JOURNAL

OF THE PENNSYLVANIA ACADEMY OF SCIENCE

Founded on April 18, 1924

ISSN: 1044-6753

December 2015
Volume 89(2)

Carl R. Pratt, Ph.D.
Editor
Department of Biology
Immaculata University
Immaculata, PA 19345

PAS Home Page: http://pennsci.org

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.

Book (Select pages)-Snedecor, G. W. and W. G. Cochran. 1976. Statistical Methods. The Iowa State Univeristy Press. Ames, IA, 237-238.

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 89(2): 43-47, 2015

RESEARCH NOTE: COMPARISION OF GROWTH AMONG DIFFERENT AGE CLASSES OF LARGEMOUTH BASS (*MICROPTERUS SALMOIDES*) POPULATIONS IN TWO IMPOUNDENTS IN NORTHWEST PENNSYLVANIA[1]

MICHAEL ENRIGHT[2], FRED J. BRENNER[3], LARISSA CASSANO[4], AND KATHERINE R. BEYER-KRAMER[5]

Biology Department, Grove City College, Grove City, PA 16127

ABSTRACT

The length of largemouth bass (*Micropterus salmoides*), as determined from scale samples, was calculated based on the formula $L_n = (S_n/S)L$. L_n is length in the n^{th} year, S_n is the length of the annulus in the n^{th} year, S is the total scale length and L is the total length of the fish at capture. These values were compared for two impoundments, Lake Latonka and Lake Wilhelm in northwestern Pennsylvania. The weight (W_o) of fish at capture was compared with the estimated weight (W_e) based on a regression equation $W_e = al^n$ and the length-weight relationship $W_e = l^n$. Fish in both Lake Latonka and Lake Wilhelm exhibited a significant length-weight correlation, except for the 0+ age class in Lake Latonka. There was a significant correlation between the expected and observed weight in both impoundments. During the first year, largemouth bass in Lake Latonka, exceeded the growth of largemouth bass in Lake Wilhelm, but in succeeding years the growth of fish in Lake Wilhelm exceeded that of fish in Lake Latonka. This difference may be the result of food availability. [J PA Acad Sci 89(2): 43-47, 2015]

INTRODUCTION

For over five decades, scale annuli have been used to determine the age and growth of a variety of fish species. Although other methods (i.e. otholiths, scales, and vertebrae) (Gunn *et al*., 2008) have been used to determine the age and growth of fish species, scale annuli are least invasive. Scale annuli form annually during the life of the fish. The distance from the center of the annulus and scale length is proportional to the length of the fish at the time of annulus formation. This allows for the determination of the length of fish at the time the annulus was formed. Because the weight (W) of a fish is a function of the cube length (L^3), it is possible to predict the weight of the fish at a given age based on their length when each annulus was formed. However, environmental factors, including temperature, food availability, spawning and other stresses may affect annuli development and growth.

The survival of 0+ year class is dependent on the growth of the fry during the early life stages of the species. Food availability, temperature and other environmental factors play an integral role in the growth rate, but the effect of these factors may vary among species (Shoup *et al*., 2007). Although food is relatively abundant during the summer months, predation is a significant source of mortality in the 0+ age class (Garvey *et al*., 2004). Since survival is dependent on growth, fish with higher growth rates are more likely to survive to the next year class when predators are present (Garvey *et al*., 2004). During the winter months, mortality is dependent not only on body size, but also energy content, energy density, and mass specific metabolism (Fullerton *et al*., 2000, Garvey *et al*., 2004).

In addition to food availability, population density (Vollestad and Olsen, 2008) is also an important factor in the growth of fish, especially during the first year. According to Slaughter and coworkers (2008) for the 0+ age class, prey availability and population density are the most important factors for the growth of largemouth bass transplanted from northern or southern populations. Some authors have suggested that food availability is a major factor in fish growth. Amoah and coworkers (2008) reported that carbohydrate availability directly impacted the growth rate of largemouth bass. The largemouth bass in their study experienced a more rapid growth and more efficient metabolism when carbohydrates were maintained at < 20% of their diet. Copeland and coworkers (2008) reported that the growth rate of four fish species in two different impoundments was related to food intake. Energy storage

[1]Accepted for publication April 2015.
[2]Current address: Conservation Manager, Five Rivers Metroparks, 409 E. Monument Ave, Dayton, Ohio 45402.
[3]Corresponding Author
[4]Current address: Mercer County Conservation District, Court House, Mercer, PA 16137
[5]Current Address: 13 Woodmont Drive, Delmar, New York 12054

was positively correlated with the relative weight of all four species, including the largemouth bass. Fish species may, however, exhibit different responses to population density. The objective of the current study was to compare the growth rate of largemouth bass populations in two impoundments in northwest Pennsylvania.

STUDY AREAS

Lake Latonka

Lake Latonka is located in rural residential development consisting of 650 homes constructed on 513 hectares surrounding a 110 hectare lake. The lake was created by damming Coolspring Creek in the 1960s. Lake Latonka is located 3 km north of US Route 62 and 3 km west of Interstate 79 in central Mercer County in northwest Pennsylvania. This development comprises approximately 14.2% of the approximately 4,400 hectare watershed. Family farms comprise 47% (2,068 ha) of the land use and of that, 50% are Amish homesteads that are continuing to increase within the watershed (Brenner *et al*., 1987; Brenner and Mondok, 1995). Woodlots (880 ha) and abandoned agricultural lands (1,452 ha) comprise 20% and 30 % of the watershed, respectively.

Since agriculture is the major land use in the watershed, it has an important impact on water quality and nutrient enrichment of Lake Latonka. In 1997, the Trophic State Indices (TSI) were 61.13 for total phosphorus and 66.43 and 59.76 for chlorophyll *a* and Secchi disc reading the TSI index was 66.43 and 59.76, respectively. Likewise in 2011 the TSI for total phosphorus was 65.31 and TSI for chlorophyll *a* 58.84 and 43.38 for the Secchi disc. In 2013 and 2014 the TSIs averaged 54.57 and 45.7 and 52.9, respectively. Based on these Trophic State Indices, Lake Latonka would be classified as eutrophic (nutrient enriched) and there has been little change in these Trophic State Indices over the last 17 years. An earlier study by Steiner and coworkers (1985) reported that phosphorus concentrations in tributary streams in the Lake Latonka watershed were significantly correlated with rainfall events. Croplands and pastures are interwoven throughout the watershed on the majority of the farms, livestock having free access to streams and wetlands. Dairy cattle and beef cattle comprise approximately 50% and 25%, of the animal units, respectively. Application of manure on both croplands and pastures is the primary method of manure management within the watershed. Manure application is practiced on the majority of the farms without adequate riparian buffers to filter pollutants from entering adjacent waterways. Corn is the principal row crop on over 34% (703 ha) of the agricultural lands and the majority of farms lack crop rotation or grassland strips of nitrogen fixing legumes. Therefore, crop fields often require commercial fertilizer applications in addition to manure which may increase nutrient loading to receiving streams that discharge into Lake Latonka. The remaining 1365 hectares are comprised of approximately 341 ha (25%) for pastures and 683 ha (50%) for hay production and 341 ha (25%) being planted with small grains, soybeans, alfalfa and vegetables (Brenner and Mondok, 1995)

Lake Wilhelm

Lake Wilhelm is located in northeastern Mercer County, Pennsylvania within the Sandy Creek watershed. It was constructed as a flood control reservoir under the auspices of the U.S. Department of Agriculture Soil Conservation Service (SCS), now known as the Natural Resource Conservation Service (NRCS). Construction was initiated on January 22, 1969 and completed in May, 1970. The gates were closed on March 10, 1971 and it reached a normal pool of 158 billion liters by June 14, 1971. Lake Wilhelm is the fourth largest lake managed by the Pennsylvania Department of Conservation and Natural Resources and is one of the 12 largest lakes in Pennsylvania. Agriculture is the primary land use within the watershed, comprising approximately 64% of the 146.3 km^2 (56.5 $mi.^2$) of Lake Wilhelm watershed. As previously described for Lake Latonka, agricultural discharges impact the water quality. Phosphorous and nitrogen loading occurs from 11 streams that discharge into Lake Wilhelm. Based on a report prepared by Pennsylvania Department of Environmental Protection (PADEP) Bureau of Watershed Management (Fair 2008), the Carlson's Trophic State Index (TSI) for total phosphorous was 61.48 and for Secchi disc and Chlorophyll *a* was 63.37 and 67.81, respectively. Lake Wilhelm is classified as being eutrophic or hypereutrophic due to the influx of nutrients from the agriculture. The majority of watershed consists of family farms, including Amish homesteads, and the farming practices are similar to those described for the Lake Latonka watershed.

Approximately 30% of the area consists of a mixed oak (*Quercus* spp.) and maple (*Acer* spp.) forest. The area of the lake itself comprises approximately 7.3 km^2 (2.8 $mi.^2$) or 5% of the watershed with the remaining 1% consisting of rural communities, including the Borough of Sheakleyville.

METHODS

Scale samples were collected from largemouth bass during the summer months at Lake Latonka and Lake Wilhelm in northwestern Pennsylvania. The standard length (mm) and weight (g) of each fish was recorded on site using a fish measuring board and spring digital scale, respectively. All fish under the state size limit were returned to the lake. Legal size fish were either retained by the fisherman or returned to

the lake. Once the scales were removed, they were placed in an envelope and the standard length (mm), weight (g) and date of capture was recorded on each scale envelope.

Scales were analyzed using a Bausch and Lomb projecting microscope with a 10X objective. Each scale was rehydrated in tap water for approximately 10 minutes, placed on a microscope slide, projected onto a screen, and the distance between each of the annuli was recorded in cm. The distance in cm was converted into actual distance between annuli using the following equation: 7.62 cm observed = 1 mm scale distance. The length at the end of each growing season was estimated using the following equation: $L_n=(S_n/S)L$, where L_n is length in the n^{th} year of life, S_n is the length of the annulus in the n^{th} year, S is total scale length and L is total length of the fish at capture. The annual growth for each year class (0-1, 1-2, 2-3). The year class was determined by subtracting the length of the fish at the end of that year from the length of the fish at the end of the previous year. Using a least squared regression and correlation analysis, the expected weight (W_e) was calculated for each fish. The expected weight for each fish was also calculated according to a length weight relationship $W_e = l^n$ where W_e is expected weight based on a known length raised to n^{th} power (Carlander, 1977; Lagler, 1956). The calculated and observed lengths and weights were compared using a t-distribution analysis.

RESULTS AND DISCUSSION

There was a significant difference in both the observed average length ($t = 19.1$, $P < 0.001$ and the observed ($t = 62.1$, $P < 0.001$) and expected weights ($P < 0.001$) based on length of the largemouth bass in Lake Latonka compared to those in Lake Wilhelm (Table 1), as well as among the different estimated weights between the two lakes. In Lake Latonka, no significant difference was noted between the observed weight and different expected weights based on length (Table 1). For the largemouth bass from Lake Wilhelm, there was a significant difference between the observed weights (W_o) and expected weights (W_e) ($t= 7.30$, $P < 0.001$), as well as between the observed weight (W_o) and estimated weight ($W_o l^n$) ($t = 4.4$, $P < 0.05$). There was no difference in the estimated body weights ($P > 0.5$) (Table 1). Largemouth bass in Lake Latonka achieved between 89.4 and 99.1% of their expected weight compared with 95.7 and 96.8% in Lake Wilhelm This difference between bass from the two lakes may be due to fish in Lake Wilhelm being larger per unit length (3.2 g/mm) than those in Lake Latonka (1.9 g/mm).

