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ANNOUNCEMENT FROM THE EDITOR i

EDITORIAL POLICY & FORMAT ii

VIEW POINT: MAKING YOUR RESEARCH COUNT – HOW TO INFLUENCE PUBLIC POLICY 195

GREG CZARNECKI

COMMENTARY: LACAWAC SANCTUARY AND FIELD STATION 197

LESLEY KNOLL AND CRAIG LUKATCH

CHANGES IN MACROINVERTEBRATE ASSEMBLAGES IN A PENNSYLVANIA TROUT STREAM OVER THIRTY-FOUR YEARS: WHERE HAVE ALL THE TRICHOPTERA GONE? 204

PATRICIA T. BRADT

RESEARCH NOTE: ANTIBIOTIC RESISTANT BACTERIA ISOLATED FROM WATER POOLS AT A FORMER DUMP SITE IN PENNSYLVANIA 216

DANVAS OMARE AND JANE E. HUFFMAN

THE EFFICACY OF CLEANING BIRD FEEDERS WITH 10 % BLEACH WIPES TO REDUCE BACTERIA 220

MAKAYLA L. BOYD, TODD J. UNDERWOOD, AND DANIEL F. ARUSCAVAGE

SURVEILLANCE OF AVIAN INFLUENZA IN WATERFOWL (FAMILY ANATIDAE) WITHIN ERIE AND MERCER COUNTIES, PENNSYLVANIA 227

AMY TEGELER, DYLAN DEGOL, AND JILL D. HENNING

RESEARCH NOTE: DEER BROWSING THREATENS A LOCALLY RARE PARASITIC PLANT, BUFFALO NUT (*PYRULARIA PUBERA* MICHX.) 231

CHRISTOPHER P. RANDLE AND JOHN W. WENZEL

ASSESSING MATING READINESS IN MALE MIGRATORY BATS FROM PENNSYLVANIA 235

JENNIFER L. WHITE AND JANINE UMBERGER

RESEARCH NOTE: MORPHOLOGICAL AND DNA ANALYSIS TO DETERMINE CANID SPECIES 247

NICOLE CHINNICI AND JANE E. HUFFMAN

Journal of the Pennsylvania Academy of Science 88(4): i-i, 2014

ANNOUNCEMENT FROM THE EDITOR

The business of scientific publishing is dynamic and is currently undergoing rapid change. The editorial team for JPAS will be working to improve the journal for readers, and authors. We will also be working to maximize the exposure and access to journal content in the new and rapidly evolving information marketplace. We will be diversifying the types of content in JPAS, including review papers, featured articles, commentaries, viewpoints, case studies and others. We encourage authors and readers to contact the editorial office with ideas for papers and special-issue topics and suggestions for strengthening the journal.

I will be stepping down as Editor-in-Chief of JPAS at the end of December 2014 and wish to thank everyone who has contributed to the success of the journal. My special thanks and appreciation go to the editorial committee members and the reviewers who gave freely of their time and offered useful and courteous comments in order to upgrade the caliber of articles published in the journal. I thank the members of the academy, the academy officers, and the authors for giving me the privilege of editing the journal. Thank you again for all your help over the last few years. I also wish to acknowledge the expert support of Larry Laubach who has meticulously reviewed and prepared the manuscripts for publication.

I am very pleased to announce that Dr. Carl Pratt will be appointed as editor. I know that the journal will continue to thrive in his expert hands. Correspondence relating to publications in the Journal should be addressed to:

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My very best wishes for the future and the New Year.
Jane E. Huffman, MS, PhD, MPH
Department of Biological Sciences
East Stroudsburg University

JOURNAL OF THE PENNSYLVANIA ACADEMY OF SCIENCE

PENNSYLVANIA ACADEMY OF SCIENCE
JOURNAL INFORMATION FOR AUTHORS
EDITORIAL POLICY AND FORMAT

The *Journal* of the Pennsylvania Academy of Science publishes original papers, research notes, commentary, editorials, view points, and review articles in the natural, physical, engineering, and social sciences. All papers must discuss the relevance of the data presented and a clear interpretation of its meaning in view of current knowledge of the discipline concerned. Helpful references for the author are: (1) Day, R.A. 1983. How to write a scientific paper. 2nd ed. ISI Press, Philadelphia, xv + 181 pp.; (2) O'Connor, M. and F.P. Woodford. 1976. Writing scientific papers in English, Elsevier, Amsterdam, vii + 108 pp.; (3) MacGregor, A.J. 1979. Graphics simplified; and (4) How to plan and prepare effective charts, graphs, illustrations, and other visual aids, University of Toronto Press, Toronto, 1-64 pp.

Authors are requested to examine recent issues of the Journal in order to conform to the general style of the journal. Papers are accepted for consideration at any time. Submitted manuscripts are accepted for review with the understanding that the same work has not been published, copyrighted or submitted for publication elsewhere and that all persons cited as a personal communication have consented to be cited. Additionally, submission of the manuscript is a representation that all the authors for the said manuscript and the institution where the research was carried out have approved its publication. Signed authorization will be required as appropriate. Authors are billed for page charges to partially defray the costs of publishing.

Submit names, email addresses, as well as the professional area of expertise of 4 possible reviewers who have agreed to review your manuscript. The reviewers must be outside the author's institution, possess knowledge of current research in the area of study, and generally be professionally qualified to referee the paper. The peer reviewing process is the Editor's responsibility, and the reviewers are selected at the discretion of the Editor.

All authors are requested to conform to the following:

1. General Format. All manuscripts should be typed, and double spaced, with 3 cm margins all around. *Do not use single spacing anywhere* (including Literature Cited). Images should be submitted as jpegs or tif and in the English language. Manuscripts should be organized as follows: (1) an unnumbered cover sheet with Title, Authors, their institutions and addresses, and name, address, and telephone number of the author to receive proof, (2) an unnumbered sheet with an Abstract, (3) Introduction, (4) Materials and Methods, (5) Results, (6) Discussion, (7) Acknowledgements, and (8) Literature Cited. All pages of the text, Introduction through Literature Cited, are to be numbered, and the names of authors should appear in the upper right-hand corner of each page. The text should begin in the middle of the first numbered page. Manuscripts need to be submitted in English.

2. *Headings.* All headings are in CAPITAL letters and centered.3. *Title.* Brief and to the point. It should inform the reader of the subject of the paper.

4. *Byline.* Include author's name, name of institution, department, address and zip code.

5. *Abstract*. A clear and concise paragraph which summarizes the research.

6. *Introduction.* The introduction should be concise and offer only that information necessary to orient the reader to the purpose and scope of the paper. It should state the reasons for the work and cite only published literature relevant to the subject.

7. *Materials and Methods.* Describe materials, methods, and equipment. Avoid repeating previously published details, unless modifications are extensive. The necessity of conciseness should not lead to omission of important experimental details necessary for others to repeat the work. When applicable, describe the experimental design and justify its use.

8. *Results and Discussion.* The Results section is a clear and concise account of the findings. Data should be presented in the most efficient manner, either in text, tables, or illustrations. All tables and illustrations must be referenced in the text. The Discussion section should extend or contradict current published information on the subject. Limit the discussion to the relevant subject and avoid speculation.

9. *Acknowledgements*. The source of any financial support received for the work being published must be indicated in the Acknowledgments section. The usual format is as follows: "This work was supported by Public Health Service grant CA-01234 from the National Cancer Institute."

Recognition of personal assistance should be given as a separate paragraph, as should any statements disclaiming endorsement or approval of the views reflected in the paper or of a product mentioned therein.

10. *Appendixes***.** Appendixes that contain additional material to aid the reader are permitted. Titles, authors, and reference sections that are distinct from those of the primary article are not allowed. If it is not feasible to list the author(s) of the appendix in the byline or the Acknowledgments section of the primary article, rewrite the appendix so that it can be considered for publication as an independent article, either full-length paper or Note style. Equations, tables, and figures should be labeled with the letter "A" preceding the numeral to distinguish them from those cited in the main body of the text.

11. *Literature Cited and Footnotes.* Except for the title and author reference at the beginning of the paper, and superscript notation in tables, do not use footnotes. Create separate Appendices or an Endnotes section if additional supplementary text material is required. Place Endnotes section just before the Literature Cited section. Number each endnote within the Endnote section using Arabic numbers in the order in which they are referred to in the

other sections of the manuscript. In other sections of the manuscript, place endnotes reference numbers in parentheses, and use the text style of type and not superscript. Place appendices after the Literature Cited section. Include a Literature Cited section: list references in alphabetical order by first author. Include only published references cited in the manuscript; unpublished work normally will be cited as personal communication (pers. comm.) in other sections of the manuscript, e.g., J.R. Halma (pers. comm.) or (J.R. Halma, pers. comm.). List all authors and full citation in the Literature Cited section. Use the most recent issue of the recognized abstracting authority to determine the correct abbreviations of periodical names (e.g., for biology use BIOSIS, Bioscience Information Service, Philadelphia, PA). If in doubt, do not abbreviate serial names. Use the following format and style for the Literature Cited section:

Journal- Monmonier, M. 1987. Title. J. Pa Acad. Sci. 62:73-77.

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Book (Complete work)-Snedecor, G. W. and W. G. Cochran. 1976. Statistical Methods. The Iowa State Univeristy Press. Ames, IA, xix + 593 pp.

For *Internet* citation, choose either MLA, APA, Chicago or another appropriate style, but stay consistent in the manuscript.

In all but the Literature Cited section, cite all works by author and year. For works with one or two authors, include names in each citation, e.g., (Smith and Reif 1984), or, if authors' names are used in the text- Smith and Reif (1984); for works by three or more authors, use et al. after the first author, e.g., (Gur et al. 1983), or, if the authors' names are used in the text- Gur et al. (1983). Research Notes with fewer than five references should be cited within the other sections of the manuscript thereby eliminating the need for a Literature Cited section. When references are cited within the text of other sections, include authors by last name only, and do not use et al. in the citation, e.g., for a journal article- (Genys, Harman and Fuller 1984, Proc. Pa. Acad. Sci. 58:67-69), or if authors are used in the text- Genys, Harman and Fuller (1984, Proc. Pa. Acad. Sci. 58:67-69); for a book-(Snedecor and Cochran, 1976, Statistical Methods, The Iowa State University Press, Ames, IA, 237-238), or, if authors are used in the text-Snedecor and Cochran (1976, The Iowa State University Press, Ames, IA, 237-238).

12. *Research Notes.* Papers submitted as short communications with an abstract are classified as Research Notes. Research Notes must contain the same basic quality of content and order of presentation as more substantial papers having content separated by section. Citations must follow the same format as articles.

13. *Case Reports.* While a full-length article or a Note may contain a case report section when the report is incidental to the rest of the paper, a specific Case Report format must be used when the report constitutes the entire article.

A Case Report must include an abstract of no more than 50 words. The text starts with presentation of the case under the section heading "Case Report"; there is no introductory text before the Case Report heading. After the case is presented, the rest of the text follows in a separate section after a ruled line to separate the sections. No separate head is used for this short discussion section, but paragraph lead-ins are permitted. The total number of tables and figures (combined) must not exceed 3.

14. *Editorials.* These are commissioned only articles. Original papers should not be submitted under this article type.

15. *Commentaries.* These are commissioned-only articles. Original papers should not be submitted under this article type. They are generally not peer-reviewed.

16. *View point.* These articles are academic papers which address an issue of current public or professional debate and/or approach an issue from a more personal perspective than standard academic writing.

17. *Tables and Illustrations.* Tables must have a title, be numbered, and typed on a separate page. Tables must be created using the tables tool in Microsoft Word or as text with tabs separating columns and paragraph returns separating rows. Computer generated images or scans should be saved as Tiff, EPS, or JPEG files. Resolution (at the dimensions to be published) should be at an absolute minimum of 300 dpi. Provide the legends for all illustrations in consecutive order on a separate page.

18. If animals are used in the research, the author(s) must state in the material and method section that the study was conducted in accordance with the guidelines laid down by the U.S. Office of Laboratory Animal Welfare (or individual country of origin) and that the research was approved by the Institutional Animal Care and Use Committee. All human subject research, and all other activities, which in part involve human subject research, must be reviewed and approved by the institutions IRB committee.

19. There are no page charges for publication in the journal, however one of the authors must be a member of the Pennsylvania Academy of Science. Galley proofs will be sent to the authors for checking; they must be returned to the Editor within a week after receipt.

20. *Editorial Policy.* Every paper is reviewed by the Editor and selected professional referees. Manuscripts will be returned or rejected if considered unsuitable for publication.

21. *Manuscripts and Correspondence*. Address all inquiries relating to publication in the *Journal* to the Editor: Dr. Carl Pratt, Immaculata University, Department of Biology, 1145 King Road, 210B Loyola Hall, Immaculata, PA 19345, **CPratt@immaculta.edu**

Journal of the Pennsylvania Academy of Science 88(4): 195-196, 2014

VIEW POINT: MAKING YOUR RESEARCH COUNT – HOW TO INFLUENCE PUBLIC POLICY[1]

GREG CZARNECKI

Executive Director, Wild Resource Conservation Program
Pennsylvania Department of Conservation and Natural Resources

As far back as I can remember, I wanted to be a scientist. It all started when my uncle gave me a book about dinosaurs. From the second I laid eyes on those Mesozoic swamps full of hulking reptiles and giant dragonflies, I knew I wanted to be a paleontologist. As the years went on I never gave up on my dream of becoming a scientist. I changed disciplines many times along the way eventually choosing wildlife biology.

Back then, in the 1960s, the space race was in the news every night and the technological discoveries that accompanied it mesmerized the public. It was a time when science was revered and being a scientist was a noble calling. It was an age when scientific discovery was heralded, admired, and eagerly accepted for the betterment of society.

Sadly, that same sense of wonder and respect is not as widespread today, not through any fault of the scientific community, but due mostly to the influence of special interest groups willing to discredit science to further their own agenda.

As I've moved through my career I've transitioned from doing research to using it for policy development, and along the way I've seen the right and wrong ways scientists try to influence the process. With that in mind, here are a few things we can do as scientists to bolster our credibility and help ensure that the important discoveries we make are used to formulate meaningful policy decisions.

Beware The Curse of Knowledge. Whether you are a new professional or a seasoned researcher, you are an expert in your field and have probably forgotten how little the rest of us know about your area of study. It's called the "curse of knowledge" and refers to the information imbalance between experts and the people they're trying to communicate with. It's a concept born out of the study of psychology and economics (Camerer, Colin; George Loewenstein & Mark Weber. "The curse of knowledge in economic settings: An experimental analysis," Journal of Political Economy 97 (1989): 1232–1254), but it applies to any scientific discipline and often leaves experts unable to communicate the basic essence of their research.

[1]Accepted for publication November 2014.

Greg Czarnecki

Policy makers need clearly articulated and interpreted data and results, and if you can't provide it, then your voice won't be heard. To avoid the "curse", think about your work from the perspective of your audience. Forget what you know, and try to explain your research and its relevance in a way that your mother would understand.

Policy is based on more than science. If we want policy makers to embrace science, we need to embrace the realities they face as well. We need to recognize that science is just one of the factors that are considered when developing public policy. Decisions need to take into account economic, social, and political factors as well. Just as policy decisions should not be based solely on economic benefit or job creation, scientific data should not be the sole determining factor either.

Extrapolate at your own risk. Stick to the facts. Extending a conclusion to a new set of circumstances is risky business if not accompanied by experimentation and analysis. On too many occasions I've seen competent scientists extend or interpret data too broadly, only to have it come back and bite them. It's also important to remember that there are no absolute truths in science, so avoid presenting it as

such. Instead, explain that good scientific research provides enough certainty to help formulate good policy. This is critical, because lack of certainty is one of the primary arguments special interest groups use to discredit science. Just look at the public debate over climate change to see how disingenuous, but effective, this tactic can be.

Be an advisor. If you provide data, expert opinion, or scientific advice that is sound, unbiased, and useful, policy makers will remember you and quite likely come to you again. If you're lucky, you may even be asked to serve in a more formal capacity, such as on an advisory committee. This is an invaluable way to make your research and your voice heard. These committees often include stakeholders with opposing philosophies and interests, thereby allowing you to be a voice of reason while at the same time gaining an appreciation for other points of view.

It's OK to be an advocate, but not a lobbyist. It's all too easy to cross the line from objective advisor to that of an activist lobbying for a particular course of action. Elizabeth Hadley of Stanford and Anthony Boranski of the University of California at Berkley characterize this difference as informative vs. descriptive advocacy (Hadley, Elizabeth. Boranski, Anthony. Beyond Science Communication: Informative versus prescriptive advocacy. ConsensusForAction blog. January 2014. http://consensusforaction.stanford.edu/blog/beyond-science-communicatio.html).

Informative advocacy involves listening to what policy makers need and providing the data, information, and expertise that fills that need. Descriptive advocacy, on the other hand, is endorsing a specific policy action. This approach narrows the options for policy makers, who need to evaluate all options and make the best choice for their constituents. We need to recognize that there are multiple paths to any desired condition, and your role as a scientist is to help define the consequences of each. It's not to dictate which should be taken.

Policy makers are continuously bombarded with information, which all too often comes from biased, uninformed sources. As scientists we're obligated to provide clear, objective information that will help them make the best decisions possible. Hopefully, by following these simple guidelines, we can integrate science into policy and help it regain the respect it deserves.

Journal of the Pennsylvania Academy of Science 88(4): 197-203, 2014

COMMENTARY: LACAWAC SANCTUARY AND FIELD STATION: A LONG-STANDING TRADITION OF RESEARCH, EDUCATION, AND PRESERVATION

LESLEY KNOLL[1] AND CRAIG LUKATCH[2]

[1]Director of Research and Education, Lacawac Sanctuary and Field Station
[2]President, Lacawac Sanctuary and Field Station

Lake Lacawac, a National Natural Landmark.

Lake Lacawac, a National Natural Landmark.

Just as Northeastern Pennsylvania residents and tourists enjoy the forests and lakes of the region, so do many students learning about environmental science and ecology. Lacawac Sanctuary and Field Station, a non-profit biological field station, nature preserve, and environmental education center located on the southwestern shores of Lake Wallenpaupack in the Poconos, has been educating and hosting students of all ages for decades. Lacawac was founded in 1966 with the mission of "Research, Education, and Preservation" and includes a well-protected glacial lake, Lake Lacawac as well as more than 500 acres of forest, hiking trails, and historic buildings. These features make Lacawac an ideal outdoor learning laboratory for educating students from kindergarten through graduate school.

For nearly 50 years, Lacawac has accomplished its mission by offering a diverse set of natural areas, facilities, and programs for K-12 and post-secondary educators and students, area residents, scientific researchers, and visitors to the region. By bringing scientists together with the general public, Lacawac provides an excellent opportunity for unique interactions.

[1]Accepted for publication December 2014.

Lake Lacawac is a National Natural Landmark and two locations on the property (natural bog and natural ledges area) are designated as Wild Plant Sanctuary's by the Pennsylvania Department of Conservation and Natural Resources. Lacawac Sanctuary allows public visitors and environmental program attendees to experience unaltered natural areas and to interact with scientists to learn about the natural world and conservation efforts.

Faculty and students from higher education institutions are working on many environmental issues of relevance to the region. Recent research projects at Lacawac have focused on how climate change impacts Pennsylvania lakes, how high deer populations are altering forest communities through their feeding activities, and whether ozone depletion can negatively affect aquatic organisms. Lacawac is also part of the ecologically and economically important Upper Delaware River watershed which delivers drinking water to more than 15 million people.

Research and Education Consortium

Lacawac Sanctuary, Miami University (Oxford Ohio), the Academy of Natural Sciences of Drexel University and Drexel University announced in the fall of 2013 an agreement to form an environmental research and education

consortium. The consortium builds on existing partnerships with leading universities and focuses on cutting-edge global climate-change and water-quality research. The consortium is comprised of higher education institutions, regional school districts, and affiliate partners (state lands, regional land trusts, non-profit conservation organizations).

The consortium currently includes the following colleges and universities: Miami University (Oxford, OH), Drexel University (Philadelphia, PA), The Academy of Natural Sciences of Drexel University (Philadelphia, PA), The University of Scranton (Scranton, PA), and Lackawanna College (Scranton, PA). Land trust members are numerous and offer more than 65,000 acres of lands and waters throughout Pennsylvania that can be accessed by consortium members for research and education purposes.

The Consortium collaborates with its members in conducting scientific research, creating education programs for K-16 audiences, and offering resources and support for early-career scientists, including graduate students and postdoctoral fellows.

Facilities

Lacawac is an ideal location for universities and colleges looking for a research site, a place to study the natural environment, and to bring a class for a field ecology trip. In the past five years, nearly 40 institutions of higher education have used Lacawac for education or research. Lodging for individuals or groups up to 27 people is available in the Historic Great Camp-an Adirondack style hunting lodge and vacation home and complex built in 1903 and listed on the National Register of Historic Places.

The Historic Lodge at Lacawac listed on the National Register of Historical Places.

Inside the Carriage House at Lacawac.

The dining room in the Lodge.

A new year-round, analytical laboratory, supported by funding from the National Science Foundation, was built in 2014. The new lab includes equipment such as a fume hood, water purification system, incubators, fluorometer (to measure algal abundance), UV Vis scanning spectrophotometer, drying oven, muffle furnace, analytical balance, and microscope with camera system. A seasonal lab and classroom are also available on the second level of the historic Carriage House which is in close proximity to Lake Lacawac.

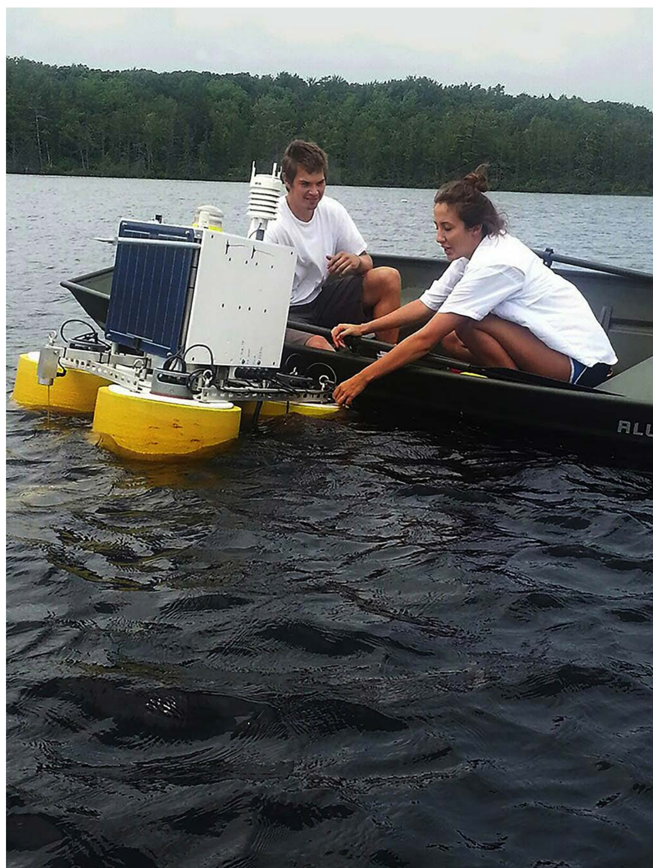
Lake Lacawac

Early lake scientists on Lake Lacawac. Clyde Goulden from the Academy of Natural Sciences of Philadelphia (left), a graduate student (center), and Alan Tessier from the University of Pennsylvania (right).

The ecological value of Lake Lacawac was recognized by scientists at the Academy of Natural Sciences in Philadelphia during visits in the 1950's. Because lake access is strictly controlled and the watershed has never been developed, the lake supports an unexploited fish community and a diverse native plant community, including several rare species. In 1968 the National Park Service designated Lake Lacawac as a National Natural Landmark. The lake is an ideal site to use as a control for comparisons with human-impacted lakes. Lake Lacawac is a site used by lake scientists because of its excellent water quality and long-term data record. Since the late 1960's, the lake has been the focus of many scientific papers, graduate theses, and undergraduate projects. Lake Lacawac is considered by many educators and scientists as a "living laboratory".

Under the direction of Lehigh University faculty in the 1980s, Lacawac became a base for many research and educational projects involving investigators and students from multiple colleges and universities. Funding from the Andrew W. Mellon and Geraldine R. Dodge Foundations was used to develop and support the Pocono Comparative Lakes Program (PCLP), an informal consortium of scientists from several institutions. The primary focus of the PCLP was a long-term sampling program on three local lakes (including Lake Lacawac) across a productivity gradient. Much of the sampling was conducted by undergraduates supported through the National Science Foundation's REU program. The lake data from these efforts and continued research projects are the core of the long-term dataset.

