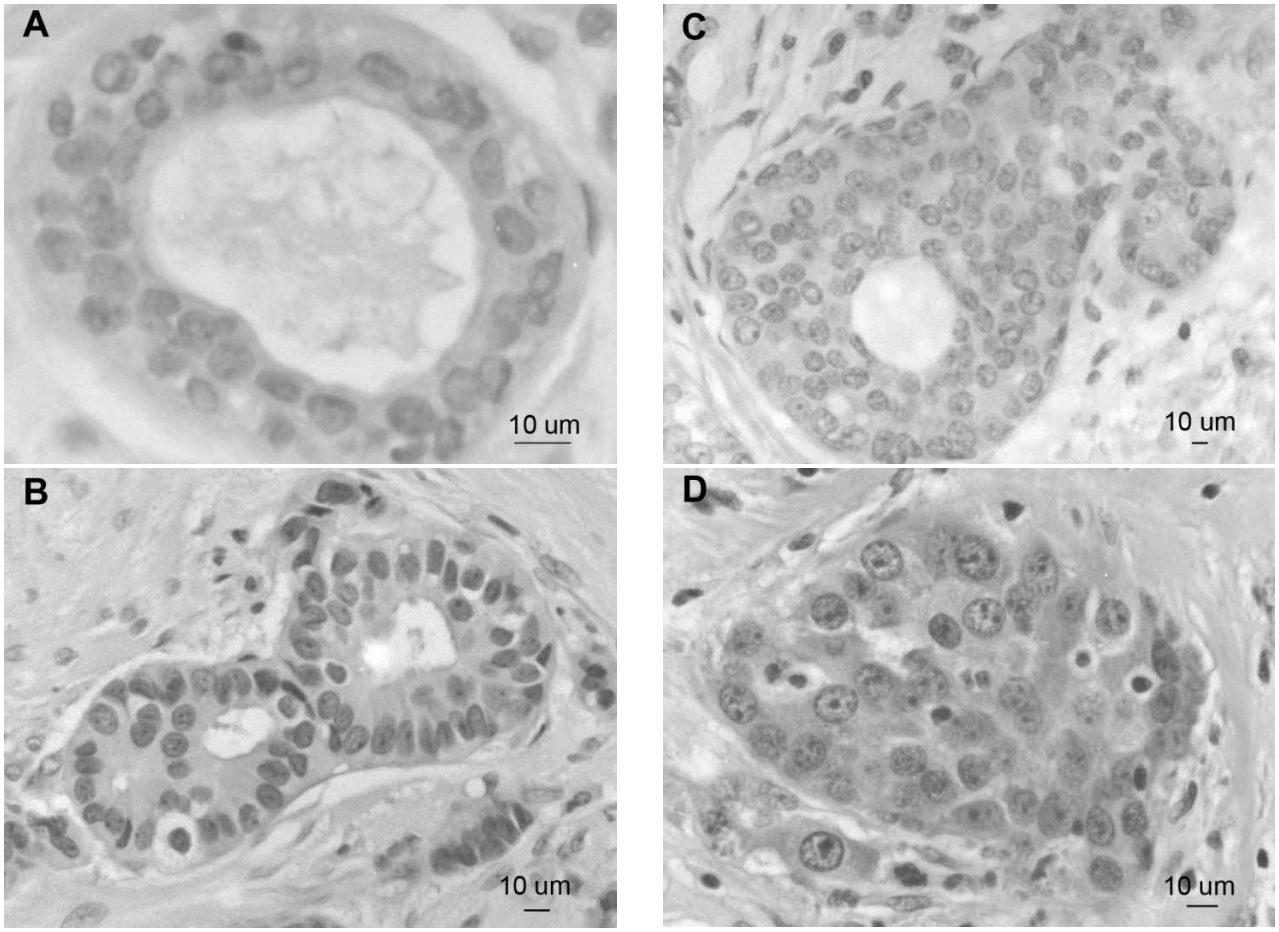

Figure 9. Variation in duct shape across grades of ductal carcinoma at 400X and stained with H&E. (A) normal tissue, (B) grade one, (C) grade two, (D) grade three.

obvious lumens are seen in the ducts of the tumor, the ducts are defined in structure, and the cells are not aggressively penetrating the stroma. While nuclear diameter data alone may suggest that case three is a higher-grade tumor, the standard deviation as well as the other grading factors are consistent with assignment to grade one. This emphasizes the importance of all three grading elements.