A comparison of the mean length (189.0 ± 9.1 mm) weight (284.2 ± 9.1 g) of largemouth bass in the 0-l age class were significantly larger in Lake Latonka in both length (152.5 ± 9.0 mm) ($t = 8.59$, $P < 0.001$) and weight (131.3 ± 34.0 g) ($t = 5.99$, $P < 0.001$) than in Lake Wilhelm. Except for the comparison of the observed weight (W_o) and expected weight based on length ($W_o l^n$) ($t = 0.35$, $P > .5$), there

Table 1. Comparison of the length and observed weights of largemouth bass (*Micropterus salmoides*) at Lake Latonka and Lake Wilhelm, Mercer County, Pennsylvania

	Lake Latonka (N=24)		Lake Wilhelm (N=23)			
Parameter	Mean	SE	Mean	SE	t value	P
Length (mm)	370.8	10.7	463.9	13.1	19.1	<0.001
Weight (W_o)(gm)	712.6	53.7	1526.1	116.4	62.6	<0.001
$W_e = al^n$	721.1	64	1460.9	66.8	11.4	<0.001
n	2.1		3			
$W_o l^n$	713.3	64.9	1458	115	64.4	<0.001
n	1.9	0.14	3.1	0.15		
$W_e l^n$	705.2	49.7	1467	101.1	60.4	<0.001
n	1.9	0.045	3.1	0.06		
W_o/W_e x 100	98.8	8.5	96.8	4.1		
$W_o/W_o l^n$x 100	98/1	14.1	97	4.1		
$W_o/W_e l^n$x 100	99.1	3.7	95.7	0.19		
$W_o l^n/W_e l^n$x 100	89.4	6	96.8	3.7		

W_o = observed weight, We = expected weight based on length,

Latonka: W_o vs W_e $t = 0.79$, $P > 0.5$, W_o vs $W_o l^n$ $t = 0.64$, $P > 0.50$, W_o vs $W_e l^n$ $t = 0.66$, $P > 0.50$ W_e vs $W_o l^n$ $t = 1.93$, $P > 0.10$, $W_o l^n$ vs $W_e l^n$ $t = 0.75$. $P > 0.50$

Wilhelm: W_o vs W_e $t = 7.4$, $P < 0.05$, W_o vs $W_o l^n$ $t = 4.4$, $P < 0.05$, W_o vs $W_e l^n = 0.47$, $P > 0.50$, W_e vs $W_o l^n$ $t = 0.21$. $P > 0.50$, $W_o l^n$ vs $W_e l^n$ $t = 0.61$, $P > 0.50$

Table 2. Comparison of length (mm) and weight (gm) of different year classes of largemouth bass (*Micropterus salmoides*) between Lake Latonka and Lake Wilhelm, Mercer County, Pennsylvania.

	LATONKA						WILHELM					
	0-1 age Class		1-2 age Class		2-3 age Class		0-1 age Class		1-2 age Class		2-3 age Class	
Parameter	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Length	189.0	9.1	318.0	9.6	391.1	16.1	152.5	9.0	273.9	49.3	404.6	16.2
Weight (W_o)	284.2	1.8	471.7	3.8	496.5	5.4	131.3	34.0	537.9	34.0	1016.0	5.6
W_e = a + n1 (W_e)	427.3	3.1	472.2	3.4	496.8	5.4	123.6	28.4	566.8	28.4	1002.7	47.3
n	2.1		3.4		3.4		3.1		3.5		3.3	
$W_o = l^n$	283.2	4.8	470.1	2.9	489.7	19.1	148.1	28.6	538.9	33.1	1016.3	41.6
n	1.5	0.1	1.5	0.0	1.4	0.0	0.9	0.06	2.1	0.07	2.4	0.03
$W_e = l^n$	416.4	1.5	472.5	3.7	493.9	0.3	160.9	47.3	563.4	0.97	973.6	39.0
n	2.0	0.1	1.5	0.0	1.3	0.0	1.0	0.06	2.0	0.2	2.3	0.03
W_o/W_e x 100	65.8	10.0	100.1	0.2	100.0	0.0	93.6	4.2	94.0	0.94	96.0	1.27
$W_o/W_o l^n$x 100	100.3	0.3	100.4	0.7	114.3	0.8	95.1	4.2	94.9	1.46	100.3	0.79
$W_o/W_e l^n$x 100	67.1	2.3	99.9	1.1	100.1	0.9	89.0	3.0	93.0	1.41	99.2	0.76
$W_o l^n/W_e l^n$ x 100	66.8	1.6	99.2	0.6	99.1	0.2	113.0	8.9	92.1	2.86	98.7	0.4

W_o = observed weight, W_e = expected weight based on length

was a significant difference among observed and estimated weights of 0-1 age class of largemouth bass in Lake Latonka, but not among the observed and estimated weights of fish in Lake Wilhelm. Likewise, in the 1-2 year old age class, there was a significant difference in the mean length (318.0 ± 9.0 mm) ($t = 5.75$, $P < 0.001$) and weight (471.7 ± 3.8 g) ($t = 6.1$, $P < .001$) of largemouth in Lake Latonka compared with the mean length (273.9 ± 49.3 mm) and weight (537.9 ± 34.0 g) of fish in Lake Wilhelm (Tables 2 and 3). Although there were not significant differences among the observed weights and expected weights of largemouth bass, Lake Latonka, there were significant differences among the observed and expected weights of largemouth bass in Lake Wilhelm (Table 3). In the 2-3 year old age class, there was a significant difference between the estimated weights W_e and $W_e l^n$ ($t = 2.67$, $P > 0.05$). Among the observed and other expected weights of largemouth bass in Lake Latonka, there were significant differences between the expected weights W_e and $W_e l^n$ ($t = 3.1$, $P < 0.05$) and $W_o l^n$ and $W_e l^n$ of fish in Lake Wilhelm (Table 3). However, the 0 -1 year class only achieved between 65.8 and 67.8 percent of their expected weights in Lake Latonka compared to between 89 and 113 percent of their expected weights in Lake Wilhelm (Table 2). But the 1-2 and 2-3 year classes achieved between 99.2 and 110.4 percent and 89 and 113 percent of their expected weights in Lake Latonka and Lake Wilhelm, respectively.

The larger size of the fish in Lake Latonka during the first year may be attributed to abundant phytoplankton and zooplankton communities during the spring and early summer months. As the fish increased in size and began to

Table 3. Comparison of observed weight (W_o) and expected weight (W_e) based on length (mm)

	Latonka						Wilhelm					
	0-1 age Class		1-2 age Class		2-3 age Class		0-1 age Class		1-2 age Class		2-3 age Class	
Parameter	t	P	t	P	t	P	t	P	t	P	t	P
W_o vs W_e	29.2	P <0.001	0.2	P >0.50	0.09	P >0.50	1	P >0.50	3.6	P <0.01	1.8	P >0.10
W_o vs $W_o l^n$	0.35	P >0.50	0.6	P >0.50	1.37	P >0.10	2.1	P >0.1	0.1	P >0.50	0.4	P >0.50
W_o vs $W_e l$	40	P <0.001	0.3	P >0.50	1.7	P >0.10	3.2	P <0.05	4.4	P <0.01	0.6	P >0.50
W_e vs $W_o l^n$	51.5	P <0.001	0.8	P >0.50	2.67	P <0.05	1.1	P >0.10	4.2	P >0.01	1.4	P >0.10
W_e vs $W_e l^n$	5.51	P <0.05	0.1	P >0.50	1.13	P>0.10	1.5	P >0.10	0.5	P >0.50	3.1	P <0.05
$W_o l^n$ vs $W_e l^n$	4.4	P <0.05	0.7	P >0.50	0.84	P >0.50	4.2	P <0.001	0.9	P >0.50	4.8	P <0.01

feed on larger prey, there may have been insufficient forage fish to support the size of the largemouth bass population. In Lake Wilhelm there was an abundance of forage fish to support the largemouth bass and walleye (*Stizostedion vitreum*) populations. In 2004, 11 gizzard shad (*Dorosoma cepedianum*) were captured in trap nets during a survey by the Pennsylvania Fish and Boat Commission (PFBC) and 10 years later over 1900 gizzard shad were captured in trap nets by personnel of the PFBC. In response to the rapid increase in gizzard shad, largemouth bass, and walleye fry and fingerlings were introduced into Lake Wilhelm to increase the predator populations. The impact of gizzard shad or the potential increase in predators will have on the growth and size of the largemouth bass populations is not known at this time, but over the last several years, there has been sizable winter kills of gizzard shad. It would be advisable to do a follow up study to evaluate the potential impact of gizzard shad and the increase of predators on the growth of both largemouth bass and walleye. But based on the results of this study that was completed when both largemouth bass and walleye were the top predators in Lake Wilhelm, there did not appear that walleyes impacted the growth of 1-2 and 2-3 age classes of largemouth bass. Although walleye were released in Lake Latonka, they did not become established and, therefore, they did not compete with largemouth bass for available forage fish.

LITERATURE CITED

Amoah, A., S.D. Coyle, C.D. Webster, R.M. Durborow, L.A. Bright, and J.H. Tidwell. 2008. Effects of Graded Levels of Carbohydrate on Growth and Survival of Largemouth Bass, *Micropterus salmoides*. J. World Aquaculture Society 39 (3): 397–405.

Brenner, F. J., and J.J Mondok. 1995. Nonpoint Source Pollution Potential in an Agricultural Watershed in Northwestern Pennsylvania. JAWRA J. American Water Resources Association 31(6): 1101–1112.

Brenner, F. J., R.P. Steiner, and J.J. Mondok. 1987. Use of Water Quality Models to Predict Non-point Source Pollution Parameters in an Agricultural Watershed. American Water Resources Association technical publication series TPS 87(2): 295-301.

Carlander, K.D. 1977. Handbook of Freshwater Fishery Biology. Volume 2. The Iowa State University Press, Ames Iowa. 431 pp.

Copeland, T. B.R. Murphy, and J.J. Ney. 2008. A Comparison of Relative Weight and Nutritional Status among Four Fish Species in Two Impoundments. J. Freshwater Ecology 23(3): 373-385.

Fair, J. 2008. Lake Wilhelm Water Quality Report. Pennsylvania Department of Environmental Protection

Fullerton A.H., J.E. Garvey, R.A. Wright and R.A. Stein. 2000. Overwinter Growth and Survival of Largemouth Bass: Interactions Among Size, Food, Origin, and Winter Severity. Transactions of the American Fisheries Society. 129:1-12.

Garvey, J.E., K.G. Ostrand, and D.H. Wahl. 2004. Energetics, Predation and Ration Affect Size-Dependent Growth and Mortality of Fish During Winter. Ecology 85(10):2860-2871.

Gunn, J.S., N.P. Clear, T.I. Carter, A.J. Rees, C.A. Stanley, J.H. Farley and J.M. Kalish. 2008. Age and Growth in Southern Bluefin Tuna, *Thunnus maccoyii* (Castelnau): Direct Estimation from Otoliths, Scales and Vertebrae. Fisheries Research 92(2-3): 207-220.

Lager, K.F. 1956. Freshwater Fishery Biology. 2nd Ed. Wm. C. Brown Company Publishers. Dubuque, Iowa. 421 pp.

Shoup, D.E., S.P. Callahan, D.H. Wahl, and C.L. Pierce. 2007. Size-Specific Growth of Bluegill, Largemouth Bass, and Channel Catfish in Relation to Prey Availability and Limnological Variables. J. Fish Biology 70(1):21–34.

Slaughter, J. E., R. A. Wright, and D. R. DeVries. 2008. Latitudinal Influence on First Year Growth and Survival of Largemouth Bass. North American J. Fisheries Management 28:993-1000.

Steiner, R. P., F.J. Brenner, and C. M. Steiner.1985. Time series modeling of some eutrophication Dynamics of a manmade lake in western Pennsylvania. Proceeding of the Fourth Annual Conference and International Symposium. North American Lake Management Society. pp 191-195.

Vøllestad, L.A. and E.M. Olsen. 2008. Non-Additive Effects of Density-Dependent and Density-Independent Factors on Brown Trout Vital Rates. Oikos 117(11):1752–1760.