In 1992, a Lehigh University faculty member began a continuous electronic weather and lake monitoring program that has been expanded several times, continues today, and contributes data to many projects. Numerous Lacawac publications have resulted from work focusing on the impact of ultraviolet radiation (UV) on aquatic ecosystems. Lake mercury evasion research was conducted in the mid to late



Summer undergraduate interns on Lake Lacawac. Chris Cassel from Bloomsburg University (left) and Anne Morgan from Miami University (right).

Lacawac's environmental laboratory.

2000's at Lacawac. Many advances in dissolved organic matter quality and optical metrics have come from research supported by Lacawac Sanctuary. Many Lacawac users have been advancing the concept of using lakes as sentinels of climate change. Lakes are at the lowest position in the landscape and thus provide chemical, biological and physical signals of change including those from the surrounding landscape.

Research and Hub for EONs

Lacawac Sanctuary strives to enhance its national reputation as a field station that facilitates meaningful discovery in the natural sciences and serves as a training ground for field research. Monitoring and conservation-focused research will contribute to a fuller understanding of our ecosystems and environmental change through published works, conferences and other means. Our goals for the upcoming years includes attracting new and established scientists and fostering a stronger field station culture; increasing resources for the research program; enhancing Lacawac's reputation as an institution that helps to launch the careers of new scientists; integrating field station research with educational programs; and organizing more scientific workshops.

Lacawac is a leader in the emerging frontier of large-scale, long-term, ecological observatory networks (EONs). In 2012, the lake became part of the Global Lake Ecological Observatory Network (GLEON, www.gleon.org), a global network of lakes and scientists. GLEON scientists address large-scale questions about lakes using their global network. Lacawac promotes and facilitates high quality training, research, and interdisciplinary interaction in the development and application of EON science. The field station provides a physical facility for EON-related work and runs training workshops to train the next generation of scientists on these emerging approaches. Lacawac's philosophy is to provide hands-on research and educational opportunities for scientists of all ages.

Lacawac has also been used extensively as a testing ground for new environmental sensors and sensor platforms. Lake Lacawac is the testing site for a prototype of a small, portable profiling buoy equipped with a suite of physical, chemical, and biological sensors that will enable small lakes to be networked to understand regional to continental scale influences of climate change.

Forest

Lacawac has supported a long-term field experiment on the impacts of deer browsing on forest understories. A pair of 0.7 hectare deer exclosures in two different forest types were created in 1994. Exclosure maintenance has been maintained over the past 20 years by local volunteers and undergraduates. Long-term data show differences in plant abundance and diversity inside vs outside the exclosures. In 2011, two additional exclosures were erected (0.8 hectare). The exclosures were placed in areas damaged by high winds and *Adelges tsugae* (hemlock woolly adelgids) with high tree mortality and little regeneration due to heavy deer browsing. One of the new exclosures serves as a control site. The other exclosure is experimental and uses approaches to restore old-growth forest characteristics.

Conferences and workshops

Each year Lacawac hosts numerous scientific conferences and workshops such at the annual fall Lacawac Ecology Conference and Lacawac Ecological Observation Workshop. The Lacawac Ecology Conference began in 2012 and is held annually the end of September. The conference is designed

Lacawac's environmental laboratory.

Entrance to Lacawac Sanctuary and Field Station.

to bring together scientists from across the region. LEC gives scientists at all levels from undergraduate to PhD-level an opportunity to discuss research ideas in an informal setting and to build new collaborations in the Northeast region. The conference is open to faculty and students, and past conferences have been attended by participants from nearly 20 different institutions.

The Lacawac Ecological Observatory Workshop brings together experts on buoy technologies, advanced ecological sensors, continental scale ecology approaches, and management/analyses of large ecological datasets to train students and early career faculty on these cutting-edge approaches in ecology. First held in 2012, LEOW has welcomed a diverse group of participants from many countries and states across the US. Many leaders in the EONs (ecological observatory networks) have presented at LEOW representing many different EONs including NEON (National Ecological Observatory Network) and GLEON (Global Lake Ecological Observatory Network).

Participants at the 2013 Lacawac Ecological Observatory Workshop.

Educational Outreach

Lacawac's YES Program complements and enhances students' classroom curriculum year round mainly in a four county geographical area: Lackawanna, Luzerne, Pike and Wayne. These programs meet Pennsylvania content standards while they encourage youth to develop and maintain an awareness, competence and enthusiasm to understand important environmental concerns impacting our local watersheds, bodies of water, and forests.

Lacawac offers various opportunities for schools and school districts:

- Pathways in Ecological Research (PiER): Pathways in Ecological Research is a program for high school juniors to explore science as a career track in emerging areas such as green technologies, ecology, and environmental studies. It aims to prepare students for the rigors of college and increase their chances for admission to top colleges and universities. Students will participate in field trips, learn research methods, conduct and present a research project of their own, and attend a week long summer residency camp.
- Women in Science Project (WiSP): The mission of the Women in Science Project is to attract and retain more women in science fields while fostering their future success. The project components include: Student Role Models (High School), Women in Science Career Camp (High School), Women and Science Conference and Women in Science Luncheon Series.
- Field Trips and Mobile Lacawac: Students participate in an in-depth investigation of the natural sciences at Lacawac's field station and research center. Through these interactive classroom experiences, students will develop important scientific skills, from making observations to designing experiments and interpreting data. Lacawac's structured environmental and natural sciences lessons for either small or large groups will also come to the classroom, school auditorium, or other school venue such as an afterschool program.
- Summer Conservation and Leadership Camp: Lacawac offers an engaging and immersive experience for students ages 13-15 in a week-long summer program where they will gain first-hand experience with conservation issues and field and laboratory techniques in the environmental sciences.

Lesley Knoll, Director of Research and Education, teaching about lake zooplankton.

Kristine Wasco a summer intern from Bloomsburg University sampling Lake Lacawac.

Jennie Brentrup, a PhD student from Miami University, deploying aquatic sensors on Lake Lacawac.

Designed to provide a link between curriculum and the real world, Lacawac's outreach to K-12 schools and districts emphasizes a "watershed approach" to appreciating, achieving and sustaining environmental improvements in a hands-on fashion intended to interest and excite students. Today, many schools have limited training and resources to fully develop and explore local water-related and forestry/land issues. Lacawac's education programs are designed to fill this void by providing students, and their teachers, with a critical understanding of local water and forestry/land issues and related environmental and ecological concerns. Lacawac's education programs provide children with a basic knowledge of the water cycle, watershed management, water chemistry, aquatic life, pollution biology, forest growth and regeneration, native plant life, forest life and environmental science that should be part of every student's experience so that they become informed voters, community leaders and policy makers.

Public Education

Lacawac Sanctuary and Field Station also educates the public about our influence on natural ecosystems and the interrelationships with the environment. It does so by offering programs year-round to the general public, teachers and students, and youth and civic groups. Lacawac Sanctuary offers community programs designed to connect people of all ages to the natural world. Lacawac offers seasonal classes related to art, photography, animals, plants, and habitats.

Lacawac's recreational and public outreach programs are designed to increase general knowledge and love of nature; promote healthy outdoor activities and continue the close relationship between the sanctuary and the local community by providing access to our land and allowing enjoyment of the natural beauty of Lacawac. Our goals are to encourage a more meaningful use of Lacawac through scheduling guided and interpretive walks and other activities throughout the year; increase the capacity of staff and volunteers to maintain trails; and make the recreational programs fiscally self-sustaining.

Our vision is for every Field Station visitor to connect with our landscapes, biological diversity, and cultural history. Through their investigation, we hope visitors will develop a "sense of place" that fosters lasting stewardship of the region and of the environment in general. To accomplish this, we provide opportunities for students and visitors to immerse themselves in the area through experiential learning, research, and the practice of conservation.

Graduate students learning about aquatic sensors at Lake Lacawac.

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CHANGES IN MACROINVERTEBRATE ASSEMBLAGES IN A PENNSYLVANIA TROUT STREAM OVER THIRTY-FOUR YEARS: WHERE HAVE ALL THE TRICHOPTERA GONE?[1]

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ABSTRACT

Benthic macroinvertebrate (BMI) assemblages in Bushkill Creek, Northampton County, PA, USA were sampled at the same site in a similar manner from 1972 to 1977 (months 1- 61) and from 1994 to 2006 (months 274 - 419). The creek supports a naturally reproducing brown trout (*Salmo trutta*) population. From 1972 to 1996 this assemblage demonstrated stability and resilience in recovering from documented physical disturbances (high water, low temperatures). However, when more recent data (1997 – 2006) are included, the following significant (ANOVA, p<0.10) decreases occurred (1972 to 2006): 1) biomass (expressed as wet weight in mg per replicate); 2) Trichoptera abundance, especially Hydropsychidae (*Hydropsyche* spp. and *Cheumatopsyche* spp.); 3) abundance of filter/collectors; 4) abundance of Ephemeropteran *Stenonema* spp. (Heptageniidae); 5) abundance of all Ephemeroptera, Plecoptera and Trichoptera (EPT); and 6) abundance of the Dipteran *Antocha* spp. (Tipulidae). Among Ephemeroptera no changes were detected in Ephemerellidae or Baetidae abundances. Biodiversity as taxa richness (total, Trichoptera, Ephemeroptera, non-Insecta) and EPT index did not register significant changes over the study period. Chironomidae became the most abundant family in the 2000's (40% of total numbers) while Hydropsychidae were most abundant in the 1970's (24.9% of total numbers). Changes in assemblage composition over this sampling period, suggest that the structure of this assemblage is changing, especially among Trichoptera, as land use changes, residential and commercial development, more frequent bankfull floods, colder than normal temperatures and unknown additional stresses increase within this drainage basin. [J PA Acad Sci 88(4): 204-215, 2014]

INTRODUCTION

Lotic biodiversity is imperiled with regional aquatic resources declining across North America specifically (Wishart and Davies 2002; Strayer 2004) and worldwide generally (Dudgeon *et al.* 2006). Running waters are the most impacted ecosystems on the planet because lotic systems are in intimate contact with the drainage basin so land use changes impact these waters directly (Malmqvist and Rundle 2002). The extinction rate for North American freshwater fauna is four percent per decade, a rate similar to tropical forests (Ricciardi and Rasmussen 1999).

Freshwater ecologists are interested not only in biological diversity, but also in the stability of lotic benthic macroinvertebrate (BMI) assemblages (Neubert and Caswell 1997). Temperate BMI assemblages are well adapted to predictable physical disturbances, but changes may occur when disturbances exceed the predictable range (Reice 1994). Long-term biological data are necessary to evaluate assemblage stability (Likens and Lambert 1998; Hooper *et al.* 2005; Jackson and Fureder 2006). However, most assemblages studies (>80%) encompass less than three years (McElravy *et al.* 1989) with only a few studies exceeding five years (e. g. Ward 1975; Johnson *et al.* 1994; Grubaugh and Wallace 1995; Vinson 2001; Milner et. al. 2008).

Previous studies of the Bushkill Creek (Bradt *et al.* 1999) over twenty-five years indicated that BMI assemblages in 1972-'73, 1976-'77 and 1994 -'96 demonstrated both stability (tendency of community composition to remain constant; Pielou 1974) and resilience (ability of system to return to some previous state following perturbation; Harrison 1979). Assemblages were sampled at the same site, with similar methods and compared using various metrics. Following a moderately severe winter, recovery time for BMI total abundance and wet weight (biomass) was 2-3 and 4-5 months, respectively (Bradt and Wieland 1981). After another colder than average 1994 winter followed by flooding, recovery of BMI assemblages to 1970's composition and abundance took twenty-seven months (Bradt *et al.* 1999). However, wet weight (biomass as mg), Trichoptera abundance and one Trichopteran genus, *Psychomyia* (Psychomyiidae) did not regain 1970's numbers by 1996. Over this twenty five year study overall assemblage composition appeared relatively stable and resilient in spite of various physical disturbances

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(lower than normal temperatures, high water). The 1996 assemblage closely resembled that of 1972 (Bradt *et al.* 1999).

Over the past thirty-four years (1972-2006), the drainage basin upstream (173 km^2) of the sampling site has been subjected to many changes including:

1. increases in human population both numbers (+11,039) and density (+181/m^2), (USDC 1972; 1992; 2002, LVPC 2006)

2. 29% increase in residential units (USDC 1972; 1992; 2002, LVPC 2006)

3. various industrial discharges including 85,300 pounds of hydrochloric acid aerosols from nearby cement plants (PennEnvironment 2009)

4. five colder than normal (normal = +2 °C) winters (1977 4.18 °C below normal; 1994 4.68 °C below; 2003 3.3 °C below; 2004 2.5 °C below normal; 2005 2.5 °C below normal; NCDC 2010)

5. withdrawal of groundwater (up to 57 MGD/day) by a limestone quarry 5 km upstream of sampling site (water is returned to the stream often accompanied by fine sediments); average daily inflow from quarry to stream is 2.2 m^3/s (USGS 2007b)

6. three bankfull floods within twenty months (2003-2005). The September 18, 2004 flood was the largest recorded in the past thirty three years and was followed 6.5 months later by the third largest flood in the past thirty years (Table 1) (USGS 2007a)

The upper drainage basin, upstream of the sampling site, has experienced changing land use as agricultural lands are converted to residential development with attendant losses of riparian vegetation (Hanover Engineering Associates 2009). Upstream of the sampling site approximately 6.5% of the area had impervious cover in 1990 (Lehigh Valley Planning Commission, Allentown, PA. personal communication 1999).

Given these increasing human impacts, the objective of this study was to assess whether this BMI assemblage continues to exhibit previously recorded stability and resilience (Bradt *et al.* 1999) in spite of increasing human-induced and natural disturbances within the drainage basin.

STUDY AREA

The Bushkill Creek (Figure 1) has a drainage area of 207 km^2 and overall length of 34.4 km (USCOE 1972). It originates from springs on the southern slope (altitude 216 m) of the Blue Mountain in Northampton County, northeastern PA, USA and flows southeasterly over Martinsburg shale to its confluence with its major tributary, Little Bushkill Creek. Land use in this section includes agriculture, forests and accelerating residential development.

After its confluence with Little Bushkill Creek, the creek flows over various limestone and dolomite formations to its confluence with the Delaware River at Easton (Figure 1), 47 m above mean sea level (USCOE 1972). Numerous limestone springs enter the stream in this lower basin and stream pH (7.2-8.9), total alkalinity (40-167 mg/L $CaCO_3$) and specific conductance (170-875 µmhos/cm) increase

Table 1. Bankfull Floods (Peak Flow >14.4 m^3/s) During Sampling Period Monacacy Creek USGS Gauging Station # 01452500 (USGS 2007a) 1972-'73, '76-'77, '94-'98, '00-'06.

Date	Peak Flow m^3/s	Sampling months after peaks flows (cfs)	BMI sampling dates after peak flows
6/23/1972	15.5 (548)	-	3/73, 4/73, 5/73(2)
6/29/1973	15.0 (528)	1, 11, 12,13	No samples
1/26/1976	60.9 (2150)	No samples	No samples
2/25/1977	28.6 (1010)	59,60, 61,274	3/77, 4/77, 5/77, 4/94
7/28/1994	19.9 (702)	No samples	No samples
3/8/1995	14.5 (512)	286	4/95
1/19/1996	22.6 (799)	297, 310	3/96, 4/97
7/24/1997	28.9 (1020)	322	4/98
1/18/1999	36.0 (1270)	No samples	No samples
12/7/2000	16.7 (589)	358,382	4/01, 4/03
9/15/2003	20.8 (735)	No samples	No samples
9/18/2004	155 (5470)	No samples	No samples
4/3/2005	37.7 (1330)	406, 419	4/22/05, 5/06

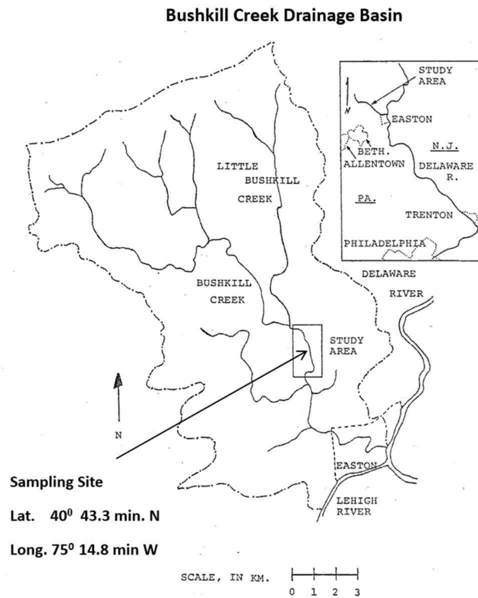

Figure 1. Bushkill Creek drainage basin, location of sampling site.

(Bradt 1974; Bradt and Wieland 1978). Even though limestone springs exert a chemical impact on water chemistry and flow at the sampling station, the stream does not meet all the requirements for a "Limestone Stream" designation according to Pennsylvania Department of Environmental Protection (Botts 2009).

The sampling site is 2.0 km downstream from the confluence with Little Bushkill Creek and 10 km upstream from the confluence with the Delaware River (Figure 1). At this site the stream is fifth order, 19 m wide with an average depth of 20 cm (Bradt and Wieland 1978). Substrate is primarily gravel and rocks (5-20 cm). Land use near the sampling site includes diminishing agriculture and growing residential and commercial development accompanied by increasing impervious cover (Browne 2005).

The Pennsylvania Fish and Boat Commission (PFBC 1997-2007) designated this creek "High-Quality-Cold Water fishery", supporting a naturally reproducing brown trout (*Salmo trutta*) population. Naturally reproducing brook trout (*Salvelinus fontinalis*) have recently been documented in the upper part of the drainage basin 11 km upstream of sampling site (Leonhardt 2008). The upper reaches of a tributary (16 km upstream of sampling site) have been designated "Exceptional Value" by the Pennsylvania Department of Environmental Protection (Hanover Engineering Associates 2009).

MATERIALS AND METHODS

BMI assemblages were sampled at the same site in a similar manner in spring, summer and fall. However, March, April and May contained the most samples so only these data are used in the statistical analyses. Table 2 indicates times of spring sampling. Two to four replicates were taken at randomly chosen sites within riffle areas with similar substratum at similar depths (Wetzel and Likens 1991; Mackie 2001). Riffle habitats were chosen because they demonstrate the strongest relationship to environmental variables (Roy *et al.* 2003).

From 1972-1977 a Surber sampler (0.093 m^2, mesh 500 μm) was used (Surber 1937; USEPA 1973) and from 1994-2006 a Hess bottom sampler (0.086 m^2, mesh 500 μm) (Hess 1941). The Hess sampler was chosen in 1994 over the Surber sampler because its design limits the escape of organisms and contamination from drift (USEPA 1996). For comparable results and statistical analyses, Hess sampler numbers were multiplied by 1.08 to account for slight differences in area sampled. Sampling methods were kept as similar as possible to have comparable results over the extended study period.

Samples were preserved in 75% ethanol in the field and invertebrates sorted from debris in the laboratory using a US Standard No. 30 mesh sieve (0.6 mm) (USEPA 1973). BMI identifications were made to the lowest possible taxon using the following keys: Pennak (1953; 1978; Smith 2001), Wiggins (1977; 1996), Peckarsky *et al.* (1990) and Merritt and Cummins (1984; 1996). All identifications were verified by the author. A reference collection is maintained at Muhlenberg College. Biomass was measured as wet weight (Needham and Needham 1962) and Ephemeroptera, Plecoptera, Trichoptera (EPT) abundance and index calculated (Barbour *et al.* 1999). Pollution tolerant ratings for BMI were developed by Hilsenhoff (1988), Barbour *et al.* (1999) and Chalfant (2007). Ratings 0-5 are considered the most sensitive, with 0 the most sensitive. Ratings of six and above are labeled "pollution tolerant". Percent intolerant BMI were calculated using Hilsenhoff tolerance values (Chalfant 2007; Barbour *et al.* 1999).

Spring (March, April, May) data were analyzed using SPSS 15.0 for Windows (2006) for ANOVA and linear regressions and Microsoft Excel (2003) for graphing. Time on the *x*-axis is represented by the number of months since the start of sampling (Months 1 – 419). Slope and significance values were obtained from SPSS analyses. Conditions for running linear regressions were met prior to analysis.

Community similarity indices (Bray and Curtis 1957; Ludwig and Reynolds 1988) for April replicates were calculated. These indices compare assemblages by designation of a fraction between 0.0 (no similarity) and 1.0 (completely similar). Means of three replicates were used in calculation of these indices for Aprils ('95, '97, '98, '01, 03, 05) and compared to April 1973.

Flood data (Table 1) were obtained from peak flow data

Table 2. Sampling Times and Number of Replicates (0.093m2)[a] Per Sampling Spring Date.

Year	‘72	‘73	‘77	‘94	‘95	‘96	‘97	‘98	‘01	‘03	‘05	‘06
Number replicates (total = 48)	4	8	9	3	3	3	3	3	3	3	3	3

[a] corrected for differences in area sampled

from a contiguous drainage basin flow gauge at the mouth of chemically similar Monocacy Creek (latitude 40038’28”, longitude 75022’47”; USGS Gauging Station 01452500, USGS 2007a). Based on discharge measurements during Hurricane Agnes (June 1972), Monocacy Creek appears less prone to flooding than Bushkill Creek (Bradt *et al.* 1999), so using discharge data from Monocacy Creek may underestimate Bushkill Creek flooding.

RESULTS

Table 3 shows water chemistry data at or within 2.5 km of the sampling site over the study period. Chemical sampling may not have been regular enough to detect any trends. Every effort was made to keep chemical sampling methods consistent. However, different personnel performed the tests over the thirty-four years, often with different reagents, making consistency difficult. Therefore, no statistical analyses were performed on water chemistry data.

Over 29,000 BMI were collected, counted and identified, but data analyses are here limited to March, April and May (11,900 BMI) because those months contained the most samples. Table 4 indicates 1) statistically significant (ANOVA $p<0.07$) changes in BMI and 2) linear regression equations and r^2 for BMI groups recording changes.

There were no detectable changes in total abundance. An abundance peak (744 = mean of three replicates) occurred in April 2003 (month 382), apparently caused by 599 small Chironomidae (Diptera) larvae. This peak was not accompanied by a wet weight increase because average wet weight per individual was the lowest recorded (0.33 mg) during this study.

Decreases in biomass as wet weight (mg) over the sampling period are indicated in Figure 2, suggesting less availability of food for the next trophic level. In sampling months 290 -350 peaks occurred in wet weight, but these peaks were not accompanied by peaks in total abundance, indicating larger bodied insects (e.g. later instars of Ephemeroptera and Trichoptera) in the samples.

The decreases in Trichoptera abundance (Figure3), including Hydropsychidae (70.1% of Trichoptera) (Figure 4), suggest an important change in assemblage composition. Among the Hydropsychidae both *Hydropsyche* spp. (pollution tolerance = 4: Barbour *et al.* 1999 =5; Chalfant 2007) and *Cheumatopsyche* spp. (pollution tolerance = 5; Barbour *et al.* 1999; = 6; Chalfant 2007), decreased over the study period (Table 4). Chalfant (2007) reported that the percentage of Trichoptera decreased with increasing

Table 3. Water chemistry at or near sampling site (within 2.5 km). Range and number of Samples (n).

Date	Temp. °C	pH	Total Alkalinity mg/L $CaCO_3$	Specific Conductance umhos/cm	NO_3 as mg/L NO_3	PO_4 mg/L as P	n	Data Source
1972-1978		6.3 – 8.5	43-139	180-875	2.6-24.2	<0.01 – 0.25	62	Bradt 1974, Bradt & Wieland 1978
1994-2007	2.9-25.0	6.5-9.3	34-340	211-510	0.08- 35	<0.01-0.50	85	Kney and Brandes 2004[a], Retired Senior Volunteer Program 2007[b] (RSVP), Bradt 2008[c]

[a]Kney, A. D. and D. Brandes. 2004. Lafayette College, Easton, PA. Unpubl. data. Site: 1.3 km upstream of sampling site.

[b]Retired Senior Volunteer Program . 2007. Bushkill Creek (Northampton County, PA). Unpublished water quality data 2005-2006. Site: 2.4 km downstream of sampling site.

[c]Bradt, P. 2008. Water quality data. Muhlenberg. College, Allentown, PA unpublished. Site: BMI sampling site.

Table 4. Benthic Macroinvertebrate Assemblages - Bushkill Creek 1972-2006. ANOVA and Regression.