Nucleoli are indicative of cell activity including mitosis and protein synthesis. Their prominence is considered along with nuclear appearance in the grading system, but frequency has traditionally not been quantified. Our results of nucleoli counts show the accelerated rate of mitosis among more invasive cancers. A strong trend in the data shows how inconspicuous the nucleoli are in grade one cancers compared to grade three cancers where they appeared in nearly every cell. Data for grade one and grade two cancers were more similar to each other than to data for grade three, which reflects an indistinct line between these stages and verifies that consideration of a multitude of more objective criteria are needed. Strong statistical differences in the proportion of nuclei with visible nucleoli among the three grades can add another level of quantification to the grading system.

The lack of statistically significant differences in lumen/duct areas between grades one and two is partly due to the difficulty of measuring undefined outlines of ducts and lumens, especially in the higher grades where the nuclei push out into the stroma, destroying the duct wall. Although a significant difference among grades was revealed when grade three ducts were included, most grade three ducts completely lack lumens, resulting in a calculated ratio of zero for grade three lumen/duct areas.

Since the difficulty in measuring duct areas is in part due to their increasing irregularity and lack of definition, one promising approach in future analyses may be a sliding semi-landmark shape analysis to compare the outlines of ducts and lumens. Structures with complex shapes, such as bones, may be compared by performing a geometric landmark analysis, in which homologous points are digitized and compared across specimens. Biological structures with curved and rounded outlines have definite shapes, but it is difficult or impossible to designate homologous points on different specimens (Bookstein 1997, Perez et al. 2006). For example, ducts and nuclei vary in their outline shapes across grades, but traditional landmarks cannot be designated (Figures 8 and 9) because they lack homologous

landmark features used in traditional geometric morphometric analyses.

In a sliding semi-landmark shape analysis, a series of points at consistent, pre-determined intervals is recorded along the curve, and the distance from the centroid of the shape to each point on the outline and/or the angles between the rays from the centroid to the points contribute to a mathematical description of the shape (Klingenberg, 2009). Traditionally, these types of data have been analyzed with an eigenshape or elliptical Fourier analysis. A newer technique uses these points as semi-landmarks that "slide" along the outline of the curve in such a way as to minimize the bending energy required to transform the shape into a reference configuration, as in a thin plate spline analysis (Bookstein, 1997; Bookstein et al., 1999; Perez et al., 2006). Designating appropriate statistical analyses for semi-landmarks is still an active area of research, but they are at least useful for visualizing shape differences among curved surfaces. We hope that in future analyses including larger sample sizes of grade three ducts with tiny lumens, this morphometric technique will enable a quantifiable comparison among the ducts of varying grades and may also be extended to nuclear shape as well.

Despite long-term use of the histological grading system for breast cancers, difficulty in clearly defining the lines between grades of carcinoma still exists. This problem originates from indefinite degrees of invasion of cells, overlapping ranges of variability in most of the factors used to assess grade, and broadly-defined and poorly-quantified categories. While grading helps to predict the extent of malignancy, its significance lies in helping to predict when a tissue will metastasize and produce symptoms or cause death in the patient, and in helping to determine which types of treatments may be appropriate (Bloom and Richardson, 1957; Dalton et al. 2000; Elston and Ellis, 1991; Pinder et al. 1998). Morphometric data such as nuclear diameters, areas of lumens and ducts, presence of visible nucleoli, and shapes of ducts and nuclei may provide objective and quantifiable verification of grade designations, when appropriate numbers of cases are analyzed for each grade. Hopefully, morphometric analyses such as those based on the preliminary data presented here may help to increase reproducibility of grading assessments among breast cancers, and in turn lead to improved prognoses and treatment decisions.

ACKNOWLEDGMENTS

We wish to thank Carmine Cerra, M.D., Laboratory Medical Director, and John Tinsley, M.D., Pathologist, at Pocono Medical Center, for their expertise, time, and advice, and for granting us access to anonymous tissue samples. We thank Matthew Wallace, Department of Biology, ESU, for use of laboratory equipment and software. This manuscript greatly benefited from the suggestions and comments made by three anonymous reviewers.