Journal of the Pennsylvania Academy of Science 89(2): 48-56, 2015

RESEARCH NOTE: EFFECT OF ROAD PROXIMITY ON REPRODUCTIVE EFFORT AND MOVEMENT PATTERNS OF THE WOOD FROG (*RANA SYLVATICA*)[1]

KATHERINE E. ENGBERG AND MEGAN B. ROTHENBERGER[2]

Biology Department, Lafayette College, Kunkel Hall, Easton, PA 18042 USA

ABSTRACT

Nearly half of the amphibian species in northeastern North America rely on vernal pools as their primary breeding habitat. The problem is that, because vernal pools are small and isolated, they are often left unmonitored and unprotected. A primary threat to both amphibians and vernal pools is habitat destruction and fragmentation, but our knowledge of the species-specific impacts of habitat loss and fragmentation on all phases of the amphibian life cycle are still rudimentary. The wood frog (*Lithobates sylvaticus*) was the focus of this research because it is considered the most common vernal pool indicator in Pennsylvania. The objectives of this study were to investigate the effect of road proximity on vernal pool hydrology and water chemistry, reproductive effort of wood frogs (i.e., numbers of egg masses deposited), and upland movement patterns of wood frogs. These parameters were compared between three isolated pools (> 1000 m from the nearest road) and two pools in a fragmented habitat (< 100 m from two roads) within a Pennsylvania state park. This study indicates that, although road proximity did not have a significant effect on vernal pool water chemistry and egg mass abundance was greater in the fragmented location, habitat fragmentation by roads did have a significant effect on the movement patterns of wood frogs in surrounding terrestrial habitat. At the isolated site where there were no barriers to movement, wood frogs were distributed randomly around the pools. However, wood frogs in the fragmented location were trapped at a lower frequency near roads than expected by chance, indicating that the presence of roads may reduce the amount of upland habitat utilized by adult wood frogs. Although this was a small and localized study, the results indicate the challenging nature of conserving species with complex life cycles in human dominated landscapes and highlight the importance of considering life-cycle and species-specific habitat requirements when designing vernal pool conservation plans. [J PA Acad Sci 89(2): 48-56, 2015]

INTRODUCTION

Vernal pools are seasonally flooded depressional wetlands that are primarily filled by precipitation but may also receive input from surface runoff and groundwater exchange (Mitsch and Gosselink 2007). In northeastern North America, these habitats are associated with forested landscapes and are characterized by absence of fish, lack of flowing water, small size, shallow depth, and presence of plants and animals that can withstand a period of drought (Calhoun and deMaynadier 2008). Because of these characteristics, vernal pools provide the primary breeding habitat for wood frogs (*Lithobates sylvaticus*), spadefoot toads (*Scaphiopus holbrookii*), ambystomatid salamanders and a variety of invertebrate taxa (Semlitsch and Skelly 2008). In fact, approximately 45% of the amphibian species that populate northeastern North America rely on vernal pools as their primary breeding habitat (Semlitsch and Skelly 2008).

Although vernal pools are recognized by scientists as valuable ecosystems that support high species richness, function as stepping stones for dispersing organisms, and export substantial secondary production in the form of amphibian and insect biomass (Comer *et al.* 2005, Hunter 2008, Semlitsch and Skelly 2008), vernal pools are often not afforded protection under wetland protection guidelines and regulations (Preisser *et al.* 2000, Calhoun *et al.* 2003). These small, temporary, isolated habitats are often overlooked as monitoring sites, and conservation of vernal pools has been hampered by a lack of consistent local knowledge concerning the function and value of vernal pools. As a result, vernal pools and the upland forests that buffer and link isolated wetlands used by pool-breeding species continue to be degraded and destroyed by human land use (Semlitsch and Bodie 2003, Mahaney and Klemens 2008). Vernal pools are now among the most threatened of freshwater wetlands globally (Range 2003).

[1]Accepted for publication June 2015.

[2]Corresponding author: Lafayette College Biology Department, 705 Sullivan Road, Easton, PA 18042, rothenbm@lafayette.edu, (610)330-5459.

Studies of vernal pool amphibians over the past two decades provide strong evidence that conservation of these species depends not only on the presence of breeding pools but also on the terrestrial landscape composition surrounding vernal pools. Although pool-breeding species are easiest to document and monitor during breeding season in vernal pools, these amphibians spend the majority of their lives foraging, resting, and hibernating in the terrestrial habitat surrounding vernal pools (Berven and Grudzien 1990, Semlitsch 1998). In addition, juvenile amphibians can disperse as far as 1000 m from their birth site and can select pools other than their birthplace for breeding (Berven and Grudzien 1990, Reh and Seitz 1990). As a result, landscape conversion that results in fragmentation, loss of connectivity, or degradation can result in disappearance of some amphibians from an area even when vernal pools remain (Semlitsch and Skelly 2008).

Roads represent one type of landscape conversion that alters and degrades the terrestrial landscape surrounding vernal pools. Studies have demonstrated that roads not only act as important barriers to the movements of amphibians (Reh and Seitz 1990, Gibbs 1998, deMaynadier and Hunter 2000) but also contribute to direct mortality (Fahrig *et al.* 1995, Hels and Bushwald 2001) and to degradation of vernal pool water quality as a result of the application of road deicing salt in winter (Forman and Deblinger 2000, Karraker *et al.* 2008). Anthropogenic activities also tend to reduce canopy cover, threatening pool-breeding specialists that perform well under shaded conditions. For example, wood frogs are declining in developing areas, especially in regions where upland forest cover has been reduced by 50% or greater (Baldwin *et al.* 2006).

Although some generalizations can be made about the impact of habitat loss and fragmentation on amphibian populations, each species will respond to landscape conversion in its environment uniquely (Cushman 2006, Semlitsch and Skelly 2008). Knowledge about the population-level implications of habitat area, edge, isolation, and road mortality relationships is still rudimentary (Cushman 2006), and it is therefore important that vernal pool monitoring studies include species-specific characterization of the habitat. In addition, many studies simply examine correlations between organism abundance and the area of various land cover types within a certain distance of a breeding pond (Cushman 2006). However, the survival of amphibian populations in fragmented landscapes depends on the interaction of multiple factors, including the distribution and quality of breeding pools, population sizes in those pools, proximity of roads, and land cover types surrounding the pools. As a result, this study attempts to address some of these research needs by analyzing the impact of fragmentation by roads on multiple environmental attributes and on a single obligate vernal pool indicator species, the wood frog, at various life cycle phases within a Pennsylvania state park. The specific objectives of this research were to compare specific physiochemical parameters, wood frog reproductive effort (i.e., numbers of egg masses deposited), and wood frog movement patterns between three isolated pools (> 1000 m from the nearest road) and two pools in a fragmented habitat (< 100 m from two roads).

STUDY AREA

Jacobsburg Environmental Education Center is a 473-ha state park located approximately 11 km northwest of Easton, PA in Northampton County (Fig. 1). The 12 species of amphibians known to inhabit this area include salamanders in the families Ambistomatidae and Salamandridae, and frogs in the families Hylidae and Ranidae. The study area was entirely within the boundaries of the state park and included five vernal pools in two locations (Fig. 1). The first location, considered isolated, is > 1000 m from the nearest paved road and includes one natural (pool 1) and two constructed (pools 2 and 3, both are unlined) vernal pools. The constructed pools were created in 2008 and 2011. These three pools are within 100 m of one another with no barriers separating the pools. The terrestrial environment surrounding the pools is comprised of white oak (*Quercus alba*), red oak (*Quercus rubra*), and maple (*Acer saccharum*) and an understory dominated by spicebush (*Lindera benzoin*) and the invasive multiflora rose (*Rosa multiflora*).

The second location, considered fragmented, is adjacent to a residential community, and the pools are ≤ 100 m from two paved roads. The fragmented location includes one constructed pool (pool 4, lined with a synthetic liner) and one natural pool (pool 5). The constructed pool was created in 2008, and these two pools are within 200 m of one another. However, a road dissects the habitat between them, and a second road dissects that habitat to the east of pool 5 (Fig. 1). The terrestrial environment surrounding these pools is dominated by shagbark hickory (*Carya ovata*), red oak (*Quercus rubra*), white pine (*Pinus strobus*), tulip poplar (*Liriodendron tulipifera*), chestnut oak (*Quercus prinus*), and spicebush (*Lindera benzoin*).

MATERIALS AND METHODS

Hydrology and Physiochemistry

Physical and chemical data were collected from vernal pools on a weekly basis from late March until the pools were dry in mid- to late July of 2014 and 2015. Physical data were collected on pool depth (free water above pool substrate), area (width and length measured to water level and calculated for area of an oval), and pool type (i.e., natural or constructed). The area and depth of each pool were used to calculate

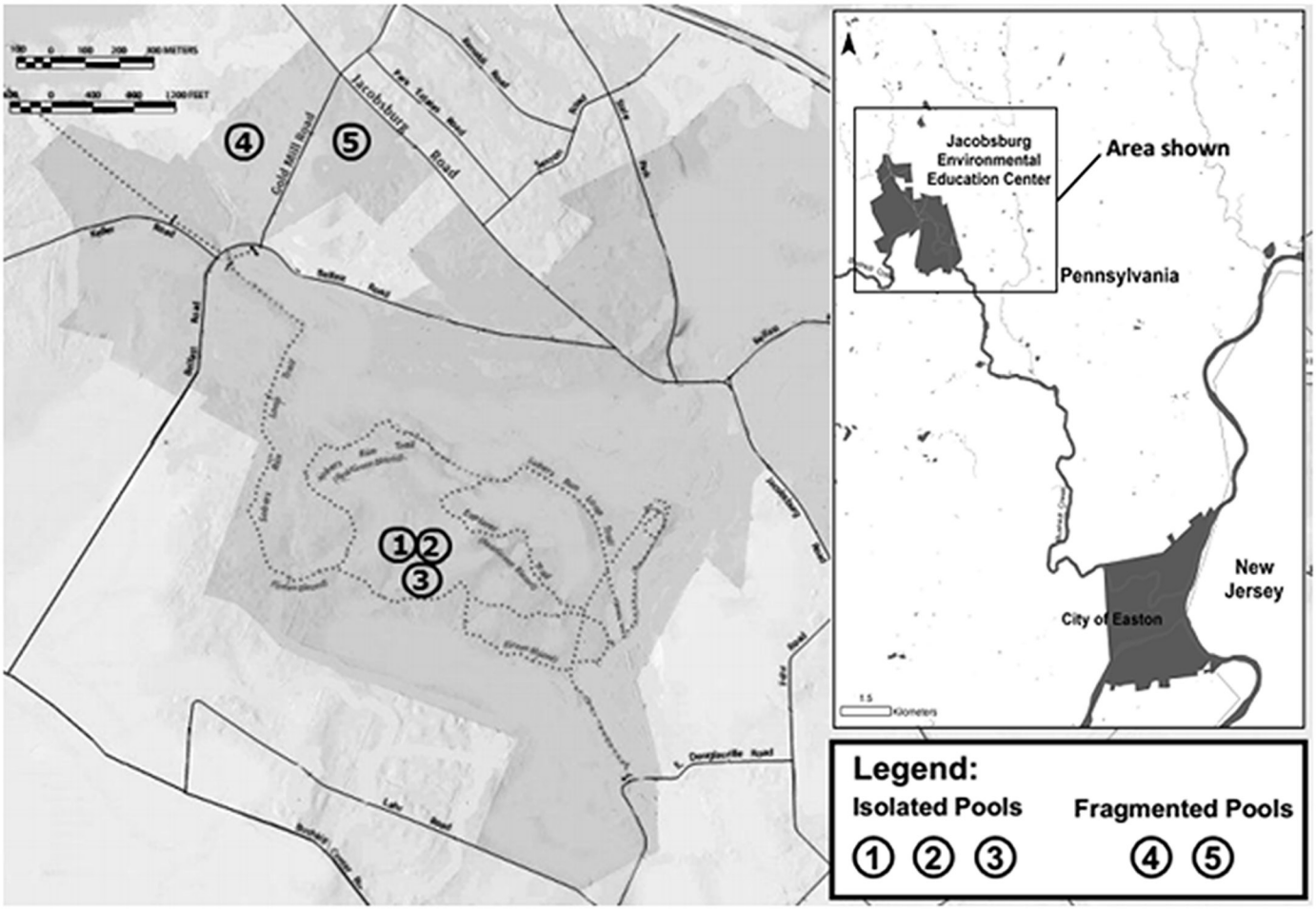

Fig.1. Map of the five vernal pools within Jacobsburg Environmental Education Center in Northampton County, PA. Pools 1-3 are at an isolated location > 1000 m from the nearest paved road. Pool 4 is 114 m from Gold Mill Road and 212 m from Jacobsburg Road, and Pool 5 is 105 m from Gold Mill Road and 90 m from Jacobsburg Road. Only pools 4 and 5 are used for educational purposes by park staff, but there are no official trails leading to these pools. Pool 3 is located 10 m from an official trail used for walking and biking.

volume throughout the season (i.e., volume (m^3) = area (m^2) x maximum depth (m)) and hydroperiod (i.e., duration of time that a pool holds water). A YSI 6820 V2 multiparameter meter was used to determine water temperature, conductivity, and dissolved oxygen (DO). Data were gathered approximately 1 m from the shoreline and at mid-depth (i.e., between the substrate and water surface) at each pool. Local precipitation data were acquired from the National Weather Service for the Lehigh Valley International Airport station, which is the nearest municipal area (i.e., 25 km southwest of the study area). Monthly temperature and precipitation averages were compared to historical averages (Fig. 2).