Variable	ANOVA F	Significance p	df	r^2	Regression Equation
Ephemeroptera, Plecoptera, Trichoptera abundance	3.46	0.069	47	0.07	Y=-0.158x+156.2
Total Trichoptera abundance	9.78	0.003	47	0.181	Y=-0.124x+80.57
Filters/Collectors abundance	10.63	0.002	47	0.188	Y=-0.134x+80.06
Hydropsychidae family abundance	14.74	0.000	47	0.243	Y=-0.136x+69.1
% Hydropsychidae/total Trichoptera	13.19	0.001	46	0.223	Y=-0.001x+0.885
Hydropsyche spp.	9.73	0.003	47	0.18	Y=-0.10x+55.4
Cheumatopsyche spp.	6.2	0.018	36	0.15	Y=-0.028x+13.96
Antocha spp.	6.33	0.016	43	0.131	Y=-0.04x+25.75
Stenonema spp.	9.51	0.005	27	0.276	Y=-0.006x+2.82

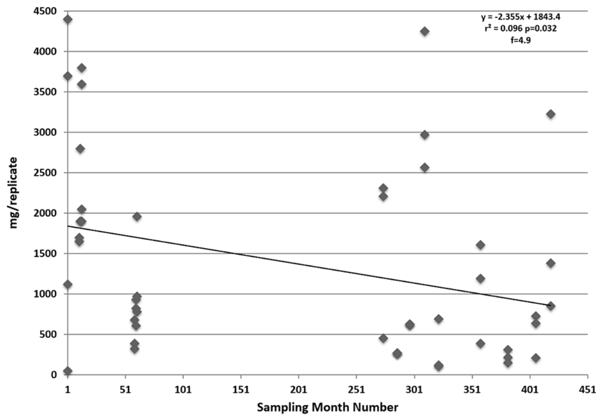

Figure 2. Total Wet Weight (mg) per replicate Bushkill Creek 1972–2006.

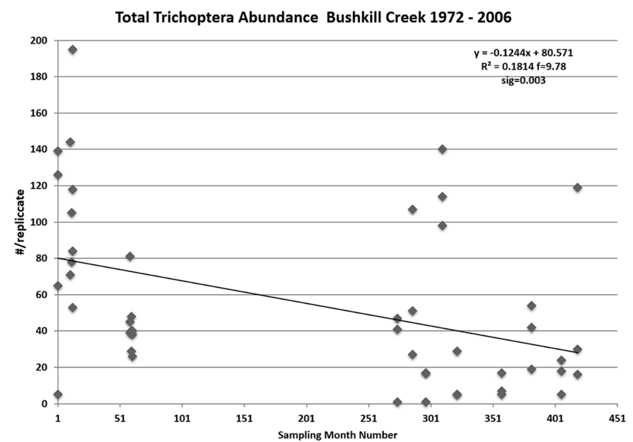

Figure 3. Total Trichoptera abundance per replicate Bushkill Creek 1972– 2006.

anthropogenic stress in Pennsylvania, a trend also detected in this study (Table 4).

Reflecting the Trichoptera decreases were decreases in EPT abundance (Figure 5) and in filter/collectors (Table 4), the Hydropsychidae functional feeding group. No measurable differences were detected in other functional feeding group (predators, scrapers, gathers/collectors, shredders). Only twenty Plecoptera were collected during this study, making it difficult to draw conclusions about their populations. Chironomidae (pollution tolerance = 6; Chalfant 2007), became the most abundant family in the 2000's (40.0% of abundance) (Figure 6), replacing Trichoptera (28.6% of abundance in 1970's) and signifying additional changes in assemblages.

Among Ephemeroptera, occasionally collected *Stenonema* spp. (pollution tolerance =3; Chalfant 2007) recorded small decreases, suggesting other assemblage changes (Table 4). However, there were no significant changes in total abundances of Ephemeroptera, or families Ephemerellidae and Baetidae.

Among Diptera *Antocha* spp. (Tipulidae) (Table 4) abundance also decreased over the study period. It is labeled 3 on the pollution tolerant scale (Chalfant (2007).

Taxa richness of Trichoptera, Ephemeroptera, Diptera and non-Insecta indicated no significant changes; neither did total taxa richness or the EPT index.

Figure 7 compares the April 1973 mean (three replicates)

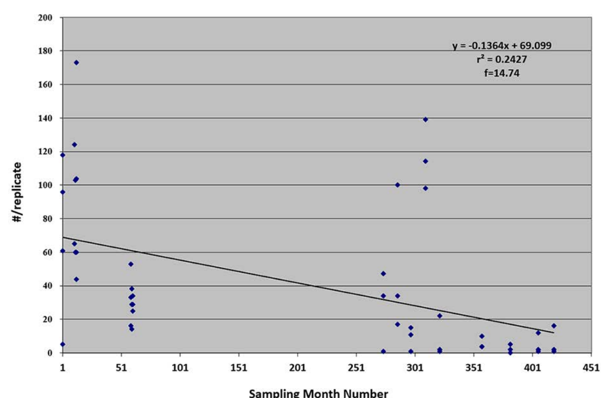

Figure 4. Total Hydropsychidae abundance per replicate Bushkill Creek 1972 – 2006.

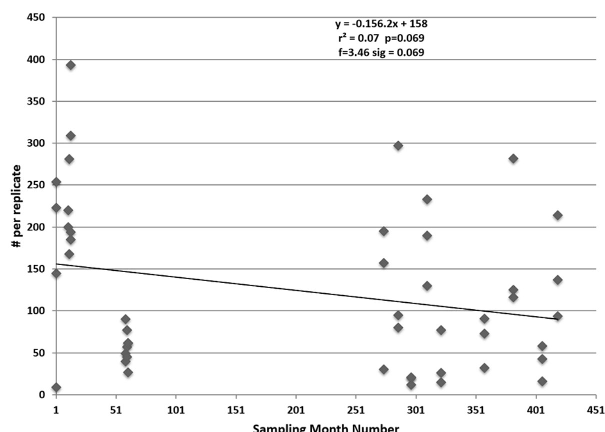

Figure 5. Ephemeroptera, Plecoptera and Trichoptera abundance per replicate Bushkill Creek 1972 – 2006.

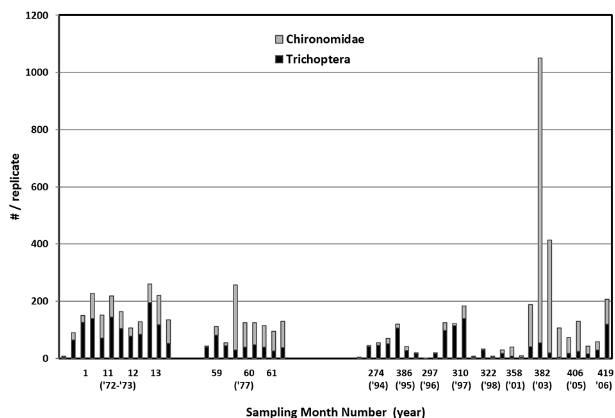

Figure 6. Diptera and Trichoptera abundance per replicate Bushkill Creek 1972 – 2006.

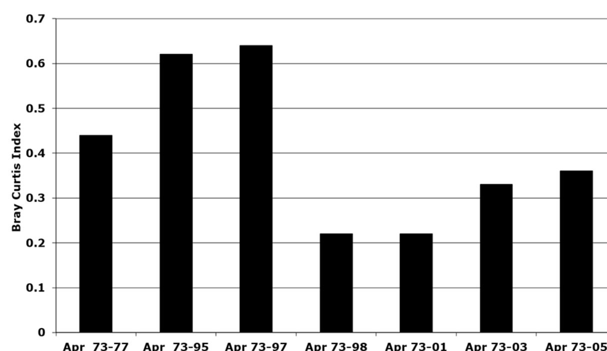

Figure 7. Bray-Curtis April Similarity Indices - 1973 Index (mean of 3 replicates) compared to 1977, 1995, 1997, 1998, 2001, 2003, 2005.

community comparison index (Bray and Curtis 1957) with indices of subsequent Aprils. The 1998 and 2001 communities appear to be the least similar to the 1973 community.

In order to determine if there was a difference in sampling methods, ANOVA's and linear regressions were performed on data from 1972-1977 (Surber sampler) and on data from 1994-2006 (Hess sampler). Decreases in *Hydropsyche* sp. were significant ($p<0.05$) from 1972–'77 and also from 1994-'06. Also, from 1972-'77 concomitant significant ($p<0.05$) decreases detected were Trichoptera abundance, EPT abundance, total abundance, wet weight (biomass) and percent intolerant BMI. The 1972-77 statistical changes detected may reflect that there were more samples (n=21) collected in a shorter amount of time during that sampling period than in the subsequent period (1994-'06; n=27).

DISCUSSION

Trichoptera

Loss of riparian cover often accompanies development in a drainage basin (Wheeler *et al.* 2005). As riparian vegetation was reduced in New England, invertebrate and fish assemblages declined (Coles *et al.* 2004; Coles *et al.* 2010). As riparian forests are destroyed and landscape is fragmented during urbanization, dispersion corridors for adult aquatic Insecta, especially Trichoptera, may be reduced or eliminated (Petersen *et al.* 1999; Urban *et al.* 2006). Certain taxa may fail to disperse across urbanizing regions, thereby contributing to assemblage changes (Petersen *et al.* 2004). Trichoptera have the same evolutionary history as terrestrial vegetation (Ross 1963) so the loss of riparian vegetation associated with land development in this drainage basin may contribute to their decrease.

Additional reasons for the decreases in Trichoptera in general and Hydropsychidae (Figures 3 and 4, Table 4) in particular are not readily apparent. This family is considered pollution intolerant by some authors (#4 Barbour *et al.* 1999, #5 Chalfant 2007), but pollution tolerant by Bellucci *et al.* (2011) and Pirvu and Pacioglu (2012). Barbour *et al.* (1999) propose that the percentage of Hydropsychidae to total Trichoptera increases with organic pollution, but in this study this percentage decreased (Table 4), suggesting that organic pollution may not be a factor in the decrease. Identification to species might solve some of these discrepancies. There may be multiple interactions contributing to this observation. Net-spinning caddisflies (in this dataset >80 percent *Hydropsyche* spp.) are resistant to flood magnitude (Hynes 1970) but sensitive to flood frequency (Robinson and Minshall 1986). Therefore numbers may have been reduced in the 1990's by a cold winter with scouring anchor and/or frazil ice (Hynes 1970) and subsequent high water. Numbers may have remained low in the 2000's and recovery delayed due to frequent floods (Table 1) and lower than normal temperatures.

During the 1970's, *Hydropsyche* spp. numbers recovered approximately two months after a colder than normal winter (Bradt and Wieland 1981). However, *Hydropsyche* spp. abundance took twenty-seven months to recover from the cold 1994 winter and subsequent flooding, emphasizing not only the disturbances' severity, but also lack of adaptations to such severity. Following the 2000's floods, Hydropsychidae had not recovered to 1970's numbers by spring 2006 (Figure 4). Trichoptera may be more easily dislodged during increased discharge (McElravy *et al.* 1989) and may take one to three years to recover after flooding (Swanson *et al.* 1998). Hydropsychidae are important ecosystem components because they not only facilitate nutrient recycling from water column to benthos, but also stabilize the substrate and moderate flood impacts (Cardinal and Palmer 2002; Cardinal *et al.* 2004). So, such a decrease in this previously numerous family may alter ecosystem functioning (Vaughn 2010) and signify impending ecological changes. Hooper *et al.* (2005) propose that biodiversity losses include declines in common species, as here observed among Hydropsychidae. Loss of any species imperils ecological resilience and increases ecosystem vulnerability to collapse (Peterson *et al.* 1998).

Ephemeroptera

The reasons for the decreases in occasionally collected *Stenonema* (Heptageniidae) (Table 4) are not apparent and are not reflected in either Ephemeroptera total abundance or taxa richness. Ephemerellidae (48.6% of Ephemeroptera) are considered pollution intolerant (tolerance = 2; Chalfant 2007), opportunistic and flood resistant (Hynes 1970; McShaffrey and McCafferty 1990). However their abundance did not change over the study period. This family, even though reported as intolerant of pollution (Bellucci *et al.* 2011), may be disturbance-adapted and therefore able to outcompete other BMI following certain disturbances (Hynes 1970). Their success could be attributed to either generally depressed numbers of other BMI, opportunistic characteristics and/or flood resistance.

As observed in this study, EPT abundance decreases with increasing human impact (Blattenberger *et al.* 2000). In this study Trichoptera indicated the most significant decrease. Song *et al.* (2008) report EPT abundance also decreased with sedimentation, but no objective measurements of sedimentation were available for this stream.

Diptera

Antocha spp. decreased over the study period (Table 4). However, Bellucci *et al.* (2011) found that *Antocha* increased as impermeable surfaces increased in Connecticut.

Chironomidae became numerically dominant (40% of abundance) in 2000's while Trichoptera (24.9% of abundance) were dominant numerically in 1970's. In Hawaii, Diptera dominated urban and mixed land-use sites while Trichoptera dominated forested sites (Brasher *et al.* 2004).

General Discussion

No statistical changes were detected in total taxa richness. Taxonomic changes may be better early detectors of ecosystem stress that various diversity indices, e.g. taxa richness (Shindler 1987).

Bray-Curtis Indices indicated that BMI assemblages in the 1970's and early 1990's were more similar to the April 1973 samples than to those after 1997 (Figure 7), reinforcing the conclusion that this assemblage has changed in the past thirty-four years.

Ecological disturbance is an event that disrupts ecosystem, community structures and/or resources (Pickett and White 1985). Such disturbances may shift species numbers and community structure, as observed in this study, and such changes may have disproportionately large impacts on stream food-web dynamics (Covich *et al.* 1999; Vaughn 2010).

Flood frequency may inflict multiple effects on BMI assemblages. During this study period fourteen bankfull floods occurred in the region, one per year until September 2004 when three large floods occurred within thirty months (Table 1). The highest flow on record (64.1 m^3/sec) for this creek was recorded in September 2004, 1.3 km above sampling site (Brandes, D., Lafayette College, Easton, PA unpublished data). The third largest flood occurred six and a half months later, April 3, 2005 (Table 1). This frequency of floods in the 2000's was not previously recorded during the study period, and was, therefore, outside the predictable

range (Resh *et al.* 1988) and may have contributed to observed 2005 and 2006 BMI assemblage changes, especially among Hydropsychidae (Figure 4; *Hydropsyche* spp. and Table 4 *Cheumatopsyche* spp.) and the Chironomidae (Figure 6). Due to their short life cycles, Chironomidae may dominate following floods (Hynes 1970) as observed in this study.

Both the surrounding land use changes and human activities within this drainage basin may affect a stream's ecological integrity, degrading streams by multiple pathways with aquatic assemblages responding in nonlinear fashion (Wang *et al.* 2001; Allan 2004). Such multiple and interacting disturbances make it difficult to match the assemblage changes to the responsible stressor(s) (Allan 2004). In early stages of urbanization, biological changes, as here observed, may be easier to detect than the physical and/or chemical changes that cause them (Wang and Lyons 2003).

CONCLUSIONS

This BMI assemblage exhibited stability and resilience from 1972 to 1996 despite colder than normal temperatures and high water. However, from 1997 to 2006 this assemblage was changing, observed among the Hydropsychidae especially, as land use changes, human population increases, more frequent floods, colder than normal temperatures and possible unidentified factors impact this assemblage.

The 2000's assemblages were not as similar as those of the 1970's and early 1990's (Figure 7). Lower than normal temperatures and the frequent, severe floods of 2004-2005 may have prolonged or prevented recovery of certain taxa to 1970's assemblage composition and abundance. There had been limited recovery by spring 2006. Hydropsychidae appear to be the most impacted family while reportedly less tolerant Ephemerellidae registered no changes. The more tolerant Chironomidae also did not register any statistically significant changes in either numbers or percentages over the study. Assemblages not commonly exposed to these particular conditions may not be adapted to such extreme physical disturbances, outside the predictable range (Reice 1994).

Additional stressors on this assemblage that may delay recovery include continued land use changes with increasing impervious cover, loss of dispersion corridors and riparian vegetation, human development and population density increases, and irregular flows due to quarry pumping. Trichoptera, also, may be impacted by feeding on pollen and/or debris blown from neighboring farms growing genetically engineered corn containing *Bacillus thuringiensis*, "Bt" toxins (Rosi-Marshall *et al.* 2006).

Bt corn, containing Cry1Ab protein, was introduced in the mid 1990's and by 2006 35 to 38% of corn acres in US were planted with Bt corn (USDA 2014). Between 2000 and 2002 an average of 149.3 km2 (15.4 % of Northampton county) were planted with corn (NASS 2014), but data on Bt corn planted are unavailable. The area upstream of the sampling site is 172.8 km^2 and approximately 56 percent of this area is agricultural (LVPC 2006). Therefore we assume that at least 3.8 km^2 of Bt corn were planted upstream of the sampling site. So pollen and/or debris from Bt corn could enter the stream via wind or runoff. Carstens *et al.* (2012) conclude that current research on the impact of Bt corn pollen and debris on aquatic invertebrates is inconclusive. Therefore, the impact of Bt products on Hydropsychidae requires additional research.

A combination of disturbances and stressors may have reduced biomass (as wet weight) (Figure 2) and Trichoptera (Figure 3), resulting in long term changes in assemblage structure, compared to the 1970's. Alterations in assemblage composition, as here observed, are a sensitive measurement of ecologically relevant change (Elmqvist *et al.* 2003) and may foreshadow ecosystem functional changes (Vaughn 2010).

Disturbance, a key factor in determining community structure, may have little impact on taxa richness, but may shift dominant taxa (Reice1994), as here observed. Coles *et al.* (2010) propose that increases in disturbances are associated with increasing urbanization as observed within this drainage basin. By 2006 Chironomidae were numerically dominant while Hydropsychidae were dominant in the 1970's and the 1990's (Figure 6), reinforcing assemblage changes.

Most studies on the effect of disturbances on BMI concentrate on flooding (Wooten *et al.* 1996; Swanson *et al.* 1998). We propose that not only may frequent flooding affect BMI assemblages, but low temperatures, changing land use and increasing human populations may also contribute to assemblage differences. Wheeler *et al.* (2005) state that the greatest threat to stream biota and habitat is the urbanizing stage within a drainage basin and that both biota and habitat are extremely sensitive to low levels of urbanization. A series of perturbations will have greater ecological impact than isolated events (Vogl 1980) and all these factors may be influencing the BMI assemblage changes here documented.

This BMI assemblage does not continue to exhibit stability and resilience, especially among Trichoptera. The changes in numerically dominant families and genera observed during this study reinforce that assemblage structure is changing.

Although we have made some progress in understanding BMI assemblages in this stream, several questions still require answers. Are continuing and increasing disturbances overwhelming the stability of this stream's Trichopteran assemblages? Will this BMI assemblage return to the 1970's structure or do these documented changes signify an impending regime shift (Biggs *et al.* 2009) signaling future BMI changes in this valued brown trout stream? Only continuing studies will be able to answer these questions. This study is ongoing to not only continue the addition of data to this long-term database, but also attempt to answer questions identified by this study.

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RESEARCH NOTE: ANTIBIOTIC RESISTANT BACTERIA ISOLATED FROM WATER POOLS AT A FORMER DUMP SITE IN PENNSYLVANIA[1]

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ABSTRACT

A bacterial survey of three water pools at different locations on a former furnace slag and casting sand dump site in East Stroudsburg, PA was conducted. Water nitrates, pH, water temperature, biochemical and chemical oxygen demand, and metals were also measured to characterize the physical and chemical features of these pools. *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella ozeanae*, *Serratia marcescens*, *Micrococcus luteus* and *Micrococcus roseus* were isolated. The reservoirs of antibiotic resistance (ROAR) for oxytetracycline, chloramphenicol, streptomycin and penicillin were assessed for the organisms isolated in the study. All isolates were resistant to penicillin, and intermediate resistance to oxytetracycline and streptomycin was found. None of the isolates were resistant to chloramphenicol. There was no difference in antibiotic resistance in the

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INTRODUCTION

Due to their unique adaptive features, bacteria and other microorganisms occupy every known habitat (Mandell, 2000). Their communities are the most complex, diverse, and important assemblages in the biosphere (Zhou et al, 2004). Many microorganisms are known to degrade toxic compounds in the soil and some have been successfully used for bioremediation. However, in soils contaminated with metals and organic pollutants, remediation has proved to be difficult because of the mixed nature of contaminants (Roane *et al*., 2001). Organisms that can degrade organic compounds are usually inhibited by toxic metals. To be successful, metal degradation must first occur before organic compounds are degraded even if the organism involved in this process is the same. Much of the information concerning the influences of heavy metals on microorganisms and the processes they mediate, is fragmentary and scattered over a wide range of scientific literature (Duxbury, 1985).

Several studies have found that metals influence microorganisms by adversely affecting their growth, morphology, and biochemical activities, resulting in decreased biomass and diversity (Baath, 1989; Dean-Ross and Mills, 1989; Hughes and Poole, 1989). Despite these toxic stresses, numerous microorganisms have evolved metal resistance and detoxification mechanisms, which include volatilization, extra cellular precipitation and exclusion, intracellular sequestration, and membrane-associated metal pumps (Hughes and Poole, 1989).

Resistance to antibiotics in bacteria is not uncommon in the environment because bacteria with intrinsic resistance to antibiotics are found in nature. It is the occurrence of bacteria that have become resistant to multiple antibiotics in recent years that has become problematic to public health officials worldwide. The widespread use of antibiotics to treat bacterial infections as well as their increased use in farming has resulted in a greater increase in the emergence of resistant bacterial strains. A study conducted by Isett and Huffman (2001) revealed how agricultural land-use practices can influence bacterial diversity and resistance to antibiotics not only in a stream ecosystem, but in many other environments as well (Meyer and Huffman 2001).

The emergence of bacteria in hospitals such as methicillin-resistant *Staphylococcus aureus* that defy even the most powerful regimen of antibiotics is disturbing. Some of these microbes are entering the environment through raw sewage discharge into receiving water and therefore horizontally transferring the resistant traits to other bacterial populations. Studies on antibiotic resistance indicate that the frequency of antibiotic resistance is significantly elevated in heavy metal-contaminated environment including sediments, benthic fish and organic foam (McArthur and Tuckfield 2000). As resistance to antibiotics spreads, the effectiveness of most antibiotics will be reduced and may become ineffective to treat infectious diseases.

Environmental contamination by heavy metals affects microbial communities. The number of single and multiple heavy metal resistant bacteria may be an indictor of the level of contamination. Heavy metals may inhibit the diversity of bacteria. This paper details the isolation and identification

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of microorganisms isolated from water samples obtained from a former dump site for furnace slag and casting sand. The isolated bacteria were assessed for reservoirs of antibiotic resistance (ROAR). The effect of water chemistry pH, temperature, biological oxygen demand and metals on the temporal and spatial distribution of the bacteria at the sampled sites was evaluated.

MATERIALS AND METHODS

Study Area

Located in East Stroudsburg, PA (N40 59'53.9'' W75 09'55.5'' elevation 62 feet), the study area was formerly used as dump site for furnace slag and casting sand in the 1950s. The area is topographically depressed and runoff from the surrounding elevated areas forms temporary pools. An environmental assessment conducted by Barr Isett and Associates Inc. in 2006, determined that there is no spring at the site and any water available is in standing ponds. Three locations with stagnant pools of water were randomly selected for sampling in May, 2007.

Sample Collection and Analysis

Two samples (250 ml) from each location were aseptically collected in T500 sterile bottles (Fisher Scientific) about five inches below the surface of the water. Water temperature at the time of sampling was measured and recorded using a digital thermometer (Taylor Model 9841). The pH was determined within one hour of sampling using the Fisher XL15 pH meter (Model X11-16298, Fisher Scientific). Water nitrate content, biochemical oxygen demand (BOD), and chemical oxygen demand (COD) analyses were performed on the samples as indicated in the Standards Methods for the examination of Water and Wastewater (1998). A scan for metals was conducted using inductively coupled plasma-atomic emission spectrometry (Thermo Electron, Madison WI) (Courtesy of Garden State labs Inc. Hillside NJ).

For bacterial analysis 50 ml of sample from each location was filtered through Millipore 0.45 micron cellulose disposable filters (Millipore Co., Bedford MA,01730) using a vacuum filtration unit. For Gram-negative bacteria, the filters were placed on MacConkey, and Thiosulfate-Citrate-Bile Salts (TCBS) agar, and incubated for 24 hours at 37 ºC. Subcultures were made on Tryptic Soy Agar (TSA) to obtain pure cultures. To isolate for *Salmonella* and *Shigella*, *Salmonella-Shigella* Agar was used while Columbia Nalidixic Acid (CNA) agar was used to isolate Gram- positive bacteria.