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Journal of the Pennsylvania Academy of Science 85(4): 183–187, 2011

A SURVEY OF THE PRION PROTEIN GENE HETEROGENEITY IN NEW JERSEY WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*)[1]

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ABSTRACT

Polymorphisms within the prion protein gene (*Prnp*) have been associated with modified susceptibility to chronic wasting disease (CWD) in white-tailed deer (*Odocoileus virginianus*). Studies of naturally infected white-tailed deer have determined that polymorphisms in the cervid *Prnp* at codons 95, 96, and 116 may provide reduced susceptibility or slowed progression of CWD. A survey was performed to determine the *Prnp* heterogeneity within New Jersey white-tailed deer. No CWD positive deer have been reported in New Jersey and all animals included in this study were determined to be CWD negative before the start of the project. The *Prnp* genes of 150 hunter-harvested deer were sequenced. All polymorphisms were identified and allelic frequencies were calculated. Seventy percent of the deer sampled in New Jersey were homozygous at codon 95 (glutamine) and codon 96 (glycine) and 94.7% had at least one copy of this allele. Only 2 % of the deer sampled had a single copy of the allele with histidine at Codon 95 and a glycine at Codon 96, which is under represented in naturally infected deer and may provide resistance to infection. These deer were found only in the southwestern portion of New Jersey. The genetic heterogeneity and distribution of the PrP gene of New Jersey white-tailed deer adds to our current understanding of the *Prnp* diversity within this species.

KEYWORDS: chronic wasting disease, CWD, New Jersey, *Odocoileus virginianus*, prion protein gene, TSE, white-tailed deer.

[J PA Acad Sci 85(4): 183–187, 2011]

INTRODUCTION

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy (TSE) of cervids in North America. Chronic wasting disease has been reported in multiple free-ranging and captive cervid species including mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), elk (*Cervus elaphus*), and moose (*Alces alces shirasi*) (Williams and Young, 1980; Spraker et al., 1997; Baeten et al., 2007). Transmissible spongiform encephalopathies are a group of neurodegenerative disorders characterized by the accumulation of protease-resistant forms (PrP^{res}) of the cellular prion protein (PrP) within the neurological tissue of infected individuals (Prusinger, 1982; 1991). Infectious PrP^{res} is formed through translational modification of the cellular PrP increasing the β-sheet content of the cellular protein (Prusinger, 1997). The PrP^{res} is thought to propagate itself by catalyzing the conversion of cellular PrP into PrP^{res} (Horiuchi and Caughey, 1999).

Currently, it is thought that TSEs require infection with a PrP^{res} that has molecular compatibility with the PrP of the host. The PrP^{res} of CWD (PrP^{CWD}) is able to convert the cellular PrP of numerous species, and transmission is believed to be related to the relative homology of the two proteins (Raymond et al., 2000). It has been documented that genetic variation within the prion protein gene (*Prnp*) affects genetic susceptibility to TSEs within and between species (Westaway et al., 1987; Hunter, 2003; Vaccari et al., 2007). Polymorphisms within the *Prnp* of sheep (*Ovis aries*) has been linked to modified host susceptibility and disease progression of both scrapie, which is a TSE of sheep and goats, and bovine spongiform encephalopathy (Hunter, 2003; Vaccari et al., 2007). In elk, a single amino acid change within the PrP protein is associated with modified susceptibility to CWD (O'Rourke et al., 1999). Studies of white–tailed deer naturally infected with CWD have determined that several *Prnp* alleles are under-represented within the infected population (Johnson et al., 2003; O'Rourke et al., 2004; Blanchong et al., 2009). Polymorphisms at codons 95, 96, and 116 may modify the susceptibility of deer or affect the overall progression of the disease (Johnson et al., 2003; O'Rourke et al., 2004; Blanchong et al., 2009). Deer with a

[1]Submitted for publication 30 June 2011; accepted 1 December 2011.