Amphibians

Wood frog egg masses were counted at the same time that physical and chemical data were collected in spring 2014 and 2015. Wood frogs are known to be explosive breeders that typically oviposit in large communal aggregates over a 5- to 8-day period (Crouch and Paton 2000). In order to capture the period immediately after the eggs were deposited and ensure that sampling encompassed the entire potential egg-laying period (i.e., microclimate conditions and cold weather can extend the period of egg deposition to 17 days or more, Crouch and Paton 2000), egg mass counting was initiated approximately one week following the first report of wood frog choruses in Jacobsburg state park (i.e., 28 March 2014 and 27 March 2015) and continued on a weekly basis until all the eggs hatched and decomposed. Egg mass counts were conducted using the methods described in Crouch and Paton (2000).

Wood frog abundance and movement patterns were monitored from September to November 2014 using drift fences and pitfall traps. Trapping arrays were oriented in each of the four cardinal directions, at 60 m and 120 m, from pools 1 (isolated and natural), 4 (fragmented and constructed), and 5 (fragmented and natural, Fig. 1). The more distant trapping arrays to the east of pool 4 and west of pool 5 were placed adjacent to Gold Mill Road at approximately 100 m from the vernal pools (Fig. 1). Drift fences consisted of 1-m lengths of polyethylene plastic suspended between two stakes and

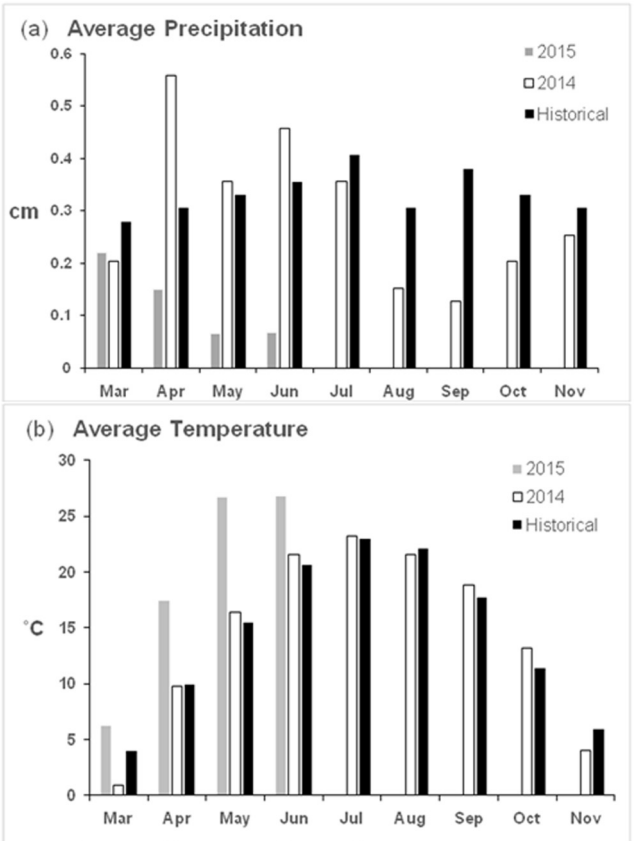

Fig.2. Monthly precipitation (a) and temperature (b) averages for the study period (March to November 2014 and March to June 2015) and historical averages for the same months (1922-2015). Data were acquired from the National Weather Service Forecast Office for the Lehigh Valley International Airport station (i.e., the nearest municipal area).

buried 5 cm deep for support. Pitfall traps (n = 4 per fence, one each direction) were 4.6-L cans with holes drilled in the bottom for drainage sunk flush to the ground. All of the drift fences and pitfall traps were located in a shaded environment, and a synthetic sponge and ground material from the site were placed in each trap and dampened at each visit to reduce probability of amphibian desiccation. Perforated bowls were placed over top of each trap to prevent amphibian escape (Gibbs 1998), and a stick was placed in each trap to allow small mammals to climb out. The trapping arrays were opened monthly for a three-day period and checked each morning between 0800 and 1200. Wood frogs were counted and immediately released. Coyote urine was used around each trapping array to repel potential amphibian predators from the traps, and traps were closed in between sampling periods. The methods for amphibian trapping and releasing were approved by the Institutional Animal Care and Use Committee (IACUC) of Lafayette College.

Statistical Analyses

To compare water quality and egg mass counts between isolated pools (i.e., n = 6, three pools x two years) and pools near roads (i.e., n=4, two pools x two years), a mixed-model approach to repeated-measures ANOVA was used with road proximity and year as independent variables and maximum depth, maximum volume, hydroperiod, mean conductivity, and maximum egg mass count as dependent variables. A separate model was run for each of the dependent variables, and pool was included as a random effect to account for samples within the same pools in two different years. A combination of chi-square analysis and three-way repeated measures ANOVA was used to compare wood frog movement between isolated and fragmented locations and to determine the significance of those movement patterns. First, the null hypothesis that wood frogs were uniformly abundant around the vernal pools was tested using chi-square tests of homogeneity of proportions (i.e., n = 72 per

Table 1. Comparison of hydrologic parameters and egg mass counts between three isolated pools (i.e., > 1000 m from the nearest road) and two pools in habitat fragmented by roads (i.e., < 100 m from two roads). Parameters were measured on a weekly basis from late March until the pools were dry in mid- to late July in both 2014 and 2015. Values are means (SEs; N = 6 for isolated and N = 4 for fragmented locations). A separate repeated-measures ANOVA (i.e., with pool included as a random effect to account for samples within the same pools in two different years) was used to determine the significance of differences between pools in the two locations. ANOVA F-statistics and P-values are shown for each comparison.

Parameter	Isolated	Fragmented	F	P
Maximum Depth (m)	0.44 (0.05)	0.86 (0.05)	25.9	< 0.001
Maximum Volume (m^3)	75.1 (42.2)	245.1 (48.9)	6.0	0.04
Hydroperiod (weeks)	11.2 (1.8)	14.5 (0.9)	2.3	0.17
Temperature (ºC)	17.0 (4.0)	16.2 (5.1)	2.0	0.10
Dissolved Oxygen (mg L^{-1})	3.8 (1.4)	2.7 (1.3)	1.8	0.08
Conductivity (µS/cm)	55.7 (9.1)	69 (7.9)	1.2	0.31
Maximum Egg Mass Count	10.5 (4.0)	103.8 (30.9)	12.6	< 0.01

Table 2. Results of the chi-square tests of homogeneity of proportions (i.e., n = 72 per pool), which were used to test the null hypothesis that wood frogs were uniformly abundant around all the vernal pools. Pool 1 is isolated from roads (i.e., > 1000 m from the nearest road), and pools 4 and 5 are located in habitat fragmented by roads (i.e., < 100 m from two roads). Rejection of the null hypothesis was interpreted as evidence of significant non-uniform orientation in movement in one or more directions.

	X^2	df	P
September			
Pool 1	4.6	3	0.20
Pool 4	11.4	3	< 0.01
Pool 5	19.1	3	< 0.001
October			
Pool 1	0.4	3	0.95
Pool 4	20.7	3	< 0.001
Pool 5	22.4	3	< 0.001
November			
Pool 1	0.8	3	0.85
Pool 4	21.1	3	< 0.001
Pool 5	15.1	3	< 0.01

pool, eight traps x three sample dates x three months). The proportions of observed captures were compared to equal expected values across directions. For this analysis, data for all wood frogs were combined at a given vernal pool (i.e., 1, 4 or 5) and one test was conducted for each pond in September, October, and November. Rejection of the null hypothesis was interpreted as evidence of significant non-uniform orientation in movement in one or more directions. Vector plots were used to depict the proportion of wood frog captures in September, October, and November in each of the four directions around pools. Finally, a three-way repeated measures ANOVA was used (i.e., n = 72 per pool) to examine the three-way interaction effect of trap distance from pool, trap orientation in relation to a road (i.e., away or toward a road), and month on frog abundance within traps. All analyses, figures, and tests of normality and homogeneity of variances were completed using R version 3.2.1.

RESULTS

Hydrology and Physiochemisty

Compared to historical means, the 2014 sampling year had comparable temperatures but considerably different precipitation totals (Fig. 2). Mean precipitation was higher in early spring 2014 and lower in autumn compared with historical data. In contrast, average temperature was much higher and precipitation was lower in spring 2015 compared with historical averages (Fig. 2). The vernal pools included in this study ranged in size from 23 to 377 m^2 with the natural pool being approximately ten times larger than the constructed pools in the isolated habitat and two times larger than the constructed pool in the fragmented habitat. Vernal pools were generally shallow, with a mean maximum depth of 0.60 ± 0.11 m in 2014 (range = 0.34 to 0.88 m) and 0.62 ± 0.11 m in 2015 (range = 0.35 to 0.97 m). In 2014, maximum volume of vernal pools occurred between the first and second week of April and ranged from 7.82 to 329 m^3 (Fig. 3a). Pool volume decreased gradually in all of the pools, but pool 2, which is the newest unlined constructed pool (i.e., constructed in 2011), was dry by the second week of May. The other pools retained water until the end of July (Fig. 3a). In 2015, maximum volume of vernal pools occurred slightly earlier between the last week of March and first week of April and ranged from 8.4 to 330 m^3 in 2015 (Fig. 3b). Pool volume decreased more rapidly in 2015, and all of the

Table 3. Results of the three-way repeated measures ANOVA (i.e., n = 72 per pool) to examine the three-way interaction effect of trap distance from pool, trap orientation in relation to a road (i.e., away or toward a road), and month on frog abundance within traps.

Source	df	Number of frogs		
		MS	F	P
Distance (D)	1	91.5	13.6	<0.001
Orientation (O)	1	469.3	69.9	<0.001
Month (M)	2	291.7	43.5	<0.001
D*O	1	0.0	0.001	0.97
D*M	1	0.1	0.01	0.92
O*M	1	23.5	3.6	0.06
D*O*M	1	13.8	2.1	0.15
Residual	60	6.5		

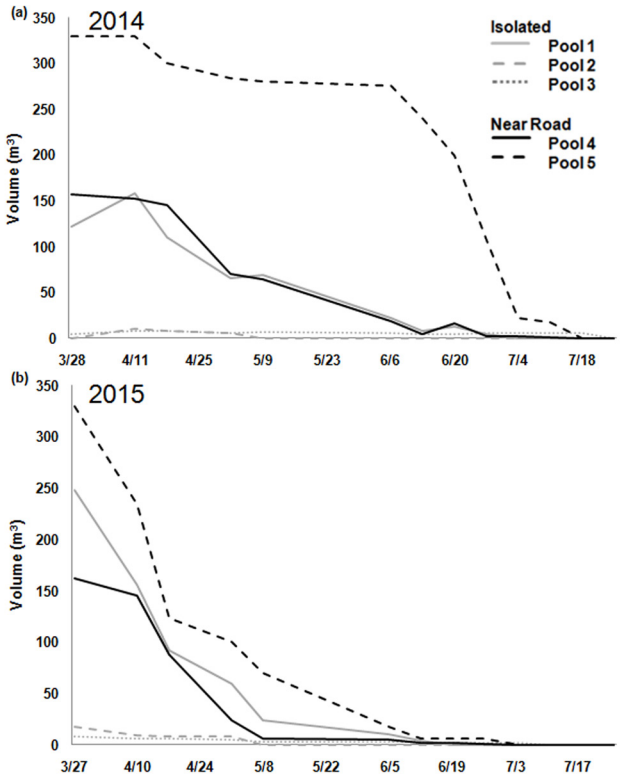

Fig.3. Volume and hydroperiod (i.e., duration of time that a pool holds water) of the five vernal pools included in this study in 2014 (a) and 2015 (b).