Bacteria Identification and Antibiotic Sensitivity Testing

To identify the Gram-negative bacteria, the Analytical Profile index (API 20E) strips for Biochemical Identification (biomerieux, Vitek, Inc.) were used. Isolated colonies were placed in 5 ml of sterilized distilled water and 2 ml was pipetted onto the API 20E wells as per the manufacture's instructions.

The bacterial isolates were screened for reservoirs of antibiotic resistance using the Kirby Bauer method. Bacterial isolates were tested on Mueller-Hinton agar by use of impregnated discs, oxytetracycline (30 µg), streptomycin (10 µg), penicillin (10 units) and chloramphenicol (30 µg) (Becton Dickson, Cockeysville, MD) on a bacterial seeded lawn.

RESULTS

Both Gram-negative and Gram-positive bacteria were isolated from all three locations at the site. No vibrios, *Salmonella* sp. or *Shigella* sp. were isolated at the sites sampled. The water temperature at the three sampling sites ranged between 20 to 20.8 °C. The water pH ranged from 7.8 to 8.3. The nitrate level was below 0.2mg/L in all three locations. Biochemical oxygen demand (BOD) ranged from 30 mg/L to 79 mg/L while chemical oxygen demand (COD) ranged from 23 mg/L to 56 mg/L. A metal scan of water samples collected at the three sites is summarized in Table 1. The highest concentrations of all metals were found in water from site 3.

Escherichia coli, *Serratia marcescens*, *Klebsiella pneumoniae* were the only Gram-negative bacteria isolated from all three sites sampled. *Klebsiella ozeanae* was isolated at site 2. The Gram-positive bacteria isolated were *Micrococcus luteus* and *M. roseus*. *M. luteus* was isolated from sites 1 and 3, and *M. roseus* from site 3. With the exception of *E. coli*, the colony forming units for each bacterial species isolated were few in all three locations. All isolates were resistant to penicillin but were susceptible to chloramphenicol. *S. marcescens* and *K. pneumoniae* isolates showed intermediate resistance to streptomycin and oxytetracycline. The *E. coli* isolates were resistant to oxytetracycline but not streptomycin.

DISCUSSION

Blast furnace slag is a nonmetallic coproduct produced in industrial processes. It consists primarily of silicates, aluminosilicates, and calcium-aluminum silicates. The furnace slag at the study site was deposited in the 1950's. The typical composition of furnace slag from the 1950's included

Table 1. Table 1. Summary of metals found in samples from the three locations at a former dump site in East Stroudsburg, PA.

Metal	Site#1 (ppb)	Site#2 (ppb)	Site#3 (ppb)
Aluminum	954	922	1366
Boron	15.7	11.8	21.5
Barium	134	146	238
Calcium	25414	23222	36295
Cadmium	<4	<4	4.99
Chromium	5.79	<4	7.66
Copper	64.5	82	125
Iron	23398	18249	68470
Magnesium	696	587	936
Manganese	235	28.4	362
Nickel	48	30.7	73.1
Lead	62.4	61.3	152
Titanium	15.2	13.1	22.7
Vanadium	8.95	11.9	19.8
Zinc	1082	1584	1696

calcium oxide (41%), silicon dioxide (36%), aluminum oxide (13%), magnesium oxide (7%), iron oxide or iron dioxide (0.5%), manganese oxide (0.8%), and sulfur (1.6%). Blast furnace slag is alkaline and exhibits a pH range of 8 to 10 (Short 1959). The alkaline property of furnace slag may have contributed to the pH range of 7.3 to 8.3 in the water samples at the sites.

Biochemical oxygen demand is the amount of dissolved oxygen needed by aerobic biological organisms in water to break down organic material present in water sample at certain temperature over a specific time period. BOD is an effective indicator of organic quality of water and wastewater treatment plants (Clair *et al*., 2003). In this study biochemical oxygen demand (BOD) ranged from 30 mg/L to 79 mg/L. A value greater than 10 mg/L indicates very poor water quality, with large amounts of organic material in the water (Streamkeeper's Field Guide: Watershed Inventory and Stream Monitoring Methods, 1991).

Chemical oxygen demand is a valuable water quality parameter. It is expressed as mg/L which indicates the mass of oxygen consumed per liter. The value of COD in this study was found in the range of 23 mg/L to 56 mg/L which is much higher than the maximum permissible limits as prescribed by World Health Organization standards (1993). The elevated COD in this study is an indicator of pollution in the samples tested.

Heavy metals generally exert an inhibitory action on microorganisms by blocking essential functional groups, displacing essential metal ions and modifying the active conformations of biological molecules (Hassen *et al.* 1998). Only five bacterial species were isolated in the sampled locations. The water was stagnant and evidence of decaying material was present which might support greater microbial diversity. One explanation for the lack of bacterial species could be the significantly high concentration of heavy metals which can inhibit bacteria.

Some metals such as aluminum, cadmium, lead, selenium, vanadium and zinc are microcidal and depending on their concentration, they can be dangerously toxic to plants and animals as their margin of safety is narrow. In fact the span between the minimum and maximum tolerable levels of some of these metals is often a matter of parts per million or even less and their contamination can present real health concerns to living organisms (Antonioli *et al.* 2007). The elevated concentrations in the sampled locations in the present study could have had a limiting effect on microbial activity and therefore led to decreased diversity.

The bacteria isolated from the sites exhibited resistance to the heavy metals that were present. Filali *et al.* (2000) reported that *K. pneumoniae* exhibits high minimal inhibitory concentrations for heavy metals such as cadmium or mercury. Rajbanshi (2008) reported on chromium resistant *Staphylococcus* spp, *E. coli*, and *Klebsiella* spp. Twelve isolates of *E. coli* were isolated from wastewater of El-Malah canal located in Assiut, Egypt and were checked for their heavy metal tolerance (Abskharon *et al.* 2008). These isolates were resistant to Cu^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cr^{6+}, Cr^{3+}, Cd^{2+} and Pb^{2+}. The results from the current demonstrate that the species isolated may be good candidates for remediation of some heavy metals and aromatic compounds in heavily polluted sites.

All isolates in this study showed complete resistance to penicillin. This is due to the presence of abundant beta-lactamase, an enzyme that breaks down the beta-lactam ring in penicillin rendering it ineffective against bacteria. In some species, resistance is due to their impermeable membrane and the efficient efflux of proteins that effectively pump the antibiotics out of the bacterial cells. Others have intrinsic abilities to resist antibiotics.

Although the results of this study may have limitations as many bacterial species are not easily cultured, the diversity of microorganisms was low at the sites sampled. The study also demonstrated that the bacteria isolated from temporary pools at the former dump site were resistant to antibiotics.

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THE EFFICACY OF CLEANING BIRD FEEDERS WITH 10 % BLEACH WIPES TO REDUCE BACTERIA[1]

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ABSTRACT

Many authorities recommend cleaning bird feeders regularly to prevent the transmission of disease at feeders, but the effectiveness of cleaning methods has not been studied. We tested the effectiveness of cleaning bird feeders with 10 % bleach wipes to reduce bacteria immediately after cleaning and over ten weeks when cleaning feeders every two weeks. Aerobic bacteria were significantly reduced on feeders immediately following cleaning with bleach wipes. Over ten weeks, aerobic bacteria on feeders were significantly reduced by cleaning with bleach wipes, but aerobic bacteria increased over time. There also was a significant interaction between cleaning and time on aerobic bacterial counts, which suggests that bleach wipes were effective in reducing aerobic bacteria in the first weeks of the study but became less effective over the long term. We suspect the loss of effectiveness was due to a buildup of organic matter on feeders over time. We found no significant relationship between animal activity (as indicated by seed consumption) and aerobic bacteria on cleaned and non-cleaned feeders, which indicates that aerobic bacteria were influenced by multiple environmental sources. By contrast, neither cleaning nor time significantly influenced Gram-negative bacteria on feeders. The lack of a significant influence on Gram-negative bacteria may have been due to the high variability in the numbers of Gram-negative bacteria on feeders and in the environment. However, we found that animal activity was significantly related to Gram-negative bacteria at non-cleaned feeders but not at cleaned feeders. The lack of a relationship at cleaned feeders suggests that cleaning with bleach wipes helped reduce Gram-negative bacteria on very active feeders. Overall, our results suggest that bleach wipes may be a simple and useful sanitization method for bird feeders if organic matter can be removed first using another cleaning technique. [J PA Acad Sci 88(4): 220-226, 2014]

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INTRODUCTION

Feeding wild birds is a popular activity throughout the western world (Jones and Reynolds 2008) and has become an important part of the economy. For example, in 2011, 50 million people maintained bird feeders in the United States and spent $4 billion on food alone (U.S Fish and Wildlife Service 2012). Individuals that feed birds gain pleasure from watching birds in their yards (Jones and Reynolds 2008) and wild birds benefit from this supplemental food source as well. Bird feeders provide food for birds when natural food sources are limited and can increase winter survival rates (Brittingham and Temple 1988, 1992). More recently, supplemental feeding has been shown to provide birds with more energy during the breeding season, which leads to increased reproductive success (Robb *et al.* 2008). Despite these benefits, most people do not realize that bird feeders may act as intermediates in the transfer of diseases (Luttrell 1997; Hess and Groskin 2006; Jones and Reynolds 2008). Bird feeders are very active locations where birds of many species are concentrated and have the potential to spread disease (Brittingham and Temple 1986).

A number of avian diseases may be transmitted among birds at feeders (Luttrell and Mead 2005). For example, major outbreaks of salmonellosis and mycoplasmosis in songbirds have been linked to feeders (Hartup *et al.* 1998; Tizard 2004). Because *Salmonella* is shed in feces, bird feeders contaminated with feces are a likely site of transmission to other birds (Friend and Franson 1999; Daoust and Prescott 2007). Although mycoplasmosis appears to be primarily spread through direct contact between infected birds (Friend and Franson 1999; Luttrell and Mead 2005), infected birds are known to linger at bird feeders where other birds are concentrated thereby increasing the likelihood of transmission (Fischer *et al.* 1997; Hartup *et al.* 1998). More recently, Dhondt *et al.* (2007) confirmed that transmission of mycoplasmosis can also occur via the physical surface of bird feeders. These major disease risks have to led some debate over whether or not the public should feed wild birds (Hess and Groskin 2006; Prescott 2002; Schreiber 2010).

To keep bird feeders clean and potentially decrease the spread of disease, many authorities recommend routine cleaning of feeders (Brittingham and Temple 1986;

Luttrell 1997; Friend and Franson 1999). These cleaning recommendations are disseminated to the public through popular bird watching organizations. For example, the Audubon Society recommends soaking bird feeders in 10 % bleach at least once or twice a month (Audubon Society 2004). Similarly, the Cornell Lab of Ornithology recommends cleaning feeders with hot soapy water every two weeks or with 10 % bleach if sick birds have been observed nearby (Cornell University 2011). Both organizations recommend removing seed hull debris on the ground below feeders as well. However, recommended cleaning intervals and methods vary and, more importantly, they are not based on any published studies of feeder hygiene. Clearly, research is needed to determine whether various cleaning methods are effective at reducing the risk of disease transmission.

In this study, our objective was to determine the effectiveness of a cleaning method and frequency at reducing bacterial populations on bird feeders over time in winter. We tested a modified version of the Audubon Society's recommended cleaning method of using 10 % bleach to clean feeders every two weeks. Instead of soaking feeders in bleach, we cleaned feeders with commercially available sanitizing wipes that contain a 10 % bleach solution. Wipes were used because these products are much easier to use than immersing a feeder in a large volume of bleach solution and may be a more feasible option for use on a regular basis. We determined whether cleaning hopper-style bird feeders every two weeks with 10 % bleach wipes reduced the counts of total aerobic bacteria and Gram-negative bacteria on feeders over time in the winter. Gram-negative bacteria were used as a sampling measure because they are good indicators of potential pathogenic bacteria that are commonly used in food and water testing (Toranzos *et al.* 2007) and because avian pathogens can be difficult to detect in the environment (e.g., Prescott *et al.* 2000). Total aerobic bacteria were also used as a sampling measure because this provided a more general measure of bacteria present to test cleaning effectiveness, which may be particularly important if potential pathogens were not present in any abundance in our study area.

MATERIALS AND METHODS

Immediate Effectiveness of Bleach Wipes at Reducing Bacteria on Feeders

We tested whether Sani-cloth 10 % bleach wipes (H24795, Professional Disposables Inc., Orangeburg, NY) were effective in cleaning feeders in the short term by measuring the reduction in counts of total aerobic bacteria before and after cleaning. Eleven hopper-style feeders (Perky-Pet No. 316, Woodstream Corp., Lititz, PA) made of plastic were placed out in Berks County, PA for six weeks from March to April 2013. When returned to the lab, the entire surfaces of the four perches on each feeder were swabbed using Q-swabs™ sample collection devices (Weber Scientific, Hamilton, NJ). The swabs were mixed by vortex for 30 seconds. The rinsate was then serial diluted, and the dilutions were plated on Luria Bertani agar (LB; Neogen Corp., Lansing, MI). LB agar was used to culture the total aerobic bacteria present per ml of rinsate. After swabbing, the four perches of each feeder were "cleaned" with Sani-Cloth bleach wipes. One to two wipes were used to remove any debris present on the surface. Another wipe was used to sanitize the perches of the feeder. The feeders were then allowed to air dry. After the feeders were completely dry, the entire perch area was swabbed using Q-swabs™ The swabs were mixed by vortex for 30 seconds. The rinsate was serial diluted and plated on LB agar. Both sets of plates were incubated at 37 °C for 24 hours. Colony counts were performed to determine whether the Sani-cloth wipes reduced the level of total aerobic bacteria present on the surface of the feeders.

Feeder Sanitization prior to Ten Week Cleaning Study

Prior to our ten-week experiment, forty hopper-style feeders (Perky-Pet No. 316, Woodstream Corp., Lititz, PA) were scrubbed using a sponge with soap and water to remove any debris. We then sprayed feeders with a 10 % bleach solution and allowed them to air dry to sanitize microorganisms present on the feeders. After cleaning, the entire perch surfaces of the feeders were swabbed with Q-swabs™ sample collection devices to ensure bacteria and fungi were not present after cleaning. The swabs were mixed by vortex for 30 seconds. The swabs were then spread across the surface of Potato Dextrose Agar (PDA; Neogen Corp., Lansing, MI) and LB agar. PDA was used to culture fungi and LB agar was used to culture total aerobic bacteria present on the feeders. Plates were incubated at 25 °C for 48 hours to ensure growth and detection of fungal colonies. The presence or absence of fungi and bacteria was determined by growth on the plates.

Feeder Placement and Maintenance

The feeders were placed in pairs at locations throughout Berks, Bucks, Lehigh and Carbon counties in Pennsylvania in late January 2012 and maintained until April by volunteers. Each pair of feeders was hung from a double-sided bird feeder post (i.e., a shepherd's hook) or from a tree. A paired design was used to ensure that the cleaned and non-cleaned feeders were exposed to the same environmental conditions. Although a paired design might facilitate the transfer of bacteria between cleaned and non-cleaned feeders, this proximity of feeders reflects actual feeder use and the environmental conditions expected in suburban and urban areas. For example, Fuller *et al.* (2008) estimated

feeder density at 925 feeders/km^2 in Sheffield, UK, which is the equivalent of one feeder per 0.27 acres. Furthermore, the average birdwatcher maintains multiple feeders in their yards, e.g., average of 5.7 feeders per household in Britain (Schreiber 2010). The feeders were filled with Feathered Friend premium black oil sunflower seeds (CHS Sunflower, Grandin, ND).

The feeders were refilled periodically as needed. The approximate volume of seed added to the feeders each time they were refilled was recorded. To measure this, four evenly spaced lines were drawn on the feeders. Volunteers noted the seed volume before and after seeds were added to each feeder. The mass of seeds within the feeders at each given increment was pre-determined and used to determine seed consumption by animals, i.e., birds and gray squirrels (*Sciurus carolinensis*), at each feeder over time. gray squirrels are regular visitors to bird feeders and are a likely source of bacteria in addition to birds. This project was ethically reviewed and approved by Kutztown University's Institutional Animal Care and Use Committee.

Feeder Cleaning Protocol

One feeder in each pair served as a control and was not cleaned throughout the experiment (hereafter non-cleaned feeders). The other feeder was cleaned once every two weeks (the weeks alternate to swabbing) using a Sani-cloth Bleach wipe that contained 10 % bleach across the entire surface and interior of the feeder (hereafter cleaned feeders). Seed was removed from each feeder prior to cleaning. One to two wipes were first used to remove dirt or debris. A final bleach wipe was then used to sanitize the surface by wiping it across surface of the feeder and allowing it to air dry. After the feeder was dry, the seed was returned to the feeder. However, we did not clean up the seed hull debris on the ground under the feeder.

Surface Sampling

Every two weeks (the weeks alternate to cleaning), the surfaces of the four perches and seed wells of each feeder were swabbed using Q-swabs™ sampling devices. The Q-swabs™ were mixed by vortex for 30 seconds and then were serial diluted in sterile buffered peptone water. Dilutions were plated on LB agar and Eosin Methylene Blue agar (EMB; Neogen, Lansing, MI). EMB agar was used to culture Gram-negative bacteria present on the feeders. The plates were incubated at 37 °C for 24 hours. Colony counts were performed on each type of media.

Statistical Analysis

To evaluate the immediate effectiveness of bleach wipes, a paired t-test was used to determine whether the wipes reduced the counts of total aerobic bacteria after cleaning a sample of feeders. Experimental cleaning data were analyzed using a two-way repeated measures ANOVA to determine whether the counts of total aerobic bacteria (hereafter aerobic bacteria) and counts of Gram-negative bacteria were significantly influenced by experimental cleaning and by time in weeks. Finally, linear regression analysis was used to determine whether there was a relationship between the mass of seed consumed at each feeder (as an indicator of animal activity) in the two weeks prior to each sampling period and the counts of bacteria on the feeders. All statistical analyses were conducted using SPSS version 19.

RESULTS

Immediate Effectiveness of Bleach Wipes at Reducing Bacteria on Feeders

The Sani-cloth bleach wipes significantly reduced aerobic bacteria from the surface of the feeders ($t = 10.91$, $n = 11$ feeders, $p < 0.001$). Before cleaning, the count of aerobic bacteria on feeders was 3.4 log CFU/ml of rinsate (± 0.24 SE), and after cleaning, the count of aerobic bacteria on feeders was 1.7 log CFU/ml of rinsate (± 0.19 SE). Thus, the bleach wipes effectively reduced the aerobic bacteria count by 1.7 log CFU/ml of rinsate.

Feeder Sanitization prior to Ten Week Cleaning Study

After the preliminary cleaning of feeders with soap and water followed by a 10 % bleach solution, no colonies were present in feeder samples on PDA or LB agar plates, which indicated that the bleach sanitization process was effective at killing microbes and that all feeders began our ten-week experiment without microorganisms present. Over the ten-week experiment, one pair of feeders was removed from the study due to squirrel damage and the week two swabbing samples were lost due to logistical problems, so 19 feeders were used and the swab sampling began at week four.

Effectiveness of Cleaning Feeders with Bleach Wipes Over Ten Weeks

Cleaning with bleach wipes significantly reduced aerobic bacteria on feeders ($F_{1, 18} = 7.52$, $p = 0.013$; Figure 1A), but aerobic bacteria significantly increased on feeders over time ($F_{3, 16} = 8.16$, $p = 0.002$; Figure 1A). In addition, there was

a significant interaction between cleaning and time that influenced aerobic bacteria on feeders ($F_{3, 16}$ = 3.47, p = 0.041; Figure 1A). This interaction suggests that cleaning became less effective over time because counts of aerobic bacteria on cleaned feeders increased from weeks 4 and 6 to weeks 8 and 10 and these counts were similar to counts on non-cleaned feeders at the end of our experiment (Figure 1A). By comparison, cleaning with bleach wipes did not significantly reduce Gram-negative bacteria on feeders ($F_{1, 18}$ = 1.88, p = 0.187; Figure 1B) and time in weeks did not significantly influence Gram-negative bacteria on feeders ($F_{3, 16}$ = 2.09, p = 0.141; Figure 1B). In addition, there was no significant interaction between cleaning and time that influenced Gram-negative bacteria on feeders ($F_{3, 16}$ = 0.61, p = 0.619; Figure 1B).

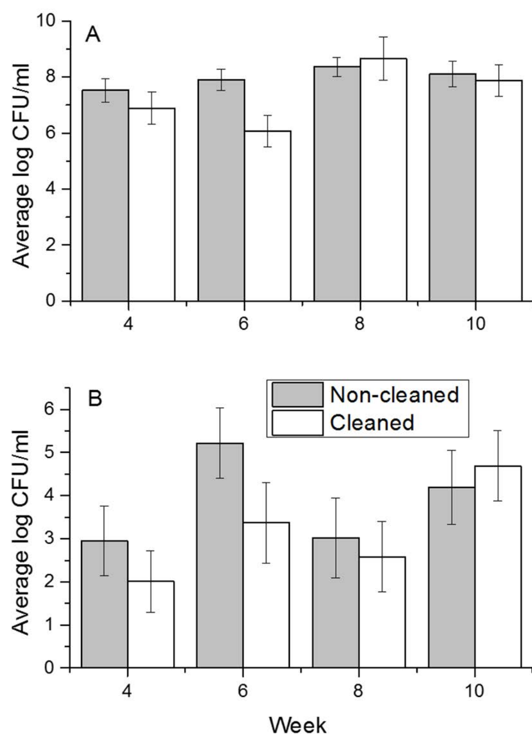

Figure 1. Average (± SE) aerobic (A) and Gram-negative (B) bacterial counts (log CFU/ml of rinsate) over ten weeks on bird feeders cleaned with 10 % bleach wipes compared with non-cleaned feeders. Bacterial counts occurred every two weeks beginning with week four.

Most of the bacteria counts on the cleaned and non-cleaned feeders were not related to the amount of seed consumed at those feeders. No relationship was found between aerobic bacteria and seed consumed at the cleaned feeders (r^2 = 0.193, n = 19, p = 0.095; Figure 2A) or the non-cleaned feeders (r^2 = 0.155, n = 19, p = 0.181; Figure 2B). There was no relationship between Gram-negative bacteria and seed consumed at the cleaned feeders (r^2 = 0.151, n = 19, p = 0.194; Figure 2C), but there was a significant positive relationship between Gram-negative bacteria and seed consumed at the non-cleaned feeders (r^2 = 0.382, n = 19, p = 0.001; Figure 2D).

DISCUSSION

We found that bleach wipes were effective at reducing aerobic bacteria on feeders, but the wipes did not completely sanitize the surface of feeders. In our ten-week cleaning experiment, we recorded high counts, i.e., > 10^7 CFU/ml, of total aerobic bacteria by week four (Figure 1A) despite beginning the experiment with no colonies detected in feeder surface samples. These levels suggest that there was a need to clean feeders after four weeks of use. Cleaning with bleach wipes every two weeks reduced aerobic bacteria on feeders, but aerobic bacteria increased over subsequent weeks, notably so after week six. The bleach wipes may have become less effective over time, i.e., by weeks eight and ten (see Figure 1A), as indicated by a significant interaction between time and cleaning on aerobic bacteria counts. By contrast, we found no significant trends between seed consumption and aerobic bacteria at cleaned and non-cleaned feeders. This suggests that counts of aerobic bacteria were influenced by multiple environmental sources and not solely by animal activity.

We suspect that the loss of cleaning efficacy for aerobic bacteria over time was due to the accumulation of organic material from soil, seed hull debris, and feces on the surface of feeders. Organic matter can potentially inactivate bleach (i.e., sodium hyperchlorite) or act as a barrier for bacteria against disinfectants (Russell 1999; Sharma *et al.* 2009). Although the Sani-Cloth wipes contain surfactants and other cleaners in addition to bleach, they are designed to disinfect surfaces and equipment for the healthcare industry and are not intended for removing large amounts of debris. Thus, our feeder cleaning protocol of using only bleach wipes likely left behind organic matter in the corners of the seed wells and in the fine grooves present on the perches of the feeders. This organic matter build up may have increased over time leading to the reduction in cleaning efficacy after several weeks.