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glutamine instead of a histidine at codon 95 and a glycine instead of a serine at codon 96 (G96S polymorphism) may have reduced susceptibility to CWD or modified disease progression (O'Rourke et al., 2004; Johnson et al., 2003; Blanchong et al., 2009). Naturally infected white-tailed deer with the G96S polymorphism have been reported, but accumulation of PrP^{CWD} in the obex was lower, indicating potentially slower progression of the disease (Johnson et al., 2006). Deer with the allele QGAS have a glutamine (Q) at codon 95, a glycine (G) at codon 96, an alanine (A) at codon 116, and a serine (S) at codon 138 are most often associated with CWD infection (O'Rourke et al., 2004; Johnson et al., 2006). Deer with the allele QSAS have a glutamine (Q) at codon 95, a serine(S) at codon 96, an alanine (A) at codon 116, and a serine (S) at codon 138 and the allele HGAS have a histidine (H) at codon 95, a glycine (G) at codon 96, an alanine (A) at codon 116, and a serine (S) at codon 138, and both of these alleles are under-represented within CWD infected deer and considered to have reduced susceptibility or slowed progression of the disease (Johnson et al., 2003; O'Rourke et al., 2004; Blanchong et al., 2009).

Chronic wasting disease is considered endemic in north central Colorado and southeastern Wyoming and has occurred in low prevalences in several other US states and Canadian provinces in captive and free-ranging herds (Spraker et al., 1997; Miller et al., 2000; Willams and Miller, 2002; Williams et al., 2002). Introductions of CWD have also occurred in multiple areas in the eastern United States including New York and West Virginia (Samuel and Richards, 2006). Since 1998, 3,054 deer have been tested and as of 2007, no CWD positive deer have been reported in New Jersey (Roscoe, 2007). The objective of this study was to determine the *Prnp* heterogeneity within New Jersey white-tailed deer and determine the geographic distribution of the observed genotypes. From these results, possible herd susceptibility to CWD can be evaluated based on previously identified susceptibility relationships (O'Rourke et al., 2004; Johnson et al., 2006).

MATERIALS AND METHODS

Hunter-harvested white-tailed deer from throughout the state of New Jersey were submitted to the Office of Fish and Wildlife Health and Forensics, New Jersey Division of Fish and Wildlife (NJDFW), from 2004 to 2006.

The obex and retropharyngeal lymph nodes were removed from the deer submitted to the NJDFW. Half of the obex and lymph nodes were fixed in 10% formalin and submitted to the University of Connecticut, TSE Laboratory for immunohistochemical testing for the presence of the CWD prion as previously described (Spraker et al., 1997). The remainder of the tissues were frozen at –80°C at the NJDFW Laboratory in the event that confirmation testing would be required.

Due to the long, undefined incubation period of CWD, deer could not be definitely determined to be CWD negative but only PrP^{CWD} free based on previously established criteria (O'Rourke et al., 2004). After the deer were determined to be PrP^{CWD} free, 150 samples representing broadly distributed regions of the state were selected from the samples maintained at the NJDFW laboratory (Figure 1). New Jersey is divided into 635 deer management units (DMUs), each approximately 14 square miles. Deer were sampled from 92 DMUs distributed across New Jersey. If available, an attempt was made to include one male and one female deer from each of the 92 DMUs represented in the survey, and of