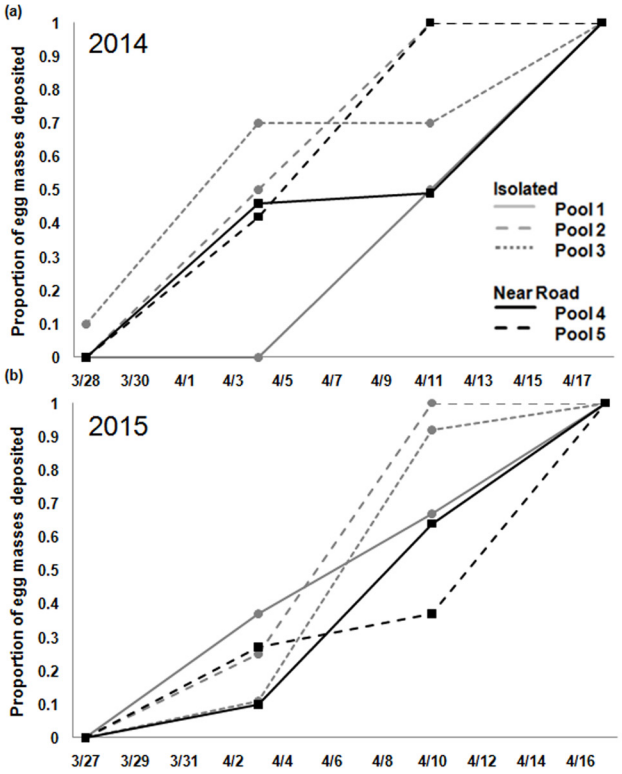

Fig.4. Chronology of wood frog egg-mass deposition at five vernal pools in 2014 (a) and 2015 (b).

pools were dry by the first week of July (Fig. 3b). The mean hydroperiod for vernal pools was 13.6 weeks in 2014 (range = 6 to16 weeks) and 11.4 weeks in 2015 (range = 6 to 13 weeks, Fig.3). Maximum depth and volume of the fragmented pools were significantly larger than that of the isolated pools, but hydroperiod, temperature (range = 9 ˚C to 25 ˚C), dissolved oxygen (range = 0.8 mg L^{-1} to 5.5 mg L^{-1}), and conductivity (range = 35 to175 µS cm^{-1}) were not significantly different between pools in the two locations (Table 1).

Amphibians

A total of 478 wood frog egg masses were counted over the two-year monitoring period, and egg masses were detected in all five vernal pools in both years. Initial egg masses were deposited at most vernal pools during the last week of March, although deposition was not initiated at pool 1 until the first week of April in 2014 (Fig.4). The deposition period ranged from 7 to 22 days, and the majority of wood frog clutches were deposited in the vernal pools between the first and third weeks of April (Fig. 4). Egg mass chronologies appear to be comparable between pools in the two locations, and all the eggs were hatched by the first week of May in both years. However, maximum number of egg masses per pool ranged from 2 to 180, and significantly more egg masses were detected in the vernal pools located in habitat fragmented by roads (Table 1).

A total of 474 adult wood frogs were captured in trapping arrays. On average, a greater number of frogs were captured at the isolated site (mean = 66 frogs) than at the fragmented site (mean = 46 frogs, Fig. 5). Results of the chi-square analysis (Table 2 and Fig. 5) indicate that the presence of roads at the fragmented location may be influencing the movements of adult frogs in the habitat surrounding the pools. Wood frogs at the isolated location were uniformly abundant in traps around the vernal pools in all three months (i.e., null hypothesis accepted, Table 2 and Fig. 5). However, there was evidence of significant non-uniform orientation in wood frog movement at the pools in the fragmented site (i.e., null hypothesis rejected, Table 2). Comparison of observed and expected values for frog abundance across directions shows that significantly more frogs than expected were found in traps located away from the roads (Fig. 5). Three-way repeated measures ANOVA also indicated that significantly more frogs were caught in traps within 60 m from pools than in traps 120 m from pools and that there was a significant decline in overall frog abundance from September to November (Table 3 and Fig. 6).

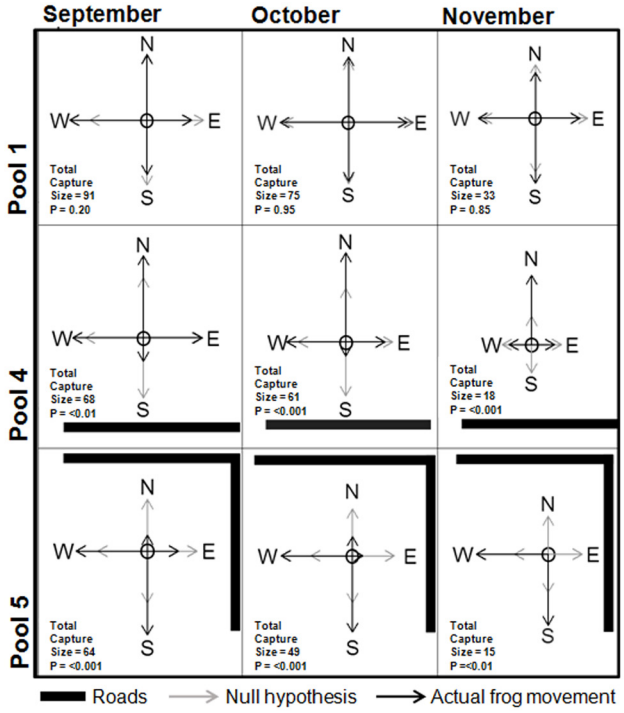

Fig.5. Vector plots depicting expected (i.e., gray arrows calculated as total sample size/4 cardinal directions) and observed (i.e., black arrows are actual frog abundance in traps) wood frog captures in September, October, and November around pools at isolated (pool 1) and fragmented (pools 4 and 5) sites. Chi-square tests of homogeneity of proportions were used to test the null hypothesis that wood frogs were uniformly abundant around the vernal pools. Table 2 provides a complete summary of the chi-square tests.

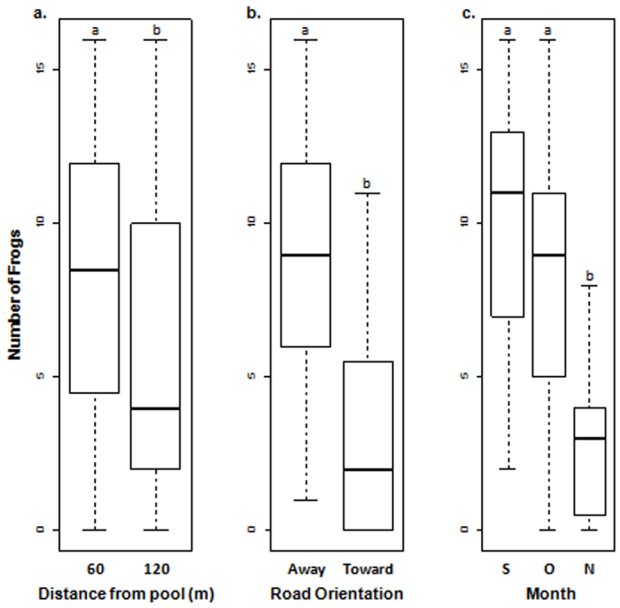

Fig.6. Boxplots depicting the direction and magnitude of relationships between wood frog abundance and distance from pool (a), road orientation (b), and month (c; September (S), October (O), and November (N)). A three-way repeated measures ANOVA was used to test significance of patterns, and Table 3 provides a complete summary of the statistical results. Different letters above boxplots indicate significant differences ($P < 0.05$).

DISCUSSION

Vernal pool volume and hydroperiod was influenced by climatic conditions in all of the pools regardless of road proximity. Hydroperiod, which is determined primarily by the balance of precipitation, evapotranspiration and groundwater input during late spring (Brooks 2004, Bauder 2005), was longer for both natural and constructed pools in both habitats in 2014 when temperatures were near average and precipitation was greater than historical averages. Examination of physical attributes also shows that the pools in the fragmented habitat are significantly larger and generally retained water longer than pools in the isolated habitat. The larger size and longer hydroperiod of the two pools in fragmented habitat most likely contributed to the significantly larger number of egg masses deposited in those pools. Other studies that have evaluated factors affecting vernal pool species distributions and reproduction also indicate that pool depth and volume tend to be positively correlated with amphibian species richness and egg mass abundance (Egan and Paton 2004, Werner 2007, Semlitsch and Skelly 2008, Korfel *et al.* 2009). Larger pools often have more open canopies, which is in turn associated with higher water temperatures, increased productivity, and higher rates of growth and development for many amphibian species (Semlitsch and Skelly 2008). However, one other interesting finding is that pool 1 had substantially lower maximum egg mass counts in both 2014 and 2015 (i.e., 2 and 20 respectively) than pool 4 (i.e., 180 and 39 respectively) even though pool 4 is comparable in size and volume to pool 1. This finding suggests that other factors such as canopy cover, within-pond woody vegetation, and the lining covering the bottom of pool 4 may be influencing the reproductive effort of wood frogs in this area. Therefore, future directions for this research will include a more thorough investigation of the relationship between within-pond parameters and wood frog oviposition.

Although physical attributes and egg mass counts differed between pools in isolated and fragmented habitats, water quality conditions were similar between the two locations and comparable to those reported for other pools in the northeast (Calhoun and deMaynadier 2008; Korfel *et al.* 2009). Although previous research indicates that roads can contribute to degradation of vernal pool water quality (Forman and Deblinger 2000, Karraker *et al.* 2008), road proximity did not seem to have a significant effect on temperature, dissolved oxygen, or conductivity in this study.

In particular, all of the pools included in this study had an initial conductivity below 100 μS, which is five times lower than the conductivities reported to reduce embryonic and larval survival rates in wood frogs (Karraker *et al.* 2008).

Although road proximity does not appear to have a significant effect on vernal pool physiochemistry, the proximity of roads in this study did have a significant effect on the movement patterns of wood frogs in surrounding terrestrial habitat. At the isolated site where there were no barriers to wood frog movement in any direction, there was no significant difference in the abundance of wood frogs among the cardinal directions. The wood frogs were distributed randomly around the pools. However, wood frogs in the fragmented location were significantly more abundant in traps further away from roads, indicating that the presence of roads may be reducing the amount of upland habitat utilized by adult wood frogs. The edge effects, or microclimate changes in light, temperature, wind, and humidity, may be having a repelling effect on this shade dependent species (Rothermel and Semlitsch 2002, Primack 2014). In order to gain a better understanding of amphibian movements in relation to forest edges and roads, it would be valuable to expand from site-specific inquiries like the study described here to larger-scale correlative studies of amphibian distribution in relation to habitat composition.

Habitat fragmentation by roads also isolates amphibian populations, decreasing dispersal and genetic diversity (Cushman 2006). Because wood frogs travel between adjacent vernal pools before overwintering, a lack of pool connectivity can lead to wood frog extirpation in an area (Semlitsch and Skelly 2008). Even within connected landscapes, amphibian populations are often at risk of local extinction. As a result, the maintenance of regional populations is dependent on migration of amphibian species from populated pools to pools with populations at risk of local extinction (Cushman 2006). Future studies on interpond dispersal rates and distances using marked populations would provide a measure of the genetic population structure of the wood frog in Jacobsburg State Park.

Over the past decade, terrestrial habitats surrounding vernal pools have been coming under increased developmental pressure (Regosin *et al.* 2003). Upland forests surrounding and linking isolated wetlands like vernal pools are rapidly degraded and destroyed for human land use (Baldwin *et al.* 2006). Very few states provide specific protection for vernal pools and their surrounding habitat, and the habitat surrounding vernal pools can be destroyed even when the pool itself remains (Lichko and Calhoun 2003). Although this was a small and localized study, these data indicate that habitat fragmentation caused by roads may not reduce wood frog larval productivity but can negatively impact the movements of adults in the habitat surrounding vernal pools. As a result, it is important that plans for vernal pool conservation and restoration consider life-cycle and species-specific effects of local and landscape-level disturbances such as road proximity on amphibians.