By contrast, neither cleaning nor time significantly influenced Gram-negative bacteria on the surface of feeders despite an apparent trend toward lower counts on cleaned feeders in three of the four sampling periods (see Figure 1B). The lack of a significant effect of cleaning was likely due to the high variability in Gram-negative bacteria at both cleaned and non-cleaned feeders. This variability is not surprising because potential pathogens have been difficult to detect at bird feeders (e.g., Prescott *et al.* 2000) and the prevalence of

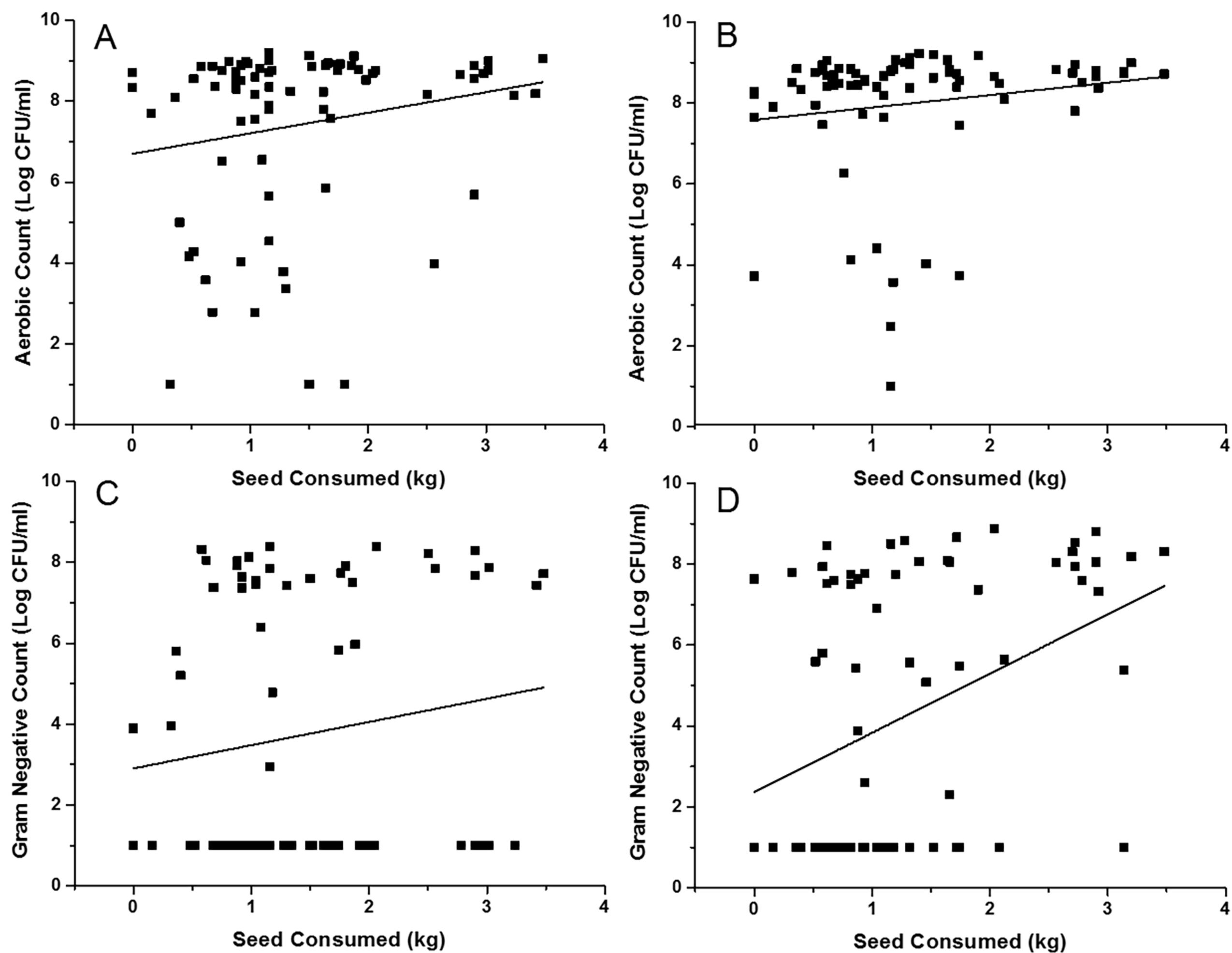

Figure 2. The influence of animal activity (as measured by seed consumption at each feeder two weeks prior to each bacterial sampling period) on bacterial counts (log CFU/ml of rinsate) on individual feeders for: total aerobic bacteria on cleaned feeders (A), total aerobic bacteria on non-cleaned feeders (B), Gram-negative bacteria on cleaned feeders (C), and Gram-negative bacteria on non-cleaned feeders (D). The trendlines represent linear regression best of fit lines.

potential pathogens is low among wild songbirds (Brittingham *et al.* 1988), but can be variable in some pathogens, such as *Salmonella* (Benskin *et al.* 2009). Interestingly, there was a significant positive relationship between seed consumption and Gram-negative bacteria at non-cleaned feeders and no significant relationship at cleaned feeders. The positive relationship for non-cleaned feeders suggests that birds and squirrels were a source of Gram-negative bacteria at feeders through either their physical contact or the deposition of feces on feeders. Although we did not measure the amount of fecal matter present on feeders, Prescott *et al.* (2000) found that fecal matter does accumulate at feeders, especially hopper-style and platform feeders. More importantly, the lack of a significant relationship for cleaned feeders also suggests that cleaning with bleach wipes helped to control Gram-negative bacteria on very active feeders.

Overall, we found that bleach wipes reduced aerobic bacteria on feeders and were able to control aerobic bacteria for a few weeks. However, this efficacy appeared to diminish after several weeks, which may have been due to the buildup of organic matter on feeders. Bleach wipes were not effective at controlling Gram-negative bacteria over several weeks but showed some ability to reduce Gram-negative bacteria on very active feeders. Our results suggest that bleach wipes might be a simple and useful sanitizing method for bird feeders if organic matter can be removed first through other means of cleaning or scrubbing. Future research should investigate the influence of organic matter on the efficacy of disinfectants to clean bird feeders and to determine whether the amount of feces or debris on feeders is related to levels of potential pathogenic bacteria.

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SURVEILLANCE OF AVIAN INFLUENZA IN WATERFOWL (FAMILY ANATIDAE) WITHIN ERIE AND MERCER COUNTIES, PENNSYLVANIA[1]

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ABSTRACT

Avian influenza virus (AIV) uses wild bird populations as a natural reservoir. Waterfowl, in particular wild dabbling ducks (Family Anatidae), are believed to constitute the main natural viral reservoir for low pathogenic influenza A virus. Over the past 30 years, only 30 studies have characterized AIV subtype distribution and prevalence in North America, and most of these studies were conducted between 1970 and 1980. Understanding AIV distribution and prevalence is critical for developing an objective risk assessment for the role that waterfowl may play as a reservoir of AIV subtypes that pose health concerns for humans and other animals. Highly pathogenic avian influenza in poultry originates from the transmission of low pathogenic viruses from wild birds. These outbreaks have occurred relatively frequently in the last decade emphasizing the need for continuous surveillance of the waterfowl populations for AIV. We collected 200 cloacal swab samples from waterfowl in Erie and Mercer counties, Pennsylvania, during August-September 2012 to determine the prevalence of AIV subtypes between species, ages, and sexes. We used RT-PCR analysis to determine that AIV was not present in the waterfowl we sampled. [J PA Acad Sci 88(4): 227-230, 2014]

INTRODUCTION

Influenza is a global public health problem (CDC 2013). Avian influenza (AIV) refers to infection of birds with avian influenza Type A viruses. These viruses occur naturally among wild aquatic birds worldwide and can infect domestic poultry and other bird and animal species (Webster *et al.* 1992). Clark and Hall (2009) have suggested that the origin of high-pathogenic AIV subtypes may be wild bird populations. Waterfowl (Family Anatidae,) are one of the main reservoirs of AIV in wild bird populations (Webster *et al.* 1992). Birds can be infected with AIV in their intestines and respiratory tract, but usually do not get sick (Clark and Hall 2009). However, AIV is very contagious among birds and some of these viruses can sicken and even kill certain domesticated bird species including chickens (*Gallus gallus*), ducks, and turkeys (*Meleagris gallopavo*) (Munster *et al.* 2005). Infected birds can shed avian influenza A viruses in their saliva, nasal secretions, and feces (Slemons *et al.* 1991). Susceptible birds become infected when they have contact with the virus as it is shed by infected birds. They can also become infected by coming in contact with surfaces that are contaminated with virus from infected birds (CDC 2013).

Relatively few studies have been done to study the prevalence of AIV in North America. Our project will provide surveillance on the subtypes of AIV that are present in wild bird populations in Pennsylvania. Our goal was to determine the prevalence of AIV in waterfowl populations from Erie and Mercer counties in Pennsylvania.

MATERIALS AND METHODS

Field Collection Sites

Pymatuning Wildlife Management Area in Mercer County and Presque Isle State Park in Erie County are two of the largest waterfowl staging areas in Pennsylvania. Pymatuning Wildlife Management Area contains the largest inland impoundment in Pennsylvania; of the 10,121 ha in the management area, 6,883 ha are covered by water and the remainder is covered by land. Pymatuning provides habitat for an estimated 25,000 to 35,000 non-resident geese and ducks during the fall and spring migration seasons (Alfonso *et al.* 1995).

Presque Isle State Park is a 3,200-acre peninsula that protrudes into Lake Erie Presque Isle is a preferred spot for migrating birds. The park's location on the Atlantic Flyway

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makes it a favorable spot for birds to stop on their migration across Lake Erie. Waterfowl migration occurs in March and in late November through December. Over 300 different species of birds have been identified on the peninsula (Presque Isle Audubon Society 2014).

Collection Methods

Peak AIV transmission among waterfowl occurs during premigratory staging in late summer (Hinshaw and Webster 1982). On August 26, 27 and September 8, 2012, we collected samples from 200 wild waterfowl at our study locations: 125 from Pymatuning and 75 from Presque Isle State Park. Birds were caught in funnel live-traps. The traps were checked every morning and evening. Captured birds were banded with steel rings and identified for species, sex, and age (adult or juvenile) based on plumage. Using sterile plastic nylon flocked swabs, we sampled each bird by swirling the swab in its cloaca. Nylon swabs were immediately put in vials containing virus transport media (Hanks balanced salt solution with bovine serum albumin, gelatin, sucrose, L-glutamic acid, HEPES buffer, phenol red, gentamicin, and amphotericin B (remel M4RT, Thermo Scientific, Lenexa, Kansas)) and kept on ice. All samples were moved to a freezer within 48 hours that stored samples at –80 °C. All animal work was reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee and the study was conducted in accordance with the guidelines of the U.S. Office of Laboratory Animal Welfare.

Avian Influenza Detection

The procedure was taken from the World Health Organization Real Time PCR protocol for the detection of Avian Influenza (WHO 2013). We used the commercially available viral RNA extraction kit from Qiagen to recover AIV RNA from the samples (Komar *et al.* 2002, Ohajuruka et al 2005). Extracted viral RNA was converted to cDNA with a commercially available kit from Life Technologies, Ag-Path One-Step RT-PCR Kit. This kit utilized published primers for AIV to determine positivity (WHO 2013). The primers used were FluA InfA Forward 5’ GAC CRAT CCT GTC ACC TCT GA C 3’ and FluA InfA Reverse 5’ AGG GCAT TYT GGA CAAA KCG TCT A 3’. The probe used was InfA Probe1 5’ FAM-TGC AGT CCT CGC TCA CTG GGC ACG-BHQ1-3’. A StepOne Real Time PCR system was used with the following conditions: 45 ˚C for 10 min, 95 ˚C for 10 min, followed by 40 cycles of 95 ˚C for 15 s and 60 ˚C for 45 s. Nuclease free water was used as a negative control and genomic RNA isolated from a preparation of pooled allantoic fluid from specific-pathogen free embryonated chicken eggs infected with an avian isolate (Kilbourne F26) of influenza A virus was provided by BEI Resources as a positive control. Positive control samples amplified at cycle 22, no detection of AIV was seen in the negative control samples.

RESULTS

We collected cloacal swab samples from 153 mallards (*Anas platyrhynchos*), 1 blue-winged teal (*Anas discors*), 43 wood ducks (*Aix sponsa*), and 2 Canada geese (*Branta canadensis*). The numbers of individuals sampled by species, age, and sex are in Table 1. Although we sampled individuals from several species and from different age and sex classes no AIV was detected.

DISCUSSION

Aquatic birds, humans and pigs are the hosts for influenza virus and the emergence of new human pandemic strains occur through the viral movement between these host species (Webster *et al.* 1992, Bouvier and Lowen 2010). Aquatic birds are reservoirs of all known AIV subtypes (Webster *et al.* 1992). That being said, waterfowl play an important role in the generation, transmission, and spread of AIV (Hinshaw *et al.* 1979, Karunakaran *et al.* 1983, Webster *et al.* 1992). AIV has most frequently been isolated from mallard ducks (Hinshaw *et al.* 1980, Hinshaw *et al.* 1986) but has also been isolated from other species of waterfowl (Nettles *et al.* 1985, Stallknecht *et al.* 1990, Graves 1992).

The Pennsylvania Game Commission (PAGC) routinely samples for AIV during Fall migration; these studies show the prevalence of AIV to be approximately 1.5% of birds sampled. The sample sizes range from 200-330 waterfowl (PAGC, 2007). Another study conducted in Pennsylvania found AIV present in mallard and American wigeon (*Anas americana*) (Alfonso *et al.* 1995). A similar study to ours for AIV in Pennsylvania at Pymatuning during 1990 and 1991 detected AIV subtypes H4N8 and H6N8 from mallard and American widgeon during fall migration (Alfonso *et al.* 1995). There have been mixed results as to whether different species are more likely to contract AIV. A study conducted in Pennsylvania did not find a difference of AIV recovery based on species (Alfonso *et al.* 1995), unlike Deibel *et al.* (1985). Prevalence levels in Pennsylvania are slightly lower than levels detected throughout North America as seen in a recent study. Huang and colleagues conducted a survey of AIV in North America and determined the prevalence of AIV to be slightly higher at 1.8% (Huang et. al, 2014).

Although we did not recover any AIV, other research has shown that waterfowl are more likely to become infected with AIV during the migration season (Hinshaw *et al.* 1980, Slemons *et al.* 1991). Alfonzo and colleagues detected AIV in breeding individuals during the fall migration (Alfonzo

et al 1995) and others have shown that sex of the individual has no effect on AIV presence (Deibel et al 1985, Slemons et. al 1991). The majority of our samples were juvenile birds and our sampling occurred pre-migration in late August and early September. This may have affected our detection rates as AIV is prevalent in waterfowl populations in the fall, (Webster *et al.* 1992). Since AIV is transmitted through water and feces, it is unlikely the many juvenile waterfowl in these flocks will remain uninfected after the autumn epidemic.

Even though we did not detect AIV in our study, the other research shows that waterfowl have been infected with AIV in these same locations (Alfonso *et al.* 1995). Given that AIV is shed from the intestinal tract between 2 days and 4 weeks, after which there is no evidence that continued shedding occurs may explain why we did not find the virus in birds we sampled (Hinshaw *et al.* 1980, Lu and Castro 2004). Also, the virus may be present in our study sites, but the birds may have recovered and thus not be shedding.

Despite our data showing no positive samples it is imperative to continue sampling aquatic birds as they are the reservoirs of all know AIV subtypes (Webster *et al.* 1992). Each of the highly pathogenic outbreaks of AIV that have occurred in recent years (Bean *et al.* 1985, Murphy 1986, Crosby 1989) likely originated from waterfowl populations (Webster *et al.* 1992). Waterfowl population surveillance continues to be an important component of AIV research. Taken together these points stress the importance for continued surveillance to assess the actual risk for AIV to both humans and aquatic birds.

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RESEARCH NOTE: DEER BROWSING THREATENS A LOCALLY RARE PARASITIC PLANT, BUFFALO NUT (*PYRULARIA PUBERA* MICHX.)[1]

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ABSTRACT

Overabundance of deer presents a threat to individual plant populations and may in the long term alter forest structure. *Pyrularia pubera*, the buffalo nut, is a plant already considered rare in Pennsylvania, the northernmost extent of its natural range. At one site in western PA, buffalo nut appears to be heavily browsed by deer. In this study, the demographic effects of deer browsing on a population of buffalo nut are investigated by comparison of growth and density inside and outside of deer exclosures. Shoot density in exclosures was approximately 40 times greater than in the surrounding forests. Shoots outside of exclosures were significantly shorter than those in exclosures. Preservation of buffalo nut in western Pennsylvania, and thus the entire state, will likely depend on efforts to curtail the negative effects of deer overabundance. [J PA Acad Sci 88(4): 231-234, 2014]

INTRODUCTION

Pyrularia pubera Michx., the buffalo nut, is a clonal, root-parasitic shrub of mesic forests of the eastern and southeastern US. It is the only North American member of the family Cervantesiaceae, a segregate of Santalaceae *s. lat.* (Nickrent *et al.* 2010). Buffalo nut occurs naturally along the Appalachian uplift from northern Alabama through western Pennsylvania (Coder 2011). Disjunct or non-native populations also exist in Suffolk and Queens counties, N.Y. (Mitchell 1986). As a hemiparasite, buffalo nut presumably obtains nutrients from the roots of other plants through a modified root called a haustorium. Buffalo nut is capable of parasitizing a wide variety of hosts, including 50 genera in 31 families of ferns, gymnosperms and angiosperms (Leopold and Muller 1981). While the life-cycle of this plant has not been studied extensively, it grows by means of underground rhizomes from which shoots arise at intervals. These rhizomes may branch producing more than one shoot simultaneously (Randle, personal observation). Plants are dioecious, bearing spikes of male or female flowers in late spring, and producing fruit from July to October, although not abundantly (Coder 2011).

Buffalo nut is listed as a conservation priority in Alabama (imperiled; Alabama Natural Heritage Program 2004), and Pennsylvania (rare; Pennsylvania Natural Heritage Program 2014). While members of a species are expected to be less abundant at the extremes of their range, browsing by white-tailed deer (*Odocoileus virginianus* Zimmerman 1780) in the northernmost known populations may present an additional conservation threat. In western Pennsylvania, buffalo nut is among the first woody plants to leaf out in the spring (late April). Throughout the growing season, browsing by deer is apparent, with few plants reaching more than 15 cm in height. In May 2014, an initial investigation at the Powdermill Nature Reserve in Pennsylvania indicated that damage from deer browsing was heavy, with most new shoots chewed to the rhizome or litter layer. Rhizomes themselves also showed signs of breakage. Breaks in rhizomes were rather clean and not accompanied by scarring as from mechanical damage not likely caused by deer, but possibly by autotomy, the spontaneous shedding of an extremity (Wenzel, personal observation). Motivated by these observations, we studied the extent of browsing damage by examining the demographics of plants both within and outside of deer exclosures.

MATERIALS AND METHODS

Study site

Data were collected from living plants present in the Carnegie Museum of Natural History, Powdermill Nature Reserve. This property in Cook Township, Westmoreland County, Pennsylvania, is characterized as an oak-maple forest, but an extensive survey included 55 woody species larger than 4 inches DBH. The full data set and web map of the most abundant 20 species of trees on the main property of the Reserve is available at http://maps.carnegiemnh.org/.

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The understory in the site consists of spicebush (*Lindera benzoin* (L.) Blume), clubmoss (*Lycopodium complanatum* L.), and various ferns and forb species that generally survive deer browsing. The entirety of the site slopes gently upward from the south. The population of buffalo nut on this property may be the northernmost natural population of this species, based on collection data from the USDA and Coder (2011). In the early spring of 2010, three deer exclosures of approximately 10 x 10 m were erected by reserve personnel on the southwest corner of the property with the intention of preserving buffalo nut in its native habitat.

Data collection

In May 2014, all shoots taller than 100 cm that were outside of deer exclosures were marked with flagging tape. Many hundreds of small shoots in the litter were observed throughout the area, easily identified by their early bud break. In September, 2014, six 60 m transects were randomly placed outside of deer exclosures and were surveyed for buffalo nut shoots. Height in inches of all shoots within 50 cm of each transect was recorded, and later converted to centimeters. Shoots were measured from the ground to an apical meristem along the longest axis of the plant. Within each deer exclosure, a 12 m transect extending from one corner of the exclosure toward the other was surveyed in the same fashion. The diameter at breast height (DBH) was also measured for all shoots reaching a height of 152 cm or more. Additionally, rhizomes of 10 shoots from outside the deer exclosure were excavated to monitor parasitic activity. Because the exclosure was built explicitly to protect plants from browsing, shoots from within exclosures were not excavated. The length along the longest axis of each excavated rhizome was measured. Major stems that had been marked in May were revisited in September and examined for signs of damage.

Analysis

Individual measurements were binned into groups representing 12.7 cm intervals from 0-254 cm to maximize statistical power while avoiding intervals in which no measurements had been taken. To test the hypothesis that samples from exclosures and surrounding forest were drawn from the same distribution, the non-parametric Kolmogorov-Smirnov two sample test was implemented using the Real Statistics Resource Pack for Microsoft Excel (Zaiontz 2014).

RESULTS

A total of 257 shoots were surveyed within deer exclosures and 66 shoots were surveyed along transects in the surrounding forest. Browsing by herbivores was strongly in evidence outside of the exclosures, resulting in many shoots missing leaves or with highly damaged leaves. No such evidence of browsing was apparent within the exclosures. Shoot density inside the deer exclosures (7.14 shoots/m^2) was notably higher than in the surrounding forest (0.18 shoots/m^2; Table 1). Further, the distribution of shoot heights within exclosures was significantly different than that of the surrounding forest (Dstat = 0.675; $p<0.001$; Fig. 1). In exclosures, the average plant height was 48.1 cm with 50% of

Table 1. Results of transect survey showing the number of plants collected along each transect, the density of plants per square meter and the mean height in cm with standard deviation.

	Number	Density/m^2	Mean height (cm)	S.E.
Exclosure				
Transect 1	109	9.08	47.0	47.1
Transect 2	70	5.83	61.9	43.7
Transect 3	78	6.50	37.3	35.6
Combined	257	7.14	48.1	43.8
Surrounding forest				
Transect 1	15	0.25	10.5	5.2
Transect 2	4	0.07	15.9	6.4
Transect 3	9	0.15	11.3	4.2
Transect 4	1	0.02	10.2	0.0
Transect 5	4	0.07	11.2	1.4
Transect 6	32	0.53	20.5	52.6
Combined	66	0.18	15.8	36.6

shoots 33 cm or less. In the surrounding forest, the average plant height was 15.8 cm, with 50% of shoots 7.6 cm or less. Two shoots measured outside of exclosures were 190.5 cm or taller; the remainder were less than 23 cm. Conversely, 63.95% of plants within exclosures were between 23 cm and 190.5 cm.

The number of surveyed plants that were greater than 152 cm in height was not enough to lend power to statistical analysis, but we report them here. In deer exclosures, 12 of 258 plants (4.65%) were 152 cm or taller in height, with an average height of 193.5 cm and an average DBH of 1.3 cm. In the surrounding forest, two of 66 plants (3.03%) were taller than 152 cm, with an average height of 218.4 cm and an average DBH of 2.2 cm. Of the 20 shoots greater than 91.4 cm marked in May, nine (45%) were completely leafless and appeared to have died as far back as the rhizome by September. On the contrary, of the many shoots taller than 152 cm in the three deer exclosures, all appeared to be healthy and in full leaf.

Rhizomes of excavated plants were in general longer than 30.5 cm. For seven of these, the distal end of the rhizome was buried deep under rocky forest soil and was not obtainable. However, in only one of the excavated plants was a haustorial connection evident, and that was at the base of a shoot that was attached to a host-root that had been severed previously and could not be identified to species.

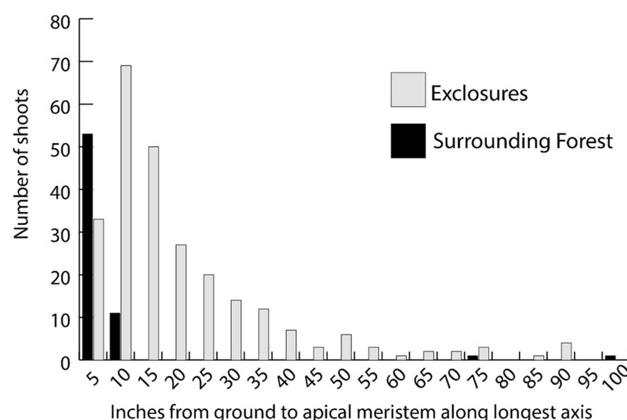

Figure 1. Distribution in shoot height from ground level to the apical meristem along the longest axis for plants surveyed in deer exclosures and those in the surrounding forest.