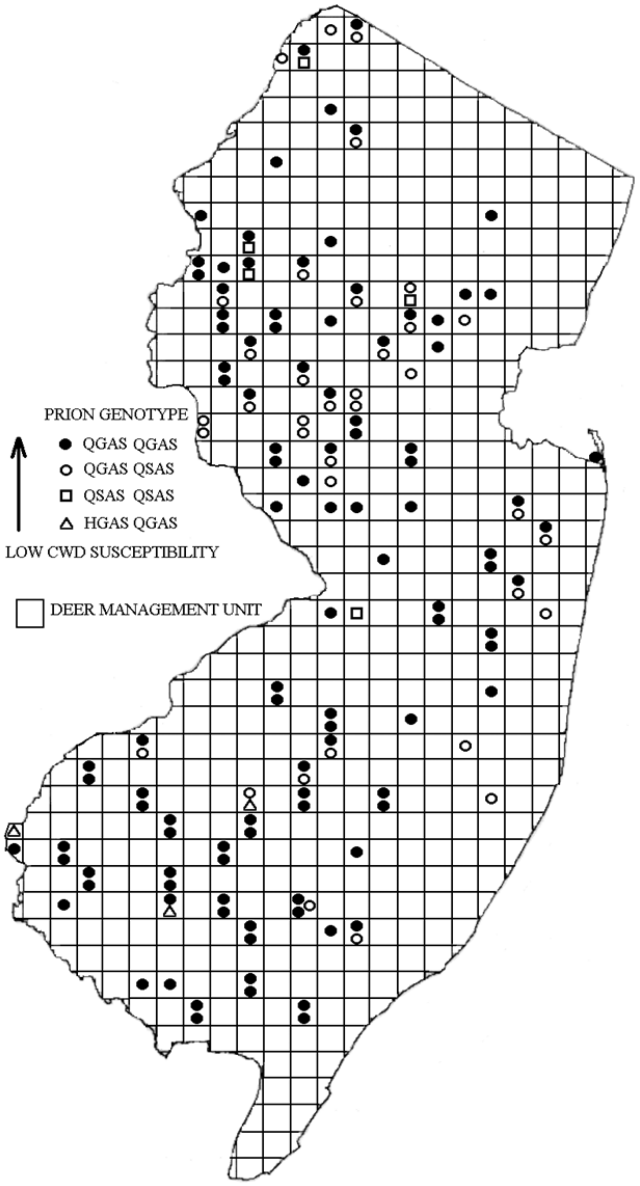

Figure 1. Distribution of the observed Prnp genotypes in the 150 deer surveyed within New Jersey. The Prnp alleles are designated by a four letter abbreviation representing the amino acids at codons 95, 96, 116 and 138 using the standard one letter abbreviations for amino acids (Q = glutamine, H = histidine, G = glycine, S = serine, A = alanine). The overall susceptibility to CWD infection is also designated based on the data of Johnson et al. (2003), O'Rourke et al. (2004), and Johnson et al. (2006).

the total 150 deer sampled, 95 were male and 55 were female. All deer were aged based on the tooth replacement and wear as defined by Severinghaus (1949); the age of the deer sampled in this study ranged from 1.5 years to 4.5 plus years with the average age being 2 years old.

To reduce the potential contamination that may have occurred during sampling, the outer portion of the tissues were removed and sterile procedures were used to section the frozen obex and lymph nodes. DNA was extracted from the samples using the MoBio Ultraclean Tissue DNA Isolation Kit (MoBio Laboratories Inc, Carlsbad, CA). Polymerase chain reaction (PCR) was performed using the primer pair 223 (5'-ACACCCTCTTTATTTGCAG-3') and 224 (5'-AGAAGATAATGAAAACAGGAAG-3'), which is specific for the functional PrP gene and produces an 830bp fragment (O'Rourke et al., 2004). PCR products were visualized using the Agilent 2100 Bioanalyzer and the Agilent DNA 1000 Chip and Reagents (Agilent Biotechnologies, Wilmington DE). PCR products were purified using ExoSap-It (USB Corporation, Cleveland, Ohio 44128) to remove unincorporated dNTPs and primers. The primer 223 was used to sequence an 830bp fragment with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Forest City, CA), on the AB 3130 Genetic Analyzer (Applied Biosystems, Forest City, CA) following the manufacturers' recommendations.

Gene Mapper v4.0 (Applied Biosystems, Forest City, CA) and BioEdit v7.0 (Hall, 1999) were used for sequence alignments and amino acid determinations. All sequences were evaluated for polypeptide polymorphisms at codons 95, 96, and 116, and any other sequence polymorphisms were noted. The amino acid at codon 138 was recorded to ensure the primers were accurately amplifying the functional *Prnp* gene instead of the pseudogene as described by O'Rourke et al. (2004).

RESULTS

No unique polymorphisms were identified within the sampled deer anywhere in the 830bp section analyzed for this study. Polymorphisms were observed at codons 95 with a histidine (cac) instead of a glutamine (caa) and at codon 96 with a serine (agt) instead of a glycine (ggt). Polymorphisms were not observed at codon 116 with all deer having the amino acid alanine (gca). All deer had the amino acid serine at codon 138, indicating proper amplification of the *Prnp* gene. Silent nucleotide polymorphisms were noted in several animals at codons 51 (cgc/cgt) and 146 (aac/aat). The QGAS allele was the most common *Prnp* allele within the New Jersey deer with a frequency of 0.83 (Table 1). Two other *Prnp* alleles were observed: QSAS had a frequency of 0.16 and HGAS had a frequency of 0.01 (Table 1). Most of the deer were homozygous for the QGAS allele (70%), with three other genotypes observed QGAS/QSAS (24%), QSAS/QSAS (3.3%), HGAS/QGAS (2.0%).