AKNOWLEDGEMENTS

We thank Trent Gaugler for statistical support, Phil Auerbach for technical support in the laboratory and with field equipment, and David Sunderlin, Caroline Ladlow, Michael Ryan, Emily McGonigle, Nikki Bauer, Tiffany Phuong, Carli Jones, Sarah Woodruff, Elizabeth Osborn, Leah Hayden, Dionne May, Rebecca Indeck, and Rachel Douglas for assistance with trapping array set-up, sampling, and data collection. We thank three anonymous reviewers for counsel on the manuscript. Funding support for this research was provided by the Lafayette College Department of Biology.

LITERATURE CITED

Baldwin, R.F., A.J.K. Calhoun, and P.G. deMaynadier. 2006. Conservation Planning for Amphibian Species with Complex Habitat Requirements: A Case Study Using Movements and Habitat Selection of the Wood Frog *Rana Sylvatica*. Journal of Herpetology 40: 443-454.

Bauder, E.T. 2005. The effects of an unpredictable precipitation regime on vernal pool hydrology. Freshwater Biology 50: 2129-2135.

Berven, K.A. and T.A. Grudzien. 1990. Dispersal in the Wood Frog (*Rana sylvatica*): Implications for Genetic Population Structure. Evolution 44: 2047-2056.

Brooks, R.T. 2004. Weather-related effects on woodland vernal pool hydrology and hydroperiod. Wetlands 24: 104-114.

Calhoun, A.J.K. and P.G. deMaynadier. 2008. Science and conservation of vernal pools in northeastern North America. CRC Press, Boca Rotan, FL.

Calhoun, A. J., T.E. Walls, S.S. Stockwell, and M McCollough. 2003. Evaluating vernal pools as a basis for conservation strategies: a Maine case study. Wetlands 23: 70-81.

Comer, P., K. Goodin, A. Tomaino, G. Hammerson, G. Kittel, S. Menard, C. Nordman, M. Pyne, Reid, L. Sneddon, and K. Snow. 2005. Biodiversity Values of Geographically Isolated Wetlands in the United States. NatureServe, Arlington, VA.

Crouch, W.B. and P.W.C. Paton. 2000. Using egg-mass counts to monitor wood frog populations. Wildlife Society Bulletin 28: 895-901.

Cushman, S.A. 2006. Effects of habitat loss and fragmentation on amphibians: A review and prospectus. Biological Conservation 128: 231-240.

deMaynadier, P.G. and Hunter, M.L., Jr. 2000. Road effects on amphibian movements in a forested landscape. Natural Areas Journal 20: 56–65.

Egan, R.S. and P.W.C. Paton. 2004. Within-pond parameters affecting oviposition by wood frogs and spotted salamanders. Wetlands 24: 1-13.

Fahrig, L., J.H. Pedlar, S.E. Pope, P.D. Talyor, and J.F. Wegner. 1995. Effect of road traffic on amphibian density. Biological Conservation 74:177-182.

Forman, R. T. T., and R.D. Deblinger. 2000. The ecological road-effect zone of a Massachusetts (U.S.A.) suburban highway. Conservation Biology 14: 36-46.

Gibbs, J.P. 1998. Amphibian Movements in Response to Forest Edges, Roads, and Streambeds in Southern New England. The Journal of Wildlife Management 62: 584-589.

Hels, T. and E. Buchwald. 2001. The effect of road kills on amphibian populations. Biological Conservation 99: 331-340.

Hunter, Jr., M.L., 2008. Valuing and conserving vernal pools as small-scale ecosystems. In: Calhoun, A.J.K., and P.G. deMaynadier (eds.). Science and Conservation of Vernal Pools in Northeastern North America. CRC Press, Boca Raton, FL, 1-8.

Karraker, N.E., J.P. Gibbs, and J.R. Vonesh. 2008. Impacts of Road Deicing Salt on the Demography of Vernal Pool-Breeding Amphibians. Ecological Applications 18: 724-734.

Korfel, C.A., W.J. Mitsch, T.E. Hetherington, and J.J. Mack. 2009. Hydrology, physiochemistry, and amphibians in natural and created vernal pool wetlands. Restoration Ecology 18: 843–854.

Lichko, L.E. and A.J.K. Calhoun. 2003. An Evaluation of Vernal Pool Creation Projects in New England: Project Documentation from 1991-2000. Environmental Management 32: 141-151.

Mahaney, W.S. and M.W. Klemens. 2008. Vernal pool conservation policy: the federal state, and local context. In: Calhoun, A.J.K., and P.G. deMaynadier (eds.). Science and Conservation of Vernal Pools in Northeastern North America. CRC Press, Boca Raton, FL, 193-212.

Mitsch, W.J. and J.G. Gosselink. 2007. Wetlands, Fourth Edition. John Wiley and Sons, Hoboken, New Jersey.

Preisser, E.L., J.Y. Kefer, J.D. Lawrence, and T.W. Clark. 2000. Vernal pool conservation in Connecticut: an assessment and recommendations. Environmental Management 26: 503-513.

Primack, R.B. 2014. Essentials of Conservation Biology, sixth edition. Sinauer Associates, Sunderland, MA.

Range, J.D. 2003. Jurisdiction under the Clean Water Act: implications of the SWANCC decision: a legal analysis of the current status of wetlands protection commissioned by the lzaak Walton League of America. lzaak Walton League of America, Gaithersburg, MD.

Regosin, J.V., B.S. Windmiller, and J.M. Reed. 2003. Terrestrial Habitat Use and Winter Densities of the Wood Frog (*Rana sylvatica*). Journal of Herpetology 37: 390-394.

Reh, W., and A. Seitz. 1990. The influence of land use on the genetic structure of populations of the common frog Rana temporaria. Biological Conservation 54: 239-249.

Rothermel, B.B. and R.D. Semlitsch. 2002. An experimental investigation of landscape resistance of forest versus old-field habitats to emigrating juvenile amphibians. Conservation Biology 16: 1324-1332.

Semlitsch, R.D. 1998. Biological delineation of terrestrial buffer zones for pond-breeding salamanders. Conservation Biology 12: 1113–1119.

Semlitsch, R.D. and J.R. Bodie. 2003. Biological criteria for buffer zones around wetlands and riparian habitats for amphibians and reptiles. Conservation Biology 17: 1219-1228.

Semlitsch, R.D. and D.K. Skelley. 2008. Ecology and Conservation of Pool-Breeding Amphibians. In: Calhoun, A.J.K., and P.G. deMaynadier (eds.). Science and Conservation of Vernal Pools in Northeastern North America. CRC Press, Boca Raton, FL, 127-148.

Werner, E.E., D.K. Skelly, R.A. Relyea, and K.L. Yurewicz. 2007. Amphibian species richness across environmental gradients. Oikos 116: 1697-1712.

Journal of the Pennsylvania Academy of Science 89(2): 57-68, 2015

OPTIMIZATION OF CELL CULTURE CONDITIONS FOR THE EARTHWORM EISENIA HORTENSIS: A STUDY INVESTIGATING THE EFFECTS OF MEDIA, CARBON DIOXIDE, TEMPERATURE, SERUM, AND ANTI-FUNGAL AGENTS[1]

SHERYL L. FULLER-ESPIE[2], DARCY R. HARRIS, JENNIFER H. DALY, AND JULIANN M. JAKEMAN

Science Department, Cabrini College, 610 King of Prussia Road, Radnor, PA 19087-3698

ABSTRACT

The aim of this study was to determine the optimal conditions for culturing coelomocytes (leukocyte-like cells) from the annelid *Eisenia hortensis*. It was of particular interest to determine if CO_2 could be omitted to permit more wide-spread use of earthworms in cell biology curricula using standard incubators. Two different types of media, DMEM and SFX-Insect Media, were used at varying conditions including: temperature, serum concentration, antimycotic concentration, CO_2, and time. Cell viability was measured using propidium iodide and flow cytometry in addition to analysis of forward and side light scatter properties. It was found that the coelomocytes of *E. hortensis* exhibit the highest level of cell viability when cultured with DMEM supplemented with 10% newborn calf serum at 25 °C. Longer incubations showed lower cell death when CO_2 was provided, but CO_2 could be omitted for shorter periods of culture without significant loss of cell viability providing 10 mM HEPES was included in the culture medium. It was also observed that SFX-Insect Medium was a suitable alternative to DMEM and was used without the need for 5% CO_2, but a minimum of 5% serum needed to be included. The toxicity of amphotericin B was tested and 0.875 µg/ml in DMEM and SFX-Insect Medium did not compromise cell viability. This information shows that earthworms can be cultured easily without the need for a CO_2 incubator, thus simplifying laboratory conditions and minimizing costs associated with using earthworms for cell biology curricula and research purposes. [J PA Acad Sci 89(2): 57-68, 2015

INTRODUCTION

Culturing mammalian cells *in vitro* has benefited from the development of various media and supplements that permit the growth and maintenance of a variety of cell types. This has enabled the investigation of molecular and cellular events associated with a vast array of biochemical and disease processes. Insect cells are also able to be cultured with ease for the purpose of synthesizing and purifying recombinant proteins using media that have been developed specifically for that purpose. The culturing of coelomocytes from earthworm species has relied heavily on the use of culture media that were developed for other cell systems and has necessitated an empirical approach to establish ideal culturing conditions. Researchers have used a variety of media to culture earthworm coelomocytes (leukocyte-like cells in the coelomic cavity) from *Eisenia* spp. For example, coelomocytes of *E. fetida* have been cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Cossarizza *et al.* 1996; Sherifa *et al.* 2005) and Dulbecco's Modified Eagle Medium (DMEM) (Engelmann *et al.* 2004; Yanqin *et al.* 2007; Gupta *et al.* 2014), while coelomocytes of *E. hortensis* have been cultured in DMEM (Fuller-Espie *et al.* 2010) and BaculoGoldTM insect medium (Cook *et al.* 2015). Furthermore, the concentration of bovine serum varies when culturing coelomocytes from these species ranging from 5% (Sherifa *et al.* 2005) to 10% (Engelmann *et al.* 2004; Cook *et al.* 2015). Although a long-standing earthworm coelomocyte buffer known as *Lumbricus* balanced salt solution (LBSS) has been described in the literature (Belmeskine *et al.* 2011), this medium is not commercially available and its preparation is therefore more labor-intensive than commericially available formulations such as RPMI, DMEM and insect media. It is of interest to note that modified culture conditions have been reported in ecotoxicological studies testing heavy metal toxicity in earthworms (Hayashi *et al.* 2012; Irizar *et al.* 2014).

Because of the ease of isolating coelomocytes from earthworms, and the fact that earthworm research does not require the need for an Institutional Animal Care and Use Committee (IACUC), this animal lends itself as an excellent model for teaching cell biology and microscopy techniques to undergraduate students in colleges and

[1]Accepted for publication October 2015

[2]Corresponding author: sfuller-espie@cabrini.edu, Professor of Biology, Cabrini College, 610 King of Prussia Road, Radnor, PA 19087-3698, Phone (610) 902-8369, Fax (610) 902-8285

in advanced placement (AP) courses in high schools as evidence-based modules. Earthworms have been utilized in a variety of college-level courses (Howard and Miskowski 2005; Melear and Lunsford 2007; Fuller-Espie 2010; Kladt *et al.* 2010; Shannon *et al.* 2014) and have much potential for teaching students important cellular mechanisms such as neurophysiology, phagocytosis, natural killer-like activity, the various aspects associated with apoptosis, and chemotaxis, to name a few. It was of interest to us to optimize *in vitro* culturing conditions for coelomocytes of *E. hortensis* and to determine if CO_2 could be omitted so that institutions that lack the capacity to invest in and maintain CO_2 incubators might be attracted to incorporating earthworms into their cell biology curricula using non-CO_2 laboratory incubators. This was established by using two different types of media, DMEM and SFX-Insect Media, culturing with and without 5% CO_2, and measuring cell viability after 24 h and 48 h using propidium iodide (PI) and flow cytometry.

In this study the effects of temperature on cell viability were also investigated. Temperatures of 25 °C, 29 °C and 37 °C were tested. We also sought to determine the optimum serum requirements for culturing coelomocytes *in vitro* by employing serial dilutions from 10% to 1.25%. To minimize costs, we used heat-inactivated newborn calf serum instead of heat-inactivated fetal calf serum, another factor that educators consider when implementing laboratory techniques into science curricula. We also tested viability in serum-free conditions. In addition, because we often experience problems with fungal contamination in culture periods exceeding 24 h, even in the presence of 0.25 µg/mL of amphotericin B and 50 µg/mL nystatin, we increased the concentration of the antifungal reagent amphotericin B and tested its toxicity over a range of concentrations from 0.25 µg/mL to 2.75 µg/mL in order to determine the maximum concentration for culturing earthworm coelomocytes without compromising cell viability.