DISCUSSION

The difference in shoot number and height between exclosures and the surrounding forest is apparently a result of browsing by deer. Deer are particularly abundant at the study site and several were sighted during the course of field study, along with copious droppings. While these results implicate deer in altering shoot height distributions in a population of buffalo nut, the broad geographical and long-term impacts of browsing are unknown. Deer browsing may affect survival, abundance, and growth patterns of plant populations, which may have broader ranging and less predictable effects on succession and plant community structure (Côté *et al.* 2004; Forrester *et al.* 2006). The direct effects of deer browsing on buffalo nut populations may include a decrease in aboveground biomass (as found in our study), thus limiting the ability to sequester carbon for growing rhizomes and preventing sexual reproduction. The latter may decrease population genetic diversity and gene flow among populations. Lapointe et al (2010) found that tolerance to simulated browsing on liliaceous species of mesic forests was positively correlated with the proportion of carbon sequestered in underground organs. Further, clonality was correlated with ability to recover from damage resulting from simulated browsing. Therefore, buffalo nut appears to be surviving in the face of heavy deer browsing because of its perennial networks of interconnected rhizomes, which in our study were often much larger and extensive than shoots. While browsing may affect an individual ramet, its genet may survive for long periods of time even under steady browsing pressure. Assuming that browsing intensity was uniform throughout the site before the construction of deer exclosures, the greater density and plant height in exclosures compared to the surrounding forest is evidence that buffalo nut can recover from browsing damage.

The lack of haustoria on excavated plants and on roots in the surrounding soil may indicate that buffalo nut is less dependent on host plants than previously believed (Leopold and Muller, 1983). An alternative explanation is that rhizome growth patterns result in shoots that emerge farther and farther from initial host plants making it very difficult to obtain productive haustoria from distal roots. The length of excavated rhizomes indicates that likely few of the shoots excavated were recently seedlings, a supposition supported by the lack of fruits observed anywhere on the property. A third explanation is that our excavation method failed to recover haustoria, either because we could not locate the youngest roots, which are rather fragile but most likely to form haustoria, or that few active haustoria may be sufficient to provide necessary nutrients.

The high mortality of large shoots outside of deer exclosures is, as far as we know, unprecedented and alarming. Of the eleven shoots that survived the summer, several had branches that were leafless and appeared to be dead. We can only speculate whether limb die-off is the direct result of browsing by deer, a response to a pathogen such as a fungal canker, that may be spread by deer browsing, or due to some other cause. Nonetheless, it represents a potential threat to a population already stressed by over-abundance of deer.

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ASSESSING MATING READINESS IN MALE MIGRATORY BATS FROM PENNSYLVANIA[1]

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ABSTRACT

Little is known about mating behaviors and seasonal reproductive conditions in migratory bats, but mating is believed to usually occur in the fall. Recent studies noting deaths of bats at wind turbines have hypothesized that bats may be attracted to these tall tree-like structures to find mates, and if so, there should be evidence that the killed bats were in a state of mating readiness at the time of death. We studied reproductive anatomy and histology of 63 male bats of 3 migratory species (*Lasiurus borealis*, *Lasiurus cinereus*, and *Lasionycteris noctivagans*) collected in eastern Pennsylvania in the summer and fall of 2007 and 2008. Testes of all three species exhibited evidence of spermatogenesis during the summer, and testis length and mass decreased as fall approached, presumably because sperm were transferred to the epididymis prior to mating. Epididymides of nearly all individuals contained sperm at the time of death, indicating that males were capable of inseminating females. Our data indicate that these bats were in a state of mating readiness at the time of death and are consistent with the hypothesis that bats are attracted to wind turbines to find mates. [J PA Acad Sci 88(4): 235-246, 2014]

INTRODUCTION

Bats of the Family Vespertilionidae are found nearly worldwide and are common throughout most of North America (Koopman 1993; Krutzsch 1979, 2000). Temperate zone vespertilionids, including those in Pennsylvania, have short reproductive cycles and only one litter per year (Willig 1985). North American male vespertilionids usually undergo spermatogenesis in the summer and copulation in the fall, but sometimes copulate in winter or spring. Female vespertilionids typically store sperm during fall and winter, and undergo ovulation, fertilization, and pregnancy in spring (Cryan *et al.* 2012, Entwistle *et al.* 1998, Kwiecinski and Damassa 2000, Myers 1977, Orr and Zuk 2013, Pearson *et al.* 1952, Rossiter *et al.* 2000). This strategy of temporally separating gametogenesis and copulation from a delayed fertilization and pregnancy has clear benefits for true hibernating bats, but it also occurs in migratory taxa such as *Lasionycteris* and *Lasiurus* (Cryan *et al.* 2012, Orr and Zuk 2013).

Most female vespertilionids exhibit either seasonal monestry or polyestry, although there is tremendous variation in breeding patterns within this family (Myers 1977). Members of the genus *Lasiurus*, along with other genera that inhabit relatively mild temperate regions, have been found to be seasonally monestrous (Krutzsch 1979), although there is no obvious behavioral indication of when estrus and ovulation may be occurring (Martin and Bernard 2000). In some bat species, sperm production, gland secretion, copulation, female ovulation, and fertilization are synchronous (Krutzsch 1979, Myers 1977). In other species, including many genera of true hibernating bats as well as the genera of migratory vespertilionids studied here, spermatogenesis, sperm storage, glandular secretion, and copulation occur at different times throughout the year (Encarnação *et al.* 2004, Entwistle *et al.* 1998, Gustafson 1979). Sperm may be produced in one season and then stored in the epididymides for the remainder of the breeding season until females are receptive to copulation (Krutzsch 2000). For example, Myers (1977) noted viable sperm in the epididymis in *Lasiurus* for up to two months past regression of the testes. Females store sperm in the uterus until fertilization later in the season or as late as the following spring (Druecker 1972, Pearson *et al.* 1952, Wimsatt 1944). Gerell and Lundberg (1985) found that female pipistrelles (*Pipistrellus pipistrellus*) joined males in the end of July and experienced several copulations, with most matings occurring in the first half of August. However, little is known about the exact timing of these events in *Lasiurus* and *Lasionycteris* (Encarnação *et al.* 2004), and the fact that most migratory bats are polygynous and often promiscuous complicates interpretations (Cryan 2008).

Racey (2009) reviewed various non-invasive methods of assessing the reproductive status of male and female bats. This can be particularly difficult due to the great variability in reproductive morphology and behaviors within Chiroptera. Changes in the size of the testes, epididymides,

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and accessory sex glands associated with spermatogenesis reflect mating readiness in males and correspond with female estrus (Gerell and Lundberg 1985, Krutzsch 1979). In vespertilionids, the testes are superficially placed against the medial thigh, and are not encased in a scrotum (Racey 2009). Therefore, they are relatively easy to locate and observe, and to dissect if the specimen is dead. In addition, testes, along with epididymides, are surrounded by a pigmented sheath, which can sometimes be noticed through the thin skin, especially in sexually immature males (Entwistle *et al.* 1998, Kermott and Timm 1988). Testes can increase drastically in size during spermatogenesis as the seminiferous tubules grow and sperm are produced, often peaking in size by mid-August or early fall (Entwistle *et al.* 1998, Gustafson 1979, Pearson *et al.* 1952); Racey and Tam (1974) reported up to a forty-fold increase in testis mass in the vespertilionid *Pipistrellus pipistrellus*. There is some evidence that increase in size of testes during spermatogenesis is associated with improved body condition or increases in body weight during seasons when food is more readily available (Entwistle *et al.* 1998, Myers 1978, Racey 2009). Signs of mating readiness in mature males include shrinkage of the testes coinciding with expansion of the caudae epididymides and reduction of the pigmentation due to stretching of the sheath (Entwistle *et al.* 1998, Pearson *et al.* 1952, Racey, 1974, 2009). Encarnação *et al.* (2004) examined epididymal expansion in *Myotis daubentonii* and categorized the size of the duct via external visual observation; they found that filling reached a maximum in mid-September and was markedly reduced by the second half of October. Gustafson (1977) made similar observations on *M. lucifugus*, and noted that reduction in size of accessory structures after mid-September coincided with the onset of copulation. Although the caudae epididymides undergo a reduction in size after sperm are ejaculated, sperm have been known to remain viable in the epididymis for over six months (Krutzsch and Crichton 1986, Pearson *et al.* 1952).

Histologically, mating readiness can be assessed by examining stages of spermatogenesis in the testes (Druecker 1972, Pearson *et al.* 1952). Pearson *et al.* (1952) also reported that adult testes of Rafinesque's big-eared bat (*Corynorhinus rafinesquii*) were characterized by reduced interstitial tissue, large seminiferous tubules, and visible meiotic stages in mid-summer. By late August to early September, testes reached their maximum size and spermatids and spermatozoa were visible, although the male was not yet capable of fertilizing a female as sperm were not yet present in the epididymis. By late September, abundant spermatids and spermatozoa were visible in the seminiferous tubules, and sperm were present in the epididymis. By October, seminiferous tubules contained either quiescent spermatogonia or mature sperm, and the testes and seminiferous tubules regressed as sperm were sent into the epididymis. Sperm were still observed in the testes in early November, but over the winter only quiescent spermatogonia and Sertoli cells were visible. Adult epididymides were found to be quite large during late fall and winter, but shrank gradually over the winter, presumably as copulations occurred (Pearson *et al.* 1952).

Druecker (1972) examined reproductive anatomy and spermatogenesis in *Lasionycteris noctivagans* and *Lasiurus cinereus* from the southwest United States and Mexico. In *L. noctivagans*, he noted the commencement of sperm production in males in May, with a peak in meiosis by late July. By early August spermatids were abundant, and their transfer to the epididymis (spermiation) occurred from late August through early October. Although sperm were noted in epididymides, males were still undergoing spermatogenesis in fall. In *L. cinereus*, most spermatogenesis began by late May and meiosis peaked by early September, although most had visible spermatids by late July. Most spermiation occurred between late July and late September; the majority of epididymides had notable amounts of sperm by early August, and all had abundant sperm by September. Druecker (1972) also found that testis length increased as meiosis and spermatogenesis increased (peaking in August), but that it decreased in late summer and fall as sperm moved into epididymides. Druecker concluded that spermiation continued into the fall in these species longer than it does in hibernating species, although most individuals already had at least moderate amounts of sperm in the epididymides by July (*L. cinereus*) or August (*L. noctivagans*). One female *L. cinereus* exhibited abundant sperm in the uterus (but not the oviduct) in September, but overall data on the timing of insemination of females were inconclusive.

Recent declines in bat populations have intensified the need for more information about breeding patterns and the timing of reproductive events in North American temperate bats (Cryan and Barclay 2009, Cryan *et al.* 2012, Myers 1977). One recently recognized factor contributing to mortality in tree-roosting migratory bats in the northeast United States is the recent expansion of commercial wind energy facilities (Arnett *et al.* 2008, Cryan and Barclay 2009, Cryan *et al.* 2012). Although the causes of bat mortality at these facilities are not fully understood, one hypothesis is that bats are attracted to wind turbines as they seek mates near these tall tree-like structures (Cryan 2008, Cryan and Barclay 2009, Cryan *et al.* 2010, 2012). If this hypothesis is correct, there should be evidence that the bats killed by turbines had been in a state of mating readiness, having already completed spermatogenesis in males or exhibiting stored sperm in females (Cryan *et al.* 2012).

To address this hypothesis, Cryan *et al.* (2012) studied testes and epididymides of 91 adult male migratory bats killed at wind energy facilities across four seasons in two U.S. states (New York and Texas) and two Canadian provinces (Alberta and Manitoba). Most were collected between mid-August and mid-October, which coincides with fall migrations and the commencement of mating activity, and the majority were either *Lasiurus cinereus* (hoary bat), *L. borealis* (eastern red bat), or *Lasioncyteris noctivagans* (silver-haired

bat). Males were examined for the presence of sperm in the caudae epididymides, the presence, length, and type of penile spines, and the length of testes. Cryan *et al.* (2012) found that in all three species the majority of bats killed in late summer through early fall had sperm present in the epididymis. Sperm increased in abundance toward the end of summer and into the fall, and appeared earliest in *L. borealis* and latest in *L. noctivagans*. Testis length also decreased significantly ($p < 0.001$) from summer into fall, at least in *L. cinereus*, while the number of penile spines increased in both species of *Lasiurus* ($p < 0.01$). The presence of sperm in one female *L. cinereus* from September, and growing ovarian follicles and evidence of uterine stimulation in several adult females of all three species (especially in *L. cinereus*), also lend support to their conclusion that many of these bats were indeed in a state of mating readiness at the time of death. Cryan *et al.* (2012) noted the need for increased sample sizes, particularly for *L. noctivagans*, especially since migration, transfer of sperm to the epididymis, and mating seem to occur later in this species than in the two *Lasiurus* species. In addition, the fact that some specimens of *L. noctivagans* had no sperm in the epididymis at the time of death (and no *L. noctivagans* females were found with sperm) suggests that mating behaviors may not have been a factor in death at wind turbines for this species. These authors conclude that there is support for the hypothesis that bats are attracted to wind turbines as a proxy for tall roosting trees (Cryan 2008, Cryan *et al.* 2012), but they noted that further studies are warranted, especially of bats from other areas of the country, to adequately test the hypothesis that mating behaviors are a factor in mortality of migratory tree bats.

The goal of the present study was to provide additional information on migratory bat reproductive biology that can be used for testing the hypothesis that mating readiness in male bats is a factor in mortality of three migratory taxa commonly killed at wind turbines. We examined 63 carcasses collected near wind turbines in eastern Pennsylvania during the summer and fall of 2007 and 2008. This study provides data on additional samples of *L. borealis*, *L. cinereus*, and *L. noctivagans*, collected in an area of the continent that has not yet been sampled, and therefore provides information on reproductive biology of vespertilionids that may aid future conservation efforts.

MATERIALS AND METHODS

Bat carcasses were collected during daily mortality surveys from May through November in 2007 and 2008 at the Locust Ridge I Wind Farm in Schuylkill County, Pennsylvania (Zellner *et al.* 2008). We examined adult male carcasses of the three migratory taxa that were found most frequently: *Lasiurus borealis* (eastern red bat), *Lasiurus cinereus* (hoary bat), and *Lasionycteris noctivagans* (silver-haired bat). A few individuals of *Myotis lucifugus*, *Eptesicus fuscus*, and *Pipistrellus subflavus* were collected, but not in sample sizes great enough for this study.

We restricted our analysis to males that were collected from July through October, which coincides with the time frame reported by Cryan *et al.* (2012) and is the period that includes spermatogenesis, spermiation, and presumably copulation. We examined 22 individuals of *L. borealis* collected between July 16 and September 30, 26 *L. cinereus* collected between July 7 and October 4, and 15 *L. noctivagans* collected between August 19 and October 12. Data were pooled across the two years because trends in collection dates and condition of reproductive organs were similar, and there were no major fluctuations in environmental conditions between the two years.

Prior to our analyses, the testes and epididymides of all carcasses had been severed from the ductus deferens and preserved in 10% buffered formalin during the preparation of voucher specimens (Fig 1A). We removed epididymides from testes under a Nikon SMZ dissecting microscope, separated the organs, and removed any obvious adipose tissue. We blotted testes on paper towels to remove excess moisture, and then weighed testes to the nearest 0.001 gram on an Acculab L series digital balance. Maximum lengths and widths of testes were measured in millimeters with Mitutoyo Digimatic calipers, and all data were recorded in Microsoft Excel (version 12.3.6). Since Orr and Zuk (2013) found no significant difference in volume between right and left testes in a large sample of multiple genera of bats, the testis in the best condition was measured for each male, and we did not distinguish between sides. Epididymides were examined for dark pigmentation of the tunica vaginalis and degree of coiling in the caudae epididymis (Figure 1B). Both testes and epididymides were placed into plastic cassettes for immediate immersion in a Tissue-Tek II tissue processor, which rotated specimens among bins of 95% ethanol, 100% ethanol, CitriSolv™ clearing agent, and paraffin for a total of 8 hours. Processed tissues were embedded in paraffin blocks using a Tissue-Tek III Dispensing Console and Cryo Console, and then solidified in a refrigerator. Samples were sectioned at 6 micrometers with a Leica RM2125 RTS manual microtome, and 4-6 consecutive cross sections of each were placed on slides and stained with hematoxylin and eosin (Galigher and Kozloff 1971). Multiple photographs of each histological preparation at 400X were taken with a Leitz Wetzlar compound microscope mounted with a Nikon DS-Fi1 digital camera accompanied by Nikon NIS Elements D imaging software. Epididymides were examined for presence and abundance of sperm in tubules, and testes were examined for overall width and thickness of tubules, presence of sperm in the lumen, and stages of spermatogenesis. Excel was used to graph both testis mass and testis length against collection date (month/day) for each species. Excel was then used to create trend lines via regression analysis, and to compute R^2 values, t-values and p-values for all specimens collected between July and October.

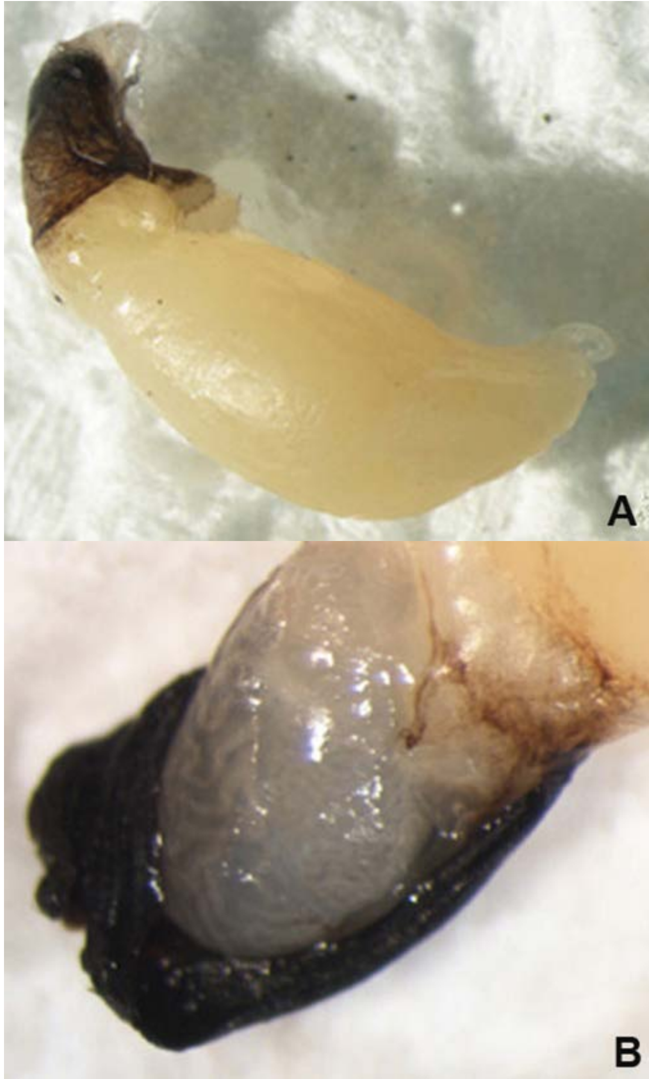

Figure 1. (A) Whole testis (right) and caudae epididymis (left) of *Lasionycteris noctivagans.* (B) Caudae epididymis of *Lasiurus borealis* exhibiting coiled tubules and pigmented tunica vaginalis.

RESULTS

Testes of all three taxa exhibited declining trends in both mass and length from mid-summer into fall. Graphs of mass and length plotted against date are presented below for each taxon. Interpretable histological slides were produced for nearly all individuals, and we chose four representative views of both testes and epididymides to document the developmental stages of sperm in seminiferous tubules and presence of sperm in caudae epididymides. Variation in expansion of tubules, prevalence of meiotic stages, and presence of sperm in the lumen was evident across the collection period in the seminiferous tubules. Nearly all hoary and eastern red bats, and most silver-haired bats, had evidence of sperm in the caudae epididymides throughout the entire collection period, although abundance in the lumen was variable.

Lasiurus borealis

Testis mass of 21 eastern red bats shows a negative trend with respect to time ($R^2 = 0.2612$, $p < 0.05$, $t = -2.59$) indicating that average testis mass began to decline near the end of summer prior to fall copulation (Fig 2A). Greatest mass was in late July to mid-August, followed by a steady decline through late September. A strong negative trend ($R^2 = 0.3433$, $p < 0.01$, $t = -3.23$) is also evident in testis length for 22 individuals (Fig 2B). The majority of testes sampled fall along the trend line in similar positions for both mass and length, suggesting a close correspondence between the two measurements. In two or three cases, the measurements for length and mass were in very different positions relative to the trend line, suggesting either an individual disconnect between the expansion of a tubule with the increase in sperm production, or a measurement error.

This trend is depicted by a series of histological slides of the testes and epididymides of four representative eastern red bats collected between mid-July and late September (Fig 3); none were found in October. One from July 22 has expanded seminiferous tubules exhibiting multiple stages of sperm cells in a multilayered arrangement, with a mostly open lumen; its epididymis exhibits some tubules with a small amount of sperm and other tubules that are empty (Fig 3A). One from approximately a month later (August 21) also exhibits greatly expanded tubules with multiple rows of sperm cells; spermatozoa are more prevalent in the lumen and the epididymis has abundant sperm in the lumen of each tubule, indicating passage of sperm from the testis to the ducts (Fig 3B). The testes of one from September 3 appear to be approaching the end of the peak period of spermatogenesis, as evidenced by fewer rows of sperm cells and more spermatids; the epididymis now contains an abundance of densely packed sperm (Fig 3C). An individual from September 30 exhibits testes with shrunken seminiferous tubules, as evidenced by the lowest mass and length, the reduced number of rows of developing sperm cells, and the mostly open lumens. The corresponding epididymis, however, exhibits expanded tubules with abundant, densely-packed sperm (Fig 3D).

Lasiurus cinereus

Testis mass of 26 hoary bats shows a negative trend ($R^2 = 0.3057$, $p < 0.01$, $t = -3.25$) indicating that the average testis mass was greatest in late July and early August and declined significantly through late September (Fig 4A). A slightly negative trend ($R^2 = 0.0798$, $p = 0.16$ [NS], $t = -1.44$) in testis length is shown for 25 bats (Fig 4B). The majority fall

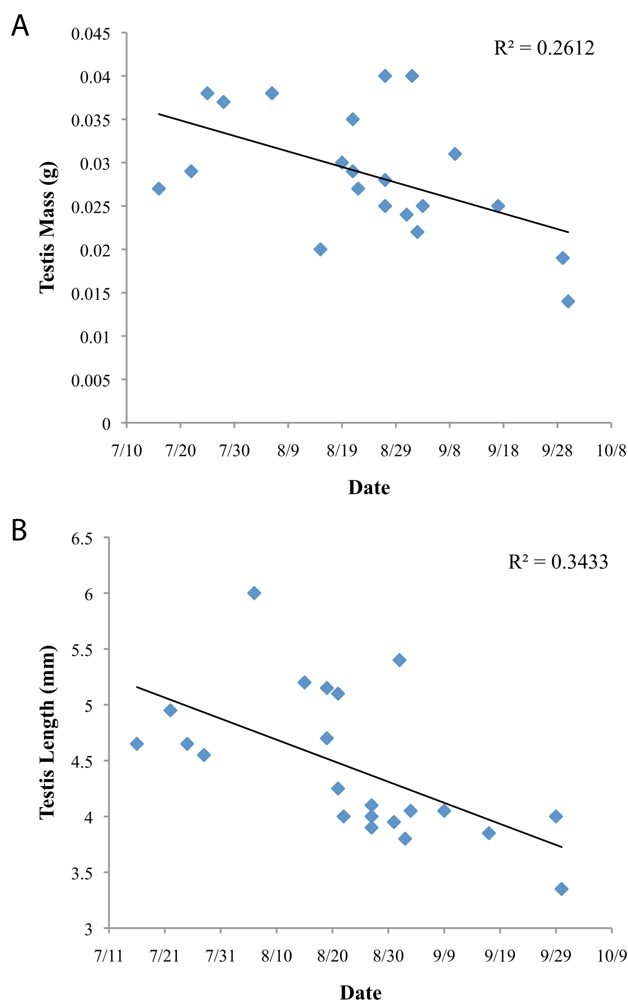

Figure 2. (A) Testis mass (g) as a function of month and day (pooled for 2007 and 2008) for 21 specimens of *Lasiurus borealis* (R^2 = 0.2612, $p < 0.05$). (B) Testis length (mm) as a function of month and day (pooled for 2007 and 2008) for 22 specimens of *Lasiurus borealis* (R^2 = 0.3433, $p < 0.01$).

along the trend line in similar positions for both mass and length, suggesting a close correspondence between the two measurements except in only two cases.