The QGAS allele was the most frequent allele within the New Jersey herd and homozygous individuals were distributed throughout the entire state (Figure 1). Within the New Jersey herd, QSAS was the second most common allele (0.16) with a majority of the individuals being heterozygous with the QGAS allele (Figure 1). The QGAS/QSAS individuals were also found throughout New Jersey, mirroring the distribution of the QGAS/QGAS individuals (Figure 1). Five QSAS homozygous individuals were identified and located primarily in the northern portion of the state, with one individual located in central New Jersey. Three individuals were heterozygous for the HGAS allele and all three were located in the southwest portion of the state (Figure 1).

DISCUSSION

Based on work in deer in Wisconsin, it has been estimated that 91–98% of white-tailed deer possess the *Prnp* alleles associated with greater genetic susceptibility to CWD (Johnson et al., 2003). Within the white-tailed deer evaluated for this project, 70% were in the most susceptible category, being homozygous for the QGAS allele. The other 30% had at least one copy of alleles associated with reduced susceptibility (QSAS or HGAS), and only 3.3% of the deer did not have any copies of the QGAS allele suggesting that at least 96.7% of New Jersey deer are genetically susceptible to CWD. The goal of this project was to establish the geographic distribution of the *Prnp* genotypes within New Jersey. Deer with the more susceptible allele occurred throughout the entire state. The small number and limited

Table 1: The prion protein (*Prnp*) genotypes of 150 white-tailed deer from New Jersey.
The *Prnp* alleles are designated by a four letter abbreviation representing the amino acids at codons 95, 96, 116 and 138 using the standard one letter abbreviations for amino acids (Q = glutamine, H = histidine, G = glycine, S = serine, A = alanine).

Prnp Genotype	CWD Susceptibility[a]	Males (n=95) No. (%)	Females (n=55) No. (%)	Overall (n=150) No. (%)
QGAS/QGAS	Increased	75 (78.9)	30 (54.5)	105 (70.0)
QGAS/QSAS	↑	16 (16.8)	20 (36.4)	36 (24.0)
QSAS/QSAS		3 (0.03)	3 (0.05)	6 (4.0)
HGAS/QGAS	Reduced	1 (0.01)	2 (0.04)	3 (2.0)

[a]Susceptibility to CWD infection is based on Johnson et al. (2003), O'Rourke et al. (2004), and Johnson et al. (2006).

geographic distribution of the less susceptible individuals (HGAS) identified in this project makes it unlikely that genetic susceptibility would impact the spread of CWD if the disease were to be introduced into the state.

Prion genotype surveys of white-tailed deer herds in Wisconsin and Nebraska that tested positive for CWD indicated that several of the *Prnp* genotypes had a reduced susceptibility or decreased progression of CWD (Johnson et al., 2003; O'Rourke et al., 2004). Several of the *Prnp* genotypes of white-tailed deer have been shown to offer reduced disease susceptibility or decreased disease progression such as those containing the alleles QGAS and HGAS, but as genotype surveys are performed within other regions of the country, new genotypes could be discovered that offer more significant protection. Although no new or unique genotypes were discovered within the white-tailed deer surveyed from New Jersey, some polymorphisms could occur and not have been detected because they occur at a very low frequency. The allele QGGS has been reported in other deer populations (O'Rourke et al., 2004), but none of the New Jersey deer surveyed had this allele. It is unknown if this allele does not exist within this population or if it occurs at a very low frequency and could not be detected based on our sample size. More intensive sampling may be required.

The genetic heterogeneity and distribution of the PrP gene of New Jersey white-tailed deer adds to our current understanding of the *Prnp* diversity within this species. Although this data may not have practical applications related to surveillance or CWD management, this information could serve as a baseline for future genetic studies in New Jersey, and be coupled with other genotype surveys across the country to contribute to our understanding of genetic susceptibility of white-tailed deer to CWD.

ACKNOWLEDGEMENTS

Partial funding for this survey was provided under the Pittman-Robertson Federal Aid to Wildlife Restoration, Fish and Wildlife Health Project. FW-69-R-10. We would like to thank G. Fromert for his contributions to this project and S. N. McGraw for review and editing of the manuscript.

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