This study shows that coelomocytes experience optimum survival when cultured in 10% newborn calf serum with no higher than 1.75 µg/mL amphotericin B added to complete medium. Serum depletion not only causes higher levels of cell death but also changes in cell morphology. Our results show that cell viability is not affected by removing CO_2 during the culture period when using complete medium supplemented with 10 mM HEPES. This information is particularly relevant to educators who are seeking an inexpensive and IACUC-independent animal model for cell biology-based laboratory courses in laboratories that are not equipped with CO_2 incubators.

MATERIALS AND METHODS

Animal Husbandry

Eisenia hortensis earthworms (Vermitechnology Unlimited, USDA Permit #52262) were maintained as short-term habitats in the dark and changed every 3-4 days. The habitats consisted of plastic boxes containing lids with HEPA filters, moistened (sterile H_2O) and autoclaved pine chips (Petco) and corn cob pellets (1/8 in, Harlan Teklad) as bottom bedding, and a top layer of shredded, autoclaved paper towels to maintain humidity. Nutrients included autoclaved slurries of Single Grain Rice Cereal or Oatmeal Banana Cereal (Gerber) and autoclaved whole oats (Quaker). A controlled temperature of 20 °C was maintained throughout the study. Euthanasia by freezing followed extrusion of coelomocytes.

Cell Culture Reagents

Phosphate buffered saline without calcium and magnesium (PBS, Hyclone), Classical Dulbecco's Modified Eagle Medium (DMEM, Hyclone), and SFX-Insect Medium with glutamine (Hyclone) were used for cell suspension and culturing. Unless otherwise indicated, media were supplemented with 10 µg/mL kanamycin (Shelton Scientific), 1X penicillin, streptomycin, amphotericin B (Gibco; amphotericin B final concentration at 0.25 µg/mL), 100 µg/mL ampicillin (Fisher BioReagents), 5 µg/mL chloramphenicol (Fluka Biochemika), 10 µg/mL tetracycline (Invitrogen), 50 µg/mL nystatin (Fisher Bioreagents), 1X non-essential amino acids (Gibco), 1X L-glutamine (Gibco), 10 mM HEPES buffer (Hyclone), and 10% heat-inactivated newborn calf serum (Sigma Aldrich, N4637) (complete media). Additional amphotericin B (Fisher BioReagents) was added as specified.

Coelomocyte Extrusion by Non-invasive Method

One day prior to coelomocyte extrusion, earthworms were transferred into Petri dishes without food containing paper towel strips saturated with 2.5 µg/mL Fungizone (Fischer Scientific). This process served to eliminate surface contamination and facilitate expulsion of gut contents prior to extrusion. Earthworms were placed individually into sterile troughs containing 3 mL of BD FACSFlow sheath fluid (BD Biosciences), the extrusion buffer which stimulated release of coelomocytes through the dorsal pores. The cell suspension was incubated 5 min with 0.5 mL of Accumax™ (Innovative Cell Technologies, Inc.) at room temperature to obtain single cell suspensions and reduce cell aggregation. After diluting samples with 5 mL of PBS, cells were collected by centrifugation (150 x g, 5 min, 4 °C)

and resuspended in 0.5 mL of PBS. Cell concentration was determined using a hemacytometer (Fisher Scientific) and phase-contrast microscopy. Samples containing the highest cell count and lowest proportion of autofluorescent eleocytes (easily identified by their large, granular appearance), and highest proportion of amoebocytes (smaller and less granular than eleocytes) were batched together in preparation for each assay. Typically 0.5 – 2 x 10^6 amoebocytes per earthworm were obtained.

Staining Coelomocytes with Propidium Iodide

After incubating coelomocytes according to the experimental condition of the assay, they were transferred to flow cytometry tubes and collected by centrifugation (150 x g, 5 min, 4 °C). After decanting the supernatant fraction, the cell pellets were resuspended in 200 µl of propidium iodide (PI, Molecular Probes) (2 µg/ml in PBS), a commonly used viability dye, placed on ice in the dark, and run immediately on the flow cytometer. Live cells did not take up PI, and were therefore not be detected by the FL-2 detector in the flow cytometer, while dead cells did take up PI, and were therefore FL-2 positive.

Coelomocyte Number, Plasticware and Post-treatment Collection

Fifty-thousand coelomocytes were cultured in 96-well, V-bottom, tissue culture-treated plates in 200 µl. Collection of coelomocytes after specified time period occurred in the plates following centrifugation (150 x g, 5 min). For all other experiments 60,000 to 150,000 coelomocytes (depending on cell yield) were cultured in 24-well tissue culture-treated plates in 2.5 mL with collection of coelomocytes after culturing period involving first the transfer of contents of wells to flow cytometry tubes, and then centrifuging as above.

Data Acquisition – Flow Cytometry List Mode Files

Data acquisition used a FACSCalibur flow cytometer equipped with an argon laser (488 nm) and Cell Quest Pro software (BD Biosciences). Voltage adjustments during instrument setup for forward scatter (FSC) which measured cell size, side scatter (SSC) which measured internal cell complexity and granularity, and FL-2, which detected the red fluorescence of PI (excitation/emission maxima of 493/636 nm), were determined by previewing the negative control samples on set-up mode. FSC threshold value (175) was adjusted to eliminate cellular debris from list mode data files. For each sample, 5,000 - 10,000 events were collected as list mode files.

Post-acquisition Data Analysis

For the analysis of post-acquisition list mode files, a region (R1) was placed around the area corresponding to the amoebocyte population on a two-dimensional histogram portraying FSC versus SSC, based on knowledge of expected size and granularity of relevant amoebocyte cells of *E. hortensis*. This region permitted the exclusion of highly auto-fluorescent eleocytes which would otherwise interfere with interpretation of PI-uptake by non-viable amoebocytes. A histogram of the baseline control gated on the R1 subpopulation was created, showing relative fluorescence intensity detected in FL-2 in log scale (abscissa) versus the cell count (ordinate). A marker (M1) was positioned to permit the discrimination of PI-negative (live) from PI-positive (dead) cells. The percent of gated cells that were PI-positive was determined for graphing and statistical analysis. Figure 1 depicts the gating and marker strategy used for data analysis.

Statistical Analysis

In Microsoft Excel 2010, the t-test: paired two-sample for means was used to determine statistical significance of the data obtained. In all assays, the percent PI positive for each triplicate set of treatments was compared to the baseline control wells as indicated. The average and standard deviation of each triplicate set of samples was determined and recorded. Statistical significance was granted to resulting p-values that were less than or equal to 0.05, indicating a 95% confidence interval.

RESULTS

Cell Viability at 24 h in DMEM or SFX-Insect Medium With and Without 10% Serum and the Effect of CO_2

The effect of culturing coelomocytes in either DMEM or SFX-Insect Medium, in the presence or absence of 10% serum with and without 5% CO_2 after 24 h at 25 °C is shown in Figures 2 and 3, respectively. Two separate assays performed on two different days were conducted with all samples run in triplicate. The graphs depict the increase in the percentage of cell death in paired treatments as indicated by brackets. The values shown represent the increase in cell death after the triplicate average of baseline (time zero) values was subtracted. When DMEM was used (Figure 2), there was a significantly higher level of cell death when cells were grown in the absence versus the presence of 10% serum but only when CO_2 was present; no significant difference

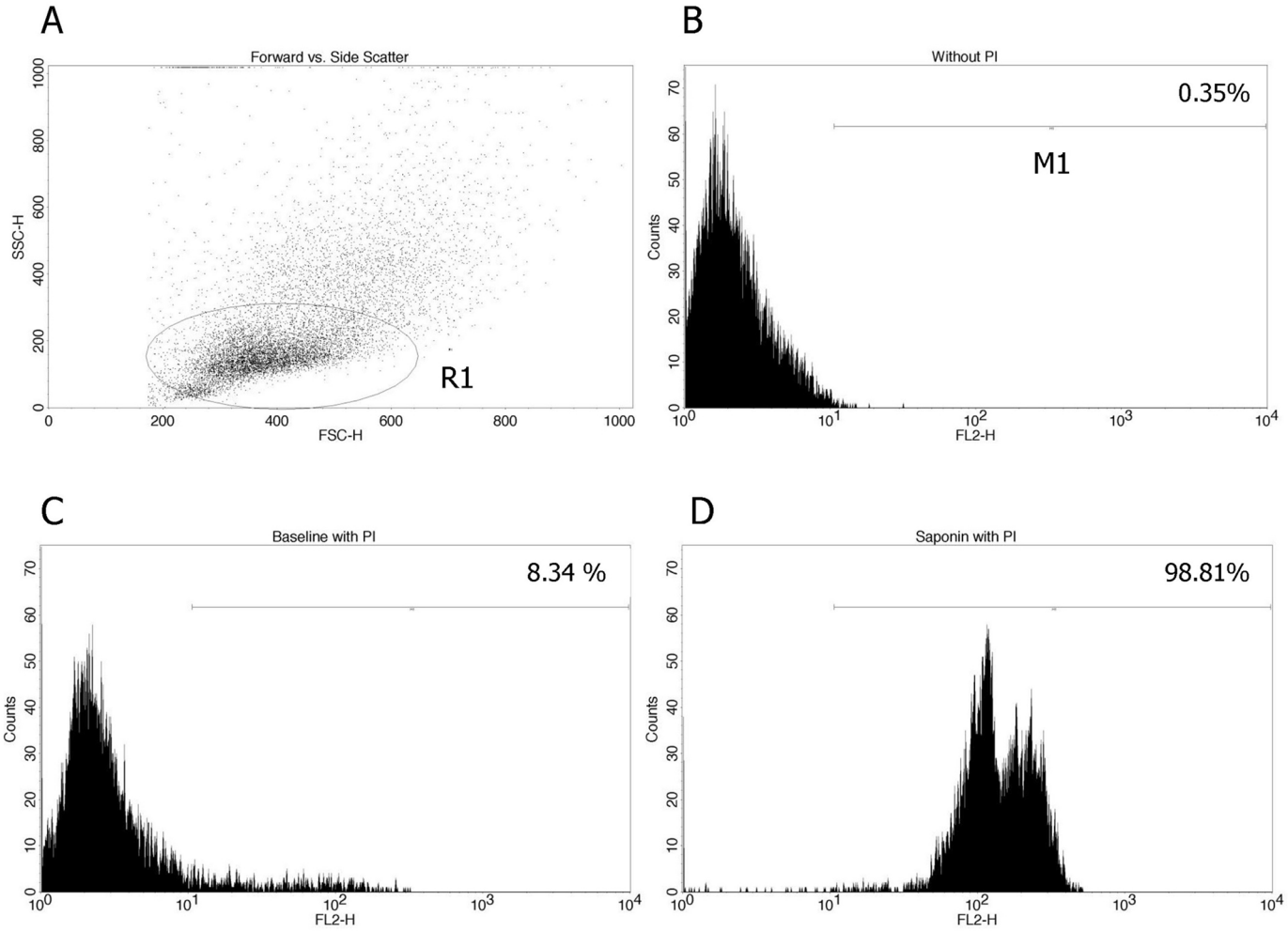

Figure 1. Representative flow cytometric analysis set-up for determination of cell viability. (A) Dot plot of forward scatter (FSC) (abscissa) versus side scatter (SSC) (ordinate) of all events collected. A region (R1) was set around the amoebocyte population to exclude auto-fluorescent eleocytes, and a high FSC threshold (175) was set to exclude cellular debris in final analysis. (B) Histogram gated on R1 of coelomocytes at time zero without the addition of propidium iodide (PI) depicting the relative fluorescence intensity (RFI) measured by the FL-2 detector (abscissa) in log scale versus cell count (ordinate). A marker (M1) was placed to facilitate the determination of the % non-viable (dead) cells in the gated amoebocyte population. Events falling within M1 correlate with PI-positive amoebocytes. (C) Histogram gated on R1 of baseline control sample at time zero plus PI. (D) Histogram gated on R1 of saponin-treated sample plus PI providing a positive control for cell death. The % PI-positive (dead) amoebocytes for (B-D) is indicated in the upper right corner of the histograms.

was observed when CO_2 was not provided (black brackets). When comparing the effect of CO_2 in the presence of 10% serum, there was no significant difference in either assay in the presence or absence of CO_2 (gray brackets). When SFX-Insect Medium was used (Figure 3), the deprivation of serum had a significant effect on cell viability whether or not CO_2 was provided (black brackets). Similar to DMEM, when 10% serum was provided, the presence or absence of CO_2 made no significant difference in cell death (gray brackets).