Histological preparations of the testis and epididymis of four representative hoary bats collected from late-July through mid-September are presented in Fig 5 (the only individual collected in October was inconclusive for presence of sperm). One from July 28 has expanded seminiferous tubules exhibiting multiple stages of sperm cells in a multilayered arrangement, with some sperm present in the lumen; this testis also exhibits one of the highest recorded masses (Fig 5A). The epididymis from this individual exhibits some sperm in each of the tubules, whereas *L. borealis* exhibited little or no sperm in the epididymis in July. One hoary bat collected on August 5 is at the peak of mass and length for testes, and multiple stages of sperm cells along with sperm in the lumen are evident; sperm are evident in the epididymis although it seems that sperm transfer is steady and gradual (Fig 5B). The testis of a bat from August 27 exhibits lower mass and length, and sperm cluster near the lumen; the corresponding epididymis exhibits sperm in every tubule (Fig 5C). The testis of one from September 20 exhibits the lowest mass and a relatively low length, with few developing sperm cells and a mostly open lumen (Fig 5D). The corresponding epididymis, however, exhibits expanded tubules with abundant, densely-packed sperm, approximately ten days earlier than in *L. borealis.*

Lasionycteris noctivagans

Seasonal trends for both testis mass (R^2 = 0.4187, $p < 0.01$, $t = -3.06$) and length (R^2 = 0.2894, $p < 0.05$, $t = -2.30$) show closely corresponding significant negative trajectories for 15 specimens of this species, indicating that both measurements peaked in mid-August and declined steadily through mid-October (Fig 6A, B). Nearly all bats fall along the trend line in similar positions for both mass and length, suggesting a close correspondence between the two measurements except in only two cases.

Histological preparations of the testis and epididymis of four representative silver-haired bats collected from mid-August (none were found in July) through mid-October are depicted in Figure 7. One from August 22 has expanded seminiferous tubules of a relatively high mass, exhibiting multiple stages and layers of developing sperm cells (Fig 7A). Its epididymis appears to have no sperm at all in the lumen, as is the case with one collected on August 23 (not depicted). By the end of August, however, sperm production appears to have increased dramatically and transport into the epididymis had occurred. A bat collected on August 31 exhibits an abundance of spermatozoa in the lumens of both the seminiferous tubules and epididymis (Fig 7B). By mid-September, spermatogenesis appears to be slowing, as evidenced by reduced seminiferous tubules (with low mass and length) of a bat collected on September 11 (Fig 7C); there are also fewer rows of developing sperm cells, less evidence of meiosis, and relatively few spermatids in the lumen. The tubules of the corresponding epididymis, however, are stretched and packed with abundant sperm (Fig. 7C). By mid-October, when testes are at their lowest masses and lengths, the seminiferous tubules appear to be reduced and mostly empty of sperm in the lumens, with less evidence of meiosis as seen in a bat collected on October 12 (Figure 7D). The epididymis of this individual still exhibited a significant amount of sperm in the lumens of the tubules, whereas the last recorded sperm-laden epididymides of *L. borealis* and *L. cinereus* in the study period were both in late September.

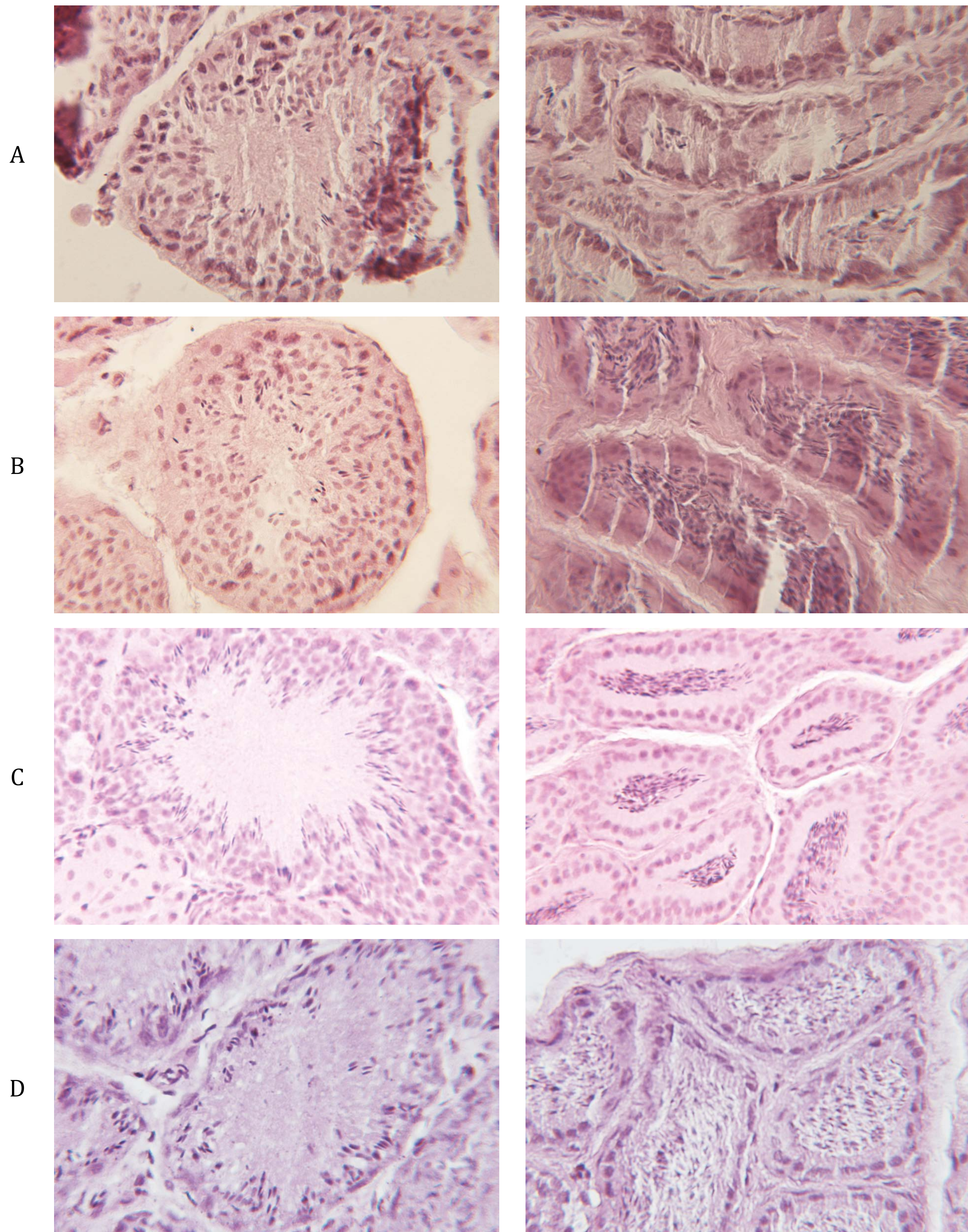

Figure 3. Seminiferous tubule of testis (left) and tubules of epididymis (right) of *Lasiurus borealis* from (A) July 22, (B) August 21, (C) September 3, and (D) September 30 (400X) (400X, hematoxylin & eosin).

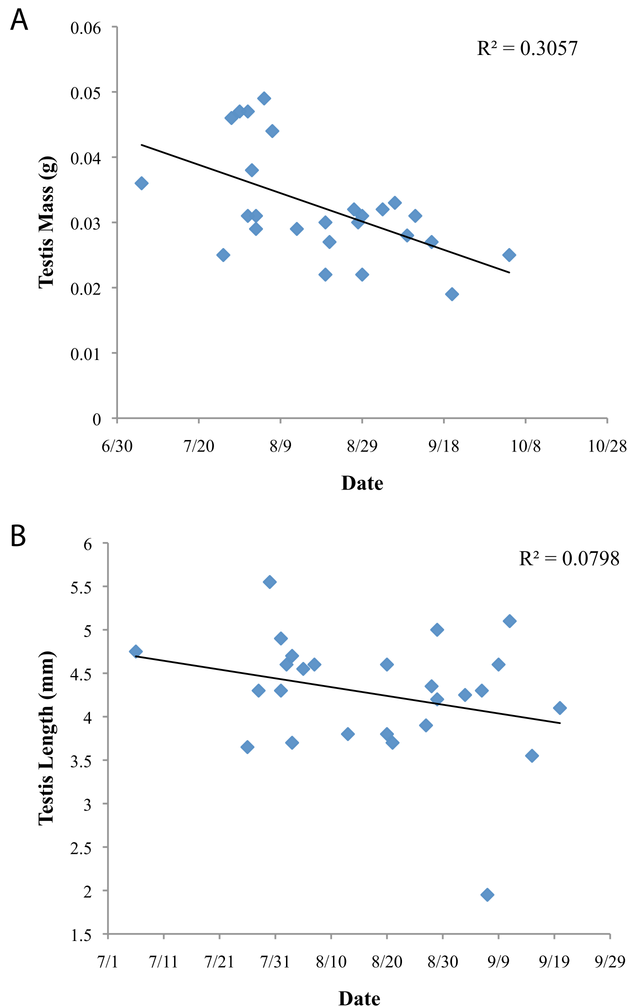

Figure 4. (A) Testis mass (g) as a function of month and day (pooled for 2007 and 2008) for 26 specimens of *Lasiurus cinereus* ($R^2 = 0.3057$, $p < 0.01$). (B) Testis length (mm) as a function of month and day (pooled for 2007 and 2008) for 26 specimens of *Lasiurus cinereus* ($R^2 = 0.0798$, NS).

DISCUSSION

Data reported here provide new information about mating readiness at the time of death for males of *L. borealis*, *L. cinereus*, and *L. noctivagans* collected at a commercial wind energy facility in eastern Pennsylvania. The majority of carcasses were found during a comparable range of time (early July through early October) to those reported by Druecker (1972) and Cryan *et al.* (2010, 2012), although the time range for *L. noctivagans* was shifted somewhat later in the season, possibly due to a later migration pattern for this species in eastern North America. All three taxa exhibited a decline in testis mass and length from a summer peak (late July in *L. cinereus* and *L. borealis*, mid-August in *L. noctivagans*) to the end of the collection period (late September in L. borealis, early October in *L. cinereus*, and mid-October in *L. noctivagans*). Active spermatogenesis, as evidenced by the presence of multiple stages and rows of sperm cells undergoing meiosis, was evident from mid-July through late August in *L. borealis*, early July through mid-August in *L. cinereus*, and mid-August through mid-September in *L. noctivagans*, with seminiferous tubules undergoing meiosis still visible in a silver-haired bat from mid-October.

The first evidence of sperm being present in the epididymis, even in small amounts, is in an eastern red bat from July 16, in a hoary bat from July 6, and in a silver-haired bat from August 31. Since few silver-haired bats were found in August, it is possible that an earlier incidence of sperm in the epididymis was missed due to lack of samples. Epididymides had abundant sperm present by August 15 in *L. borealis*, by August 13 in *L. cinereus*, and by August 31 in *L. noctivagans* (the first time it was observed, it was abundant). Last evidence of sperm consistently present in epididymides was on September 30 in *L. borealis*, September 20 in *L. cinereus*, and October 12 in *L. noctivagans*. Cryan *et al.* (2012) reported on percentage of individuals with sperm present in the caudae epididymides as a function of collection period, which they divided into three approximately one-month-long intervals: July 10 – August 12, August 13 – September 14, and September 15 – October 17 (Cryan *et al.* 2012, Table 1). Sperm were present in epididymides in each of the three time intervals for *L. borealis* and *L. cinereus*, and in only the later two intervals for *L. noctivagans*. All 15 eastern red bats had sperm present throughout the three collection intervals. Of 70 hoary bat epididymides, 70% of those from the first collection interval had sperm present, 90% had sperm in the second interval, and 100% had sperm present in the third interval. Of six silver-haired bat epididymides, none from the first collection interval had sperm present, 50% had sperm in the second interval, and 100% had sperm present in the third interval (Cryan *et al.* 2012).

Data reported in our study correspond very closely to those reported by Cryan *et al.* (2012) for *L. borealis*, *L. cinereus*, and *L. noctivagans* in Texas, New York, Alberta, and Manitoba. All sixteen specimens of eastern red bats examined in our study also all had sperm present in the epididymides throughout our entire sampling period (July 16 – September 30). Of our 21 hoary bats collected between July 6 and October 4, all had sperm present in the first interval, 91% in the second, and all in the third interval. Our 12 silver-haired bats collected between August 19 and October 12 correspond exactly to percentages reported by Cryan *et al.* (2012) for the second and third collection intervals (we did not have access to any bats of this species from the first collection interval as defined by Cryan *et al.* (2012)).

Data reported here for *L. borealis*, *L. cinereus*, and *L. noctivagans* from Pennsylvania support and strengthen the conclusions of both Druecker (1972) and Cryan *et al.* (2010, 2012). The few differences in results could easily

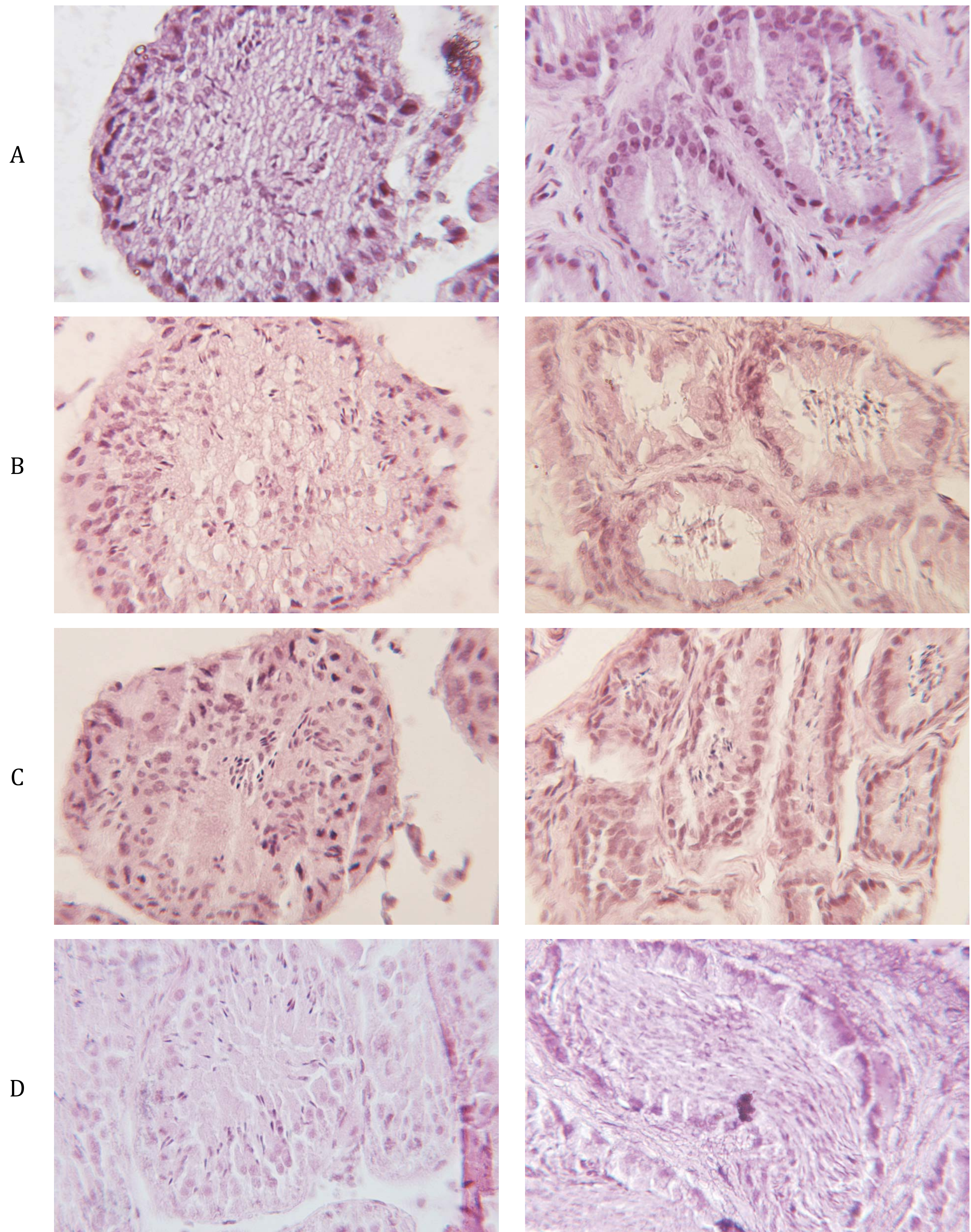

Figure 5. Seminiferous tubule of testis (left) and tubules of epididymis (right) of *Lasiurus cinereus* from (A) July 28, (B) August 5, (C) August 27, and (D) September 20 (400X) (400X, hematoxylin & eosin).

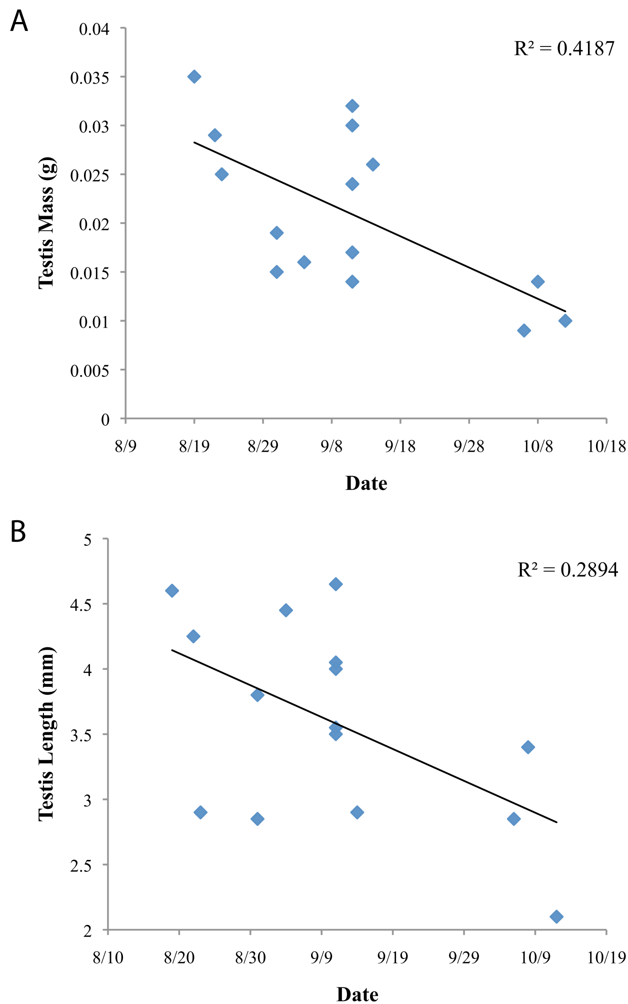

Figure 6. (A) Testis mass (g) as a function of month and day (pooled for 2007 and 2008) for 15 specimens of *Lasionycteris noctivagans* ($R^2 = 0.4187$, $p < 0.01$). (B) Testis length (mm) as a function of month and day (pooled for 2007 and 2008) for 15 specimens of *Lasionycteris noctivagans* ($R^2 = 0.2894$, $p < 0.05$).

be due to sampling error or geographic variation. For example, Druecker (1972) noted moderate amounts of sperm in epididymides of *L. noctivagans* in mid-July, whereas sperm were not noted in any of our individuals until late August. Both our study and Cryan *et al.* (2012) concluded that *L. noctivagans* showed evidence of the latest consistent occurrence of sperm in epididymides, although we were unable to examine *Lasiurus* specimens from October (no *L. borealis* were found, and the only *L. cinereus* individual was inconclusive); in addition, a single *L. cinereus* individual found outside of the study period in November revealed a trace of sperm. Cryan *et al.* (2012) reported the earliest evidence of epididymal sperm in *L. borealis*, while we found earliest evidence in *L. cinereus*. Finally, Cryan *et al.* (2012) noted the biggest decrease in testis size in *L. cinereus*, whereas we noted the steepest decline in *L. noctivagans* (evidenced by both R^2 and p values).

Minor discrepancies notwithstanding, data reported here provide important additional information about the timing of male reproductive events in Pennsylvania populations of the species of migratory tree-roosting bats that are most vulnerable to death at wind turbines, and support the conclusions of Cryan *et al.* (2012). The majority of males of the three species studied here showed evidence of active spermatogenesis during the summer, and a reduction of size of testes and number of meiotic stages as the season progressed into fall. Most individuals collected of all three taxa had at least some sperm present in the epididymides, and all exhibited abundant sperm at some point in August. These observations do not demonstrate that bats were actively copulating at this time, and in fact there is evidence in some small mammals (e.g. rodents such as *Peromyscus*) that mere proximity to females can increase gonad mass, sperm counts, and testosterone production, regardless of whether mating occurs (Demas and Nelson 1998). In addition, testis size and testosterone production increase in some vespertilionid males upon waking from hibernation in the spring (Martin and Bernard 2000). However, such factors are unlikely to have a consistent influence in bats throughout the year, as reproductive behaviors are highly seasonal (Kwiecinski and Damassa 2000) and males and females are not confined together.

The fact that most males died in a state of mating readiness is consistent with the hypothesis that bats are attracted to wind turbines when searching for mates, and warrants further evaluation. Many hypotheses that address proximate and ultimate causes of turbine deaths in bats favor coincidental migration as an ultimate cause (see Cryan 2008, Cryan and Barclay 2009, Cryan *et al.* 2012); for example, bats may be predisposed toward flying through corridors that are suitable for wind turbines. If the search for mates is actually an ultimate cause of fatality because migrating bats perceive them as tall trees, then there should be evidence that bats were in a state of mating readiness (Cryan and Barclay 2009, Cryan *et al.* 2012) at the time of death. Most hypotheses advanced have not been adequately tested, however, and this study provides additional information about reproductive status and timing of mating events needed to better understand mating behaviors and to test hypotheses about mortality. More information about the reproductive condition of female bats killed at wind turbines will also help when evaluating these hypotheses and formulating conservation efforts for vespertilionid bats.

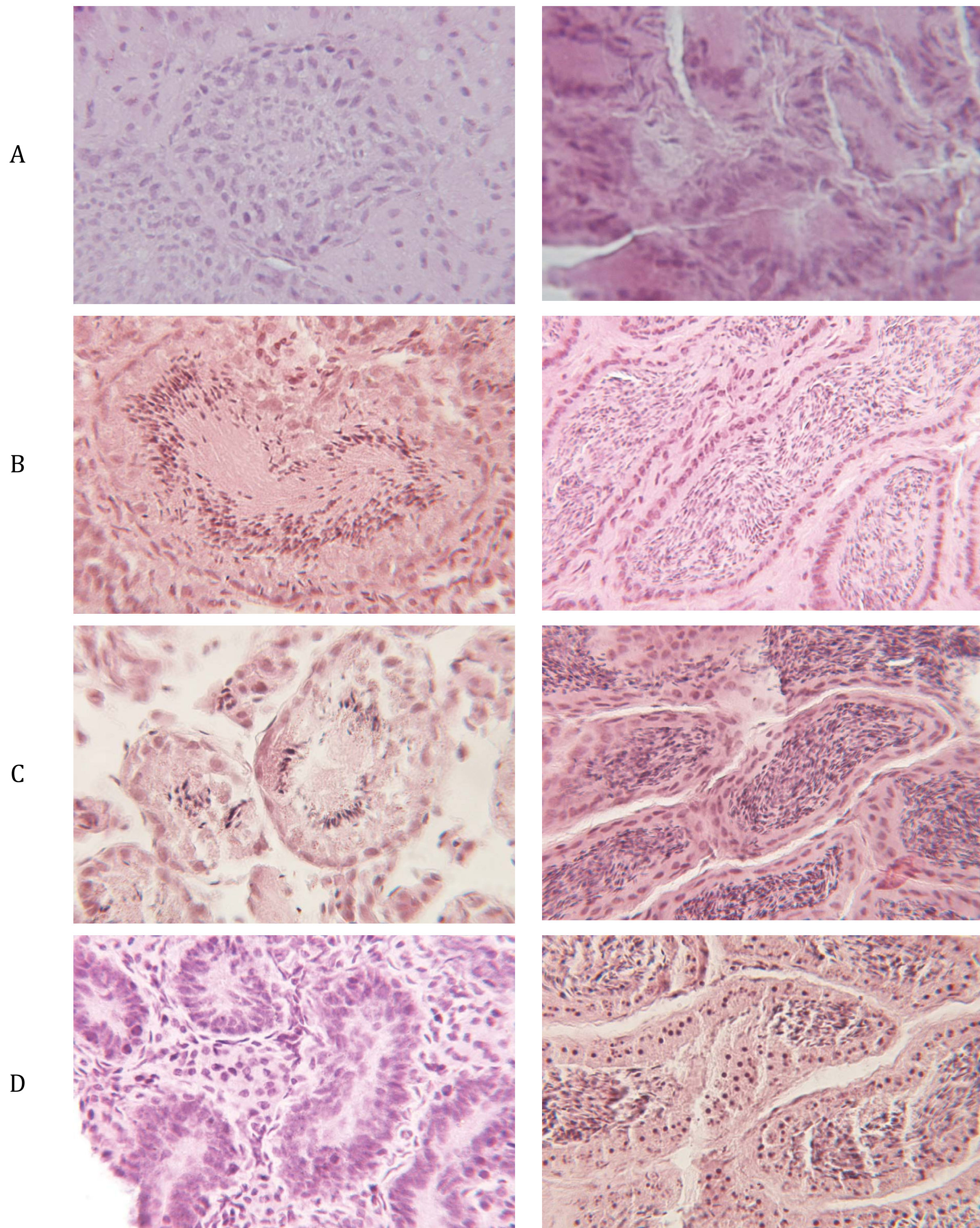

Figure 7. Seminiferous tubule of testis (left) and tubules of epididymis (right) of *Lasionycteris noctivagans* from (A) August 22, (B) August 31, (C) September 11, and (D) October 12 (400X) (400X, hematoxylin & eosin).