Cell Viability at 48 h in DMEM or SFX-Insect Medium With and Without 10% Serum and the effect of CO_2

A longer incubation period was also employed for DMEM and SFX-Insect Medium as a continuation of Assay B depicted in Figures 2B and 3B to measure the effect on cell death after 48 h in culture in order to determine optimum growth conditions for longer-term needs. Longer incubations may be desirable in studies employing timecourse experiments related to immunological or ecotoxicological processes as opposed to more immediate responses that require only short incubation periods, e.g. phagocytosis. Figure 4 depicts the

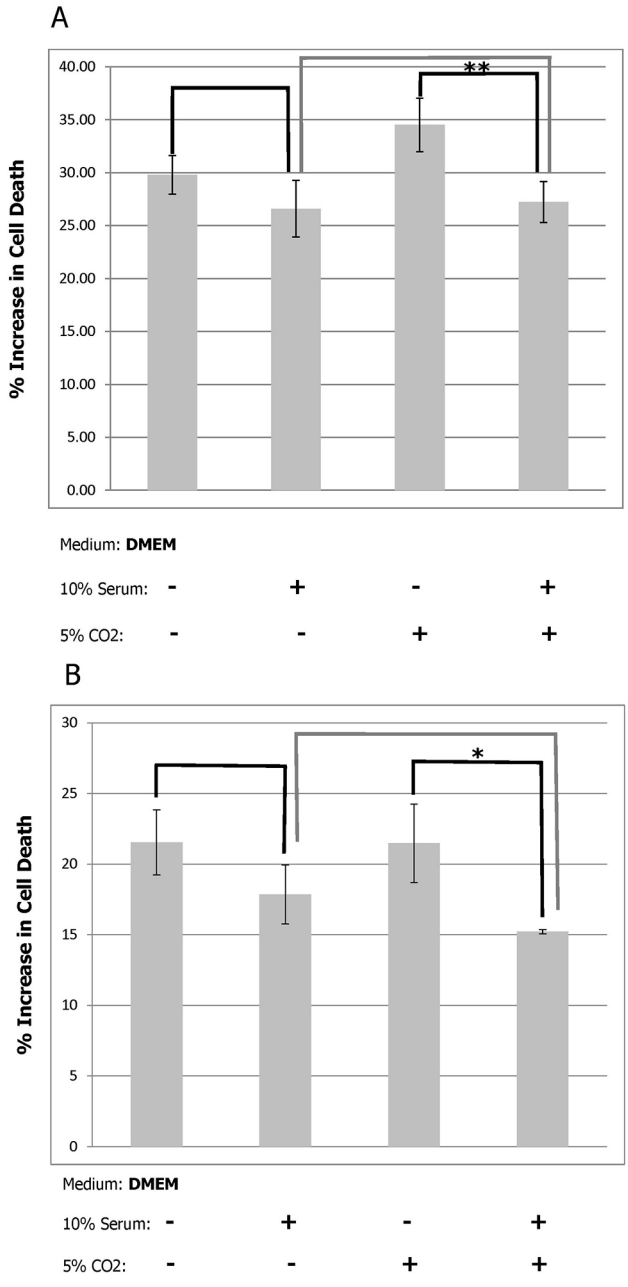

Figure 2. Cell viability in DMEM with and without 10% serum and the effect of CO_2 after 24 h. Two separate assays (A and B) were conducted at 25 °C. Culture conditions are indicated beneath the bar graphs. Percent increase in cell death after subtracting baseline average is shown. Statistical significance between paired treatments is indicated with brackets (* = $p \leq 0.05$; ** = $p \leq 0.005$). (DMEM = Dulbecco's Modified Eagle Medium)

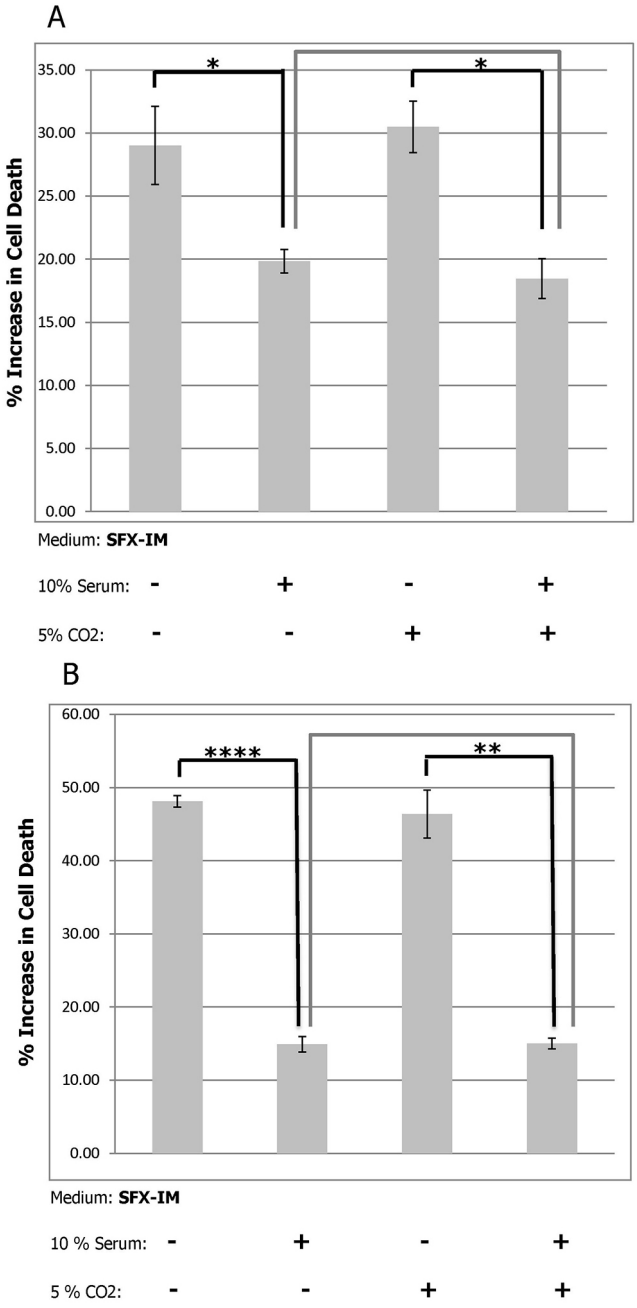

Figure 3. Cell viability in SFX-Insect Medium with and without 10% serum and the effect of CO_2 after 24 h. Two assays (A and B) were conducted at 25 °C. Culture conditions are indicated beneath the bar graphs. Percent increase in cell death after subtracting baseline average is shown. Statistical significance between paired treatments is indicated with brackets (* = $p \leq 0.05$; ** = $p \leq 0.005$; *** = $p \leq 0.0005$; **** = $p \leq 0.00005$). (SFX-IM = SFX-Insect Medium)

results at 48 h at 25 °C. Figure 4A shows that when CO_2 was not provided, there was no significant increase in cell death if serum was present or absent (black bracket, left side) when using DMEM. Likewise, when CO_2 was provided, there was no significant increase in cell death with or without serum (black bracket, right side). The level of cell death was lower, however, when CO_2 was provided. Figure 4A does show

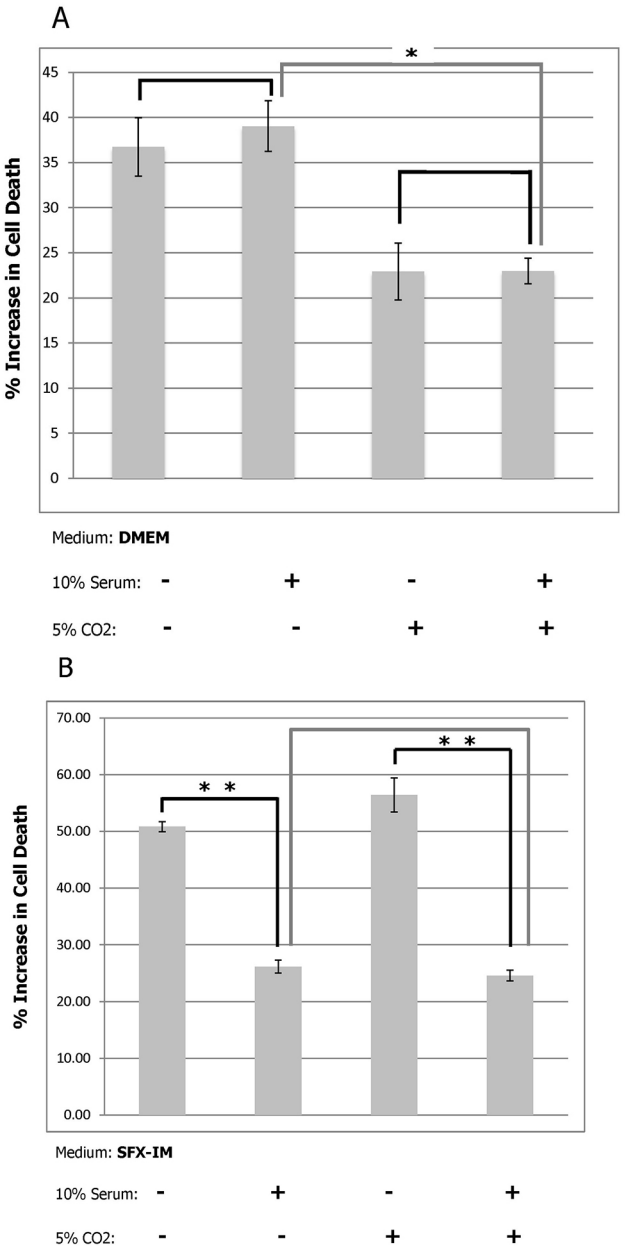

Figure 4. Cell viability in DMEM and SFX-Insect Medium with and without 10% serum and the effect of CO_2 after 48 h. Assays were conducted at 25 °C in DMEM (A) or SFX-Insect Medium (B). Culture conditions are indicated beneath the bar graphs. Percent increase in cell death after subtracting baseline average is shown. Statistical significance between paired treatments is indicated with brackets (* = $p \leq 0.05$; ** = $p \leq 0.005$).

significant differences when comparing the absence versus the presence of CO_2 when serum was present; much lower levels of cell death above baseline occurred when both serum and CO_2 were provided (gray bracket). This difference was not observed at 24 h.

When SFX-Insect Medium was used (Figure 4B) the absence of serum had a very significant effect on cell viability whether or not CO_2 was provided (black brackets). When serum was present, there was no significant change in cell viability whether or not CO_2 was provided (gray bracket).

Table 1 compares the results of 24 h with 48 h using the same batch of earthworm coelomocytes and shows that the most dramatic increases in cell death at 48 h were observed using SFX-Insect Medium +/- CO_2 with serum deprivation where increases in cell death above baseline exceeded 50%, approximately twice that observed with SFX-Insect Medium +/- CO_2 plus serum. In all cases the levels of cell death at 48 h were significantly higher compared to 24 h.

Coelomocytes Exhibit Higher Cell Death at Temperatures of 29 °C and 37 °C compared to 25 °C

The effect of temperature above 25 °C (baseline) was investigated after 24 h of culture. Two assays were conducted on two separate days with all treatments in triplicate. Either DMEM or SFX-Insect Medium containing 10% serum was used plus or minus 5% CO_2. Figure 5 demonstrates that in every case except one, the increase in cell death was statistically significant in both assays at 29 °C (black brackets) and 37 °C (gray brackets) compared to baseline with or without CO_2. The exception was SFX-Insect Medium at 29 °C without CO_2 where significance was observed in only one of the two assays.

Titration of Serum Reveals Increased Cell Death and Changes in Cellular