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RESEARCH NOTE: MORPHOLOGICAL AND DNA ANALYSIS TO DETERMINE CANID SPECIES[1]

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ABSTRACT

A partially decomposed carcass of a canid was submitted to the Northeast Wildlife DNA Laboratory (NEWDL) on September 11, 2014 for identification. A possible case of animal cruelty was suspected. The carcass was characterized by active decay. To determine if the animal was a domestic dog or coyote various identification techniques were employed. Morphological and molecular techniques were combined to determine the identification due to the state of decomposition. Various biological samples were collected, including hair, tissue and paws. Molecular techniques to compare mitochondrial DNA success and nuclear DNA success were evaluated and reported. [J PA Acad Sci 88(4): 247-253, 2014]

INTRODUCTION

In a suspected case of animal cruelty involving canids, it was necessary to determine if the carcass submitted was a domestic dog or coyote. Four species of *Canis* occur in the United States. The four include the red and gray wolf, the coyote, and the domestic dog. All four can interbreed (Whitaker and Hamilton 1998). Coyotes are a moderately sized canid; they are larger than foxes but smaller than wolves. The coyote is a carnivorous generalist feeding mainly on mammalian flesh. This generalist behavior enables the coyote to colonize and breed successfully in virtually any North American habitat (Fener *et al.* 2005). Native to western North America, coyotes have increased in numbers and have increased their geographical range during the past fifty years, due in part to anthropogenic factors. Coyotes have doubled their home ranges and are now found throughout most of North America. In addition to occurring in natural areas, coyotes are also found in a range of human-populated areas, including rural farms, suburbs and cities. According to the American Veterinary Medical Association, it is estimated that 70-80 million dogs are owned in the United States which is approximately 37-47% of all households in the United States having a dog.

For species identification, three reliable and court-accepted methods commonly employed by forensic laboratories are molecular testing, microscopic, and morphological examination (Wilson-Wilde, 2010; Linacre and Tobe 2011). The advantage of the molecular approach is its high accuracy and sensitivity; however, it is time-consuming and expensive. In the case of decomposed carcasses, molecular techniques to identify species may be hard to evaluate due to degrading of DNA evidence. Traditional hair morphological examination and classic techniques of comparative anatomy to identify animal remains to species are viable alternatives for species identification. For an initial microscopic and morphological examination, documentation of evidence characteristics are followed with detailed comparisons between the evidence and reference specimens, or standards. The verification of diagnostic characters with reference specimens or authoritative published data is essential for identifications. The disadvantage of morphological and microscopic examination is having access to a reference database specific to the probable species identification based on hair morphology has been reported for many animal species (van den Broeck *et al.* 2001; Deedrick and Koch 2004; Marinis and Asprea 2006; Sato *et al.* 2006; Espinoza *et al.* 2008; Sahajpal *et al.* 2008, 2009). A wide range of hair morphological features has also been reported in these different species. However, most studies cannot be reliably used in the forensic context, as the critical process of validation is lacking. An important aspect of validation is a large enough sample size and a reporting of scientific findings with statistics such as likelihood ratios. The conventional hair morphology-based method is subjective, relying heavily on expert opinions. Comparisons can only be made when reference samples are readily available. Also, variations in hair morphology, which are biologically meaningful (van den Broeck *et al.* 2001) have been overlooked in previous studies.

Most dogs cannot be confused with any native PA mammal. In some larger dogs such as malamutes or German shepherds, the crania are morphologically similar to coyotes and it may

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be confusing when trying to differentiate between the two (Callaway, 2001). Howard (1949) described a technique to identify coyote and dog skulls by determining the ratio of two measurements. To identify a skull the reciprocal of the ratio of the length of the upper molar tooth-row divided by palatal width between the upper first premolars. If the molar tooth-row is 3.1 or more times that of the palatal width, the specimen is a coyote, but if it is less than 2.7 times it is a dog. The snout of coyotes in comparison to that of a dog is long and thin. The ratio of the length of the molariform tooth row to the distance between the inside faces of the first two molariform teeth is usually under 3.0 in dogs, 3.1 to 3.6 in hybrids (coydogs), and greater than 3.6 in coyotes (Whittaker and Hamilton 1998).

The most common molecular genetic approach to species identification within mammals involves sequence analysis of the mitochondrial DNA cytochrome b gene. Mitochondrial DNA has greater use in forensic casework than nuclear DNA due to the abundance of mitochondrial DNA in cells compared to nuclear DNA. Most biological samples submitted to a forensic laboratory for analyses have low DNA quality thus increasing the sensitivity of molecular testing is important for sensitivity and accuracy (Branicki, Kupiec and Pawlowski 2003). Many techniques for amplification of the mitochondrial cytb gene have been validated throughout the scientific literature and have been developed specifically for forensic use (Zehner *et al.* 1998; Holland and Parsons, 1999; Parson *et al.* 2000; Branicki, Kupiec and Pawlowski 2003; Bravi *et al*., 2004). Other molecular methods such as microsatellite analysis can be used to identify the gender and individual genotypes of various mammals.

The goal of this research note is to determine the animal species submitted to the Northeast Wildlife DNA Laboratory (NEWDL). The objectives were to evaluate microscopic, morphological and molecular testing in identification of a canid species. For molecular analysis mitochondrial and nuclear DNA analysis was completed for comparison.

MATERIALS AND METHODS

Description of Evidence

A partially decomposed unknown canid was submitted to the NEWDL on September 11, 2014 for identification. The carcass was characterized by active decay. Tissue loss occurred as a result of the feeding by maggots and the purging of decomposition fluids into the surrounding environment (Carter and Tibbett 2008).

Morphological Characteristics to Distinguish Canids

The skull was removed, cleaned (Mooney *et al.* 1982) and used for morphological measurements. The snout of a coyote is known to be longer and narrower than those of dogs. A ratio incorporating this characteristic was used to determine the species. The ratio is the palatal width (between inner margins of alveoli of upper, first premolars) divided by the length of the upper molar tooth-row (anterior margin of alveolus of first premolar to the posterior margin of the last molar alveolus) (Howard, 1949).

Qualitative Hair Characteristics

Dorsal guard hairs were selected for examination. Guard hairs are important in species identification as they exhibit diagnostically reliable features (Harrison, 2002). The hair samples were cleaned before examination with sterile distilled water solution to remove dust and debris. Each dried hair strand was mounted on a microscope slide with a drop of distilled water and a cover slip. The hair was then examined under 100x and 450x magnification.

The qualitative hair characteristics that were recorded for each hair consisted of one whole- mount and the characteristics of the follicle, proximal, middle and distal parts. The whole mount characteristic was medulla type. The hair-section characteristics were hair color, cortex color, scale margin, scale separation, and scale pattern. The classification of these characteristics was based on Brunner and Coman (1974) and Teerink (1991). Hair samples were compared to reference samples of *Canis familiaris* and *Canis latrans*.

DNA Analysis

Hair, portions of muscle tissue and metacarpal pad were removed and placed into separate 1.5 mL microcentrifuge tubes. A standard DNA tissue extraction method was completed on all three samples using a Qiagen DNeasy Tissue and Blood DNA extraction kit (Fresco, California). Samples were incubated overnight at 56 °C. A 17 microsatellite multiplex was used for genotyping and a universal mitochondrial mammal specific primer (MCB398/869) was used to amplify a 472 base pair amplicon. Polymerase chain reaction mixtures were prepared in an 11 µL reaction utilizing Qiagen Multiplex PCR reagents. Genotyping was completed with a touchdown PCR protocol and DNA sequencing PCR protocol for MCB398/869 included 35 cycles of 95 °C for 45 seconds, 51 °C for 60 seconds and 72 °C for 120 seconds. Samples were analyzed using an Applied Biosystems ABI3130 genetic analyzer.

Following amplification of each sample, mitochondrial DNA was imported into NCBI blast genomic database

for species identification. Sequences were aligned using ClustalW and lengths of sequences were recorded. Nuclear DNA genetic profiles for 17 microsatellites were recorded and the success rate of amplification was calculated.

RESULTS

Morphological characteristics to distinguish canid species

This specimen had a longer, narrow muzzle than would be seen with other canid species. Examination of the skull revealed a large sagittal crest with orbits that face forward and the nuchal crest was wide and well-defined (Figure 1A). The specimen had mixed dentition with large canine teeth, crushing molars and distinctive carnassial teeth. The upper canine teeth in this study reached the imaginary lined formed by the two mandibular foramina pits in the lower jaw, which is a characteristic seen in coyotes (Elbroch, 2006). The palate to tooth row ratio was calculated to be 3.5 (A = 7.0 cm/ B= 2.0 cm) (Figure 1B).

Qualitative Hair Characteristics

The hair coloration was grayish brown on the upper parts, with the throat and abdominal hair whitish. The forelegs, side of the head, muzzle and feet were reddish brown. The back had fulvous colored underfur, and long, black-tipped guard hairs with the tail having a black tip. The follicle was spade shaped (Figure 2A), the proximal medullar was characterized as unbroken and cellular (Figure 2B), and the distal medulla was characterized as simple unbroken amorphous (Figure 2C).

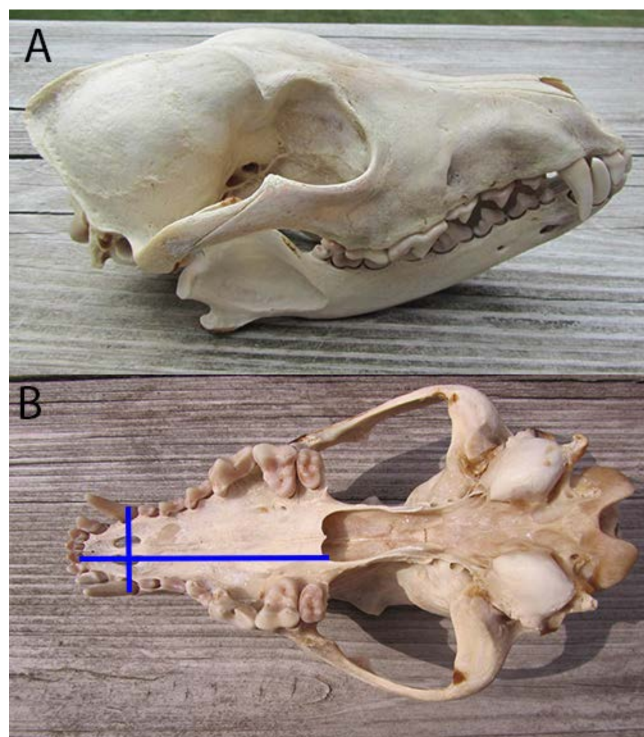

Figure 1: (A) Skull illustrating large sagittal crest, well-defied nuchal crest, orbit facing forward and large canine teeth. (B) Ventral view of the *Canis* skull. The palate to tooth row ratio calculated was and illustrated by the two perpendicular lines in photo B (A = 7.0 cm/ B = 2.0 cm).

DNA Analysis

DNA analysis was completed with mitochondrial primers and nuclear microsatellite primers. Three samples (as described in materials and methods) were compared for quality of DNA analysis. The three samples were compared to a known coyote (Coyote 2) which was represented as the control. A successful sequence length for each sample was 388/472 (82.2%) base pairs for the paw pad, 397/472 (84.1%) base pairs for the hair sample and 398/472 (84.3%) base pairs for the muscle tissue. Each sample was imported into NCBI

Table 1. Microsatellite genotype results of multiplex I for coyote H-029. Three samples collected from the partially decomposed carcass were analyzed.

Sample	Cxx121		Cxx172		Cxx103		Cxx20		Cxx377		Cxx173		Cxx109		Cxx200		Cxx250	
H-029 Tissue	93	99	142	142	76	78	120	124	160	160	90	111	X	X	X	X	X	X
H-029 Hair	93	99	142	142	76	78	120	124	160	164	99	111	X	X	X	X	X	X
H-029 Paw	93	99	142	142	76	78	120	124	160	164	99	111	X	X	X	X	X	X

Table 2. Microsatellite genotype results of multiplex II for coyote H-029. Three samples collected from the partially decomposed carcass were analyzed.

Sample	Cxx2001		Cxx2010		Cxx2062		Cxx225		Cxx403		Cxx2145		FH2054		Cxx2004	
H-029 Tissue	142	142	228	228	133	133	162	166	X	X	X	X	X	X	307	315
H-029 Hair	138	142	228	228	123	133	162	166	279	279	288	296	151	155	307	315
H-029 Paw	138	142	228	228	X	X	162	166	279	279	288	296	151	155	307	315

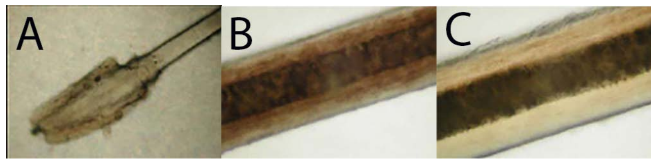

Figure 2: (A) The follicle was spade shaped; (B) the proximal medullar was characterized as unbroken and cellular. (C) The distal medulla was characterized as simple unbroken amorphous.

blast and all three samples were 100% identical to *Canis latrans*, coyote. Sequences were aligned using ClustalW for a secondary assessment. All three samples were compared to a known coyote and of the 388 base pair alignment, there was a 100% identical match made to *Canis latrans*. Figure 3 illustrates the alignment of DNA sequences of the cytb gene of the paw pad, muscle tissue and hair.

Seventeen fluorescently labeled microsatellite loci were used to analyze the nuclear DNA from the hair, muscle tissue and paw pad of the unknown canid. Based on DNA typing, the samples showed the same alleles at 10 of the 17 tested loci: Cxx121, Cxx172, Cxx103, Cxx20, Cxx173, Cxx2001, Cxx2010, Cxx225, and Cxx2004 (Tables 1 and 2) (Ostrander *et al.* 1993, 1995; Mellersh *et al.* 1997). Cxx109, CXX200 and Cxx250 did not amplify any alleles at these loci. Cxx2062 produced alleles for the muscle tissue and hair follicles tested but not for the paw pad. CXX403, CXX2145 and FH2054 loci did not produce alleles for the muscle tissue but was successful for the hair follicles and tissue from the paw pad. Allelic dropout was observed only in the muscle tissue at 3 loci, CXX377, CXX2001 and CXX2062. The success rate for the nuclear DNA microsatellite analysis was 11/17 (64.7%) for the muscle tissue, 13/17 (76.5%) for the paw pad and 14/17 (82.3%) for the hair (Table 1 and 2).

DISCUSSION

The eastern coyote is firmly established throughout PA. Coyotes thrive in suburban settings and even some urban ones, because of the availability of food and the lack of predators (Gehrt and Riley 2010; Feinstein, 2011). Due to close ancestors and similar characteristics it is possible to mistake a coyote for a domestic dog and vice versa. Various validation reports for molecular analysis (Kocher *et al.* 1989; Holland and Parsons 1999; Branicki *et al.* 2003), morphological analysis (Howard, 1949; Elbrouch, 2006) and hair analysis (Brunner and Coman 1974; Teerink, 1991) are currently documented throughout the scientific literature. This research note evaluated species identification techniques in an unknown decomposed canid carcass submitted to the NEWDL.

In this study morphological and molecular techniques were combined to determine the identification of the canid species. Despite the confusion that may still arise when differentiating between the two species, especially if the cranium is incomplete, craniometric evaluations may be utilized to establish mathematical guidelines for distinguishing the two species. The palate to tooth row ratio for the skull in this study was determined to be 3.5 (Figure 1B). If the molar tooth row is 3.1 or more times that of a palatal width, the specimen is a coyote. If the molar tooth row is 2.7 times or less, the specimen is a dog (Howard, 1949). Our results indicate along with other skull features that the unknown canid is a coyote (*C. latrans*).

Each species of animal possesses hair with characteristic length, color, shape, root appearance, and internal microscopic features that distinguish one animal from another. The root of a domestic dog is spade shaped whereas the root of the coyote is bulbous and spade shaped. The proximal medulla of a coyote is unbroken and cellular whereas a domestic dog is simple unbroken and amorphous. One of the most distinguishing hair characteristics between a domestic dog and a coyote is a banding pattern of the hair. These characteristics were all used for species identification in this analysis. Characteristics of the guard hair follicle, distal medulla and proximal medulla of the unknown canid carcass were consistent with that of a coyote when compared

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coyote2    CATCCCCTATATCGGAACCAACTTAGTAGAATGAATCTGAGGCGGCTTCTCAGTGGACAA
Paw        --TCCCCTATATCGGAACCAACTTAGTAGAATGAATCTGAGGCGGCTTCTCAGTGGACAA
Tissue     --TCCCCTATATCGGAACCAACTTAGTAGAATGAATCTGAGGCGGCTTCTCAGTGGACAA
Hair       ----CCCTATATCGGAACCAACTTAGTAGAATGAATCTGAGGCGGCTTCTCAGTGGACAA
consensus  --tcCCCTATATCGGAACCAACTTAGTAGAATGAATCTGAGGCGGCTTCTCAGTGGACAA

coyote2    AGCAACCCTAACACGATTCTTTGCATTCCACTTTATCCTCCCATTCATTATCGCAGCCCT
Paw        AGCAACCCTAACACGATTCTTTGCATTCCACTTTATCCTCCCATTCATTATCGCAGCCCT
Tissue     AGCAACCCTAACACGATTCTTTGCATTCCACTTTATCCTCCCATTCATTATCGCAGCCCT
Hair       AGCAACCCTAACACGATTCTTTGCATTCCACTTTATCCTCCCATTCATTATCGCAGCCCT
consensus  AGCAACCCTAACACGATTCTTTGCATTCCACTTTATCCTCCCATTCATTATCGCAGCCCT

coyote2    AGCAATAGTACACCTCCTATTTCTACATGAGACCGGATCCAACAACCCCTCAGGAATCAC
Paw        AGCAATAGTACACCTCCTATTTCTACATGAGACCGGATCCAACAACCCCTCAGGAATCAC
Tissue     AGCAATAGTACACCTCCTATTTCTACATGAGACCGGATCCAACAACCCCTCAGGAATCAC
Hair       AGCAATAGTACACCTCCTATTTCTACATGAGACCGGATCCAACAACCCCTCAGGAATCAC
consensus  AGCAATAGTACACCTCCTATTTCTACATGAGACCGGATCCAACAACCCCTCAGGAATCAC

coyote2    ATCAGACTCAGACAAAATTCCATTTCACCCTTACTACACAATCAAAGACATCCTAGGAGC
Paw        ATCAGACTCAGACAAAATTCCATTTCACCCTTACTACACAATCAAAGACATCCTAGGAGC
Tissue     ATCAGACTCAGACAAAATTCCATTTCACCCTTACTACACAATCAAAGACATCCTAGGAGC
Hair       ATCAGACTCAGACAAAATTCCATTTCACCCTTACTACACAATCAAAGACATCCTAGGAGC
consensus  ATCAGACTCAGACAAAATTCCATTTCACCCTTACTACACAATCAAAGACATCCTAGGAGC

coyote2    CTTACTCCTACTCCTAGCCCTAATATCACTAGTCTTATTCTCACCAGACCTATTAGGAGA
Paw        CTTACTCCTACTCCTAGCCCTAATATCACTAGTCTTATTCTCACCAGACCTATTAGGAGA
Tissue     CTTACTCCTACTCCTAGCCCTAATATCACTAGTCTTATTCTCACCAGACCTATTAGGAGA
Hair       CTTACTCCTACTCCTAGCCCTAATATCACTAGTCTTATTCTCACCAGACCTATTAGGAGA
consensus  CTTACTCCTACTCCTAGCCCTAATATCACTAGTCTTATTCTCACCAGACCTATTAGGAGA

coyote2    CCCAGATAACTACACCCCTGCAAACCCCCTAAATACCCCACCACATATCAAACCCGAATG
Paw        CCCAGATAACTACACCCCTGCAAACCCCCTAAATACCCCACCACATATCAAACCCGAATG
Tissue     CCCAGATAACTACACCCCTGCAAACCCCCTAAATACCCCACCACATATCAAACCCGAATG
Hair       CCCAGATAACTACACCCCTGCAAACCCCCTAAATACCCCACCACATATCAAACCCGAATG
consensus  CCCAGATAACTACACCCCTGCAAACCCCCTAAATACCCCACCACATATCAAACCCGAATG

coyote2    ATATTTCCTATTCGCCTATGCTATCCTACGATCCATTCCCAATAAACTAGGAGGCGTACT
Paw        ATATTTCCTATTCGCCTATGCTATCCTACG------------------------------
Tissue     ATATTTCCTATTCGCCTATGCTATCCTACGATCAATCCCT--------------------
Hair       ATATTTCCTATTCGCCTATGCTATCCTACGATCAATCCCTA-------------------
consensus  ATATTTCCTATTCGCCTATGCTATCCTACGatc-attccc--------------------
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Figure 3: DNA sequence alignments from the paw pad, muscle tissue and hair using universal mitochondrial DNA primers. Sequences were compared to a known coyote sample (Coyote 2). The sequence alignments bases A, C, T, and G highlighted in green indicate that the sequences are identical for the three sequenced samples.

to reference samples.

DNA was successfully extracted from three sources from the decaying carcass. Analysis of nuclear DNA employing 17 microsatellite markers had an average success rate of 74.5% of the DNA microsatellites amplifying. Sequence analysis of the mitochondrial cytochrome b gene resulted in an average success rate of 83.5%. Comparison of the sequence analysis used for mtDNA cytochrome b had a 9% higher success rate than the nuclear DNA analysis using microsatellites. The results illustrated in this paper are consistent with that of Parson *et al.* (2000) and Branicki *et al.* (2003) in which the mitochondrial DNA analysis of the cytochrome b gene is more efficient and accurate in species identification when using low quality DNA samples.

When analyzing a partially decomposed carcass to collect forensic evidence, knowing the type of tissue to collect for the greatest success is important. In this study three samples were collected from various areas; hair, metacarpal pad and muscle tissue. All three samples were amplified using mtDNA and nuclear DNA. Success rate of each sample was compared to determine the biological sample which would produce the best DNA quality for analysis. The metacarpal pad had an 82.2% success rate amplifying 388/472 base pairs of the cytb gene and a 76.5% success rate in amplifying 13/17 microsatellites for nuclear DNA analysis. The sample with a low standard of quality was the muscle tissue sample which

amplified 64.7% (11/17) microsatellites with three of the amplified microsatellites exhibiting allelic dropout. Allelic dropout is known to occur when DNA is degraded ultimately resulting in low DNA concentrations. Although the muscle tissue on average had a low success rate, it had the highest success rate for mtDNA analysis, 84.3% or 398/472 base pairs of the cytb gene. The sample with the highest success rate analyzed in this study was the hair sample amplifying 14/17 (82.3%) of the microsatellite and 84.1% or 397/472 base pairs of the cytb gene. Although all samples were successful in amplifying DNA, this study suggests the best biological sample to analyze when handling a partially decomposed carcass is the hair. This may be due in part to some epithelial cells being present on the follicle of the hair when removed from the carcass increasing the number of cells present for analysis. Further molecular analysis with a larger sample size is needed to compare the success of biological samples.

Overall, any one of the four methods utilized, morphological characteristics of hair and skull and molecular analysis using mitochondrial and nuclear DNA, in this case, would enable successful species identification. All four methods consistently identified the unknown carcass as a *Canis latrans* (coyote). Future analysis is needed with a larger sample size of decomposed carcasses of various species analyzed with the four methods presented in this research note to further evaluate the success.

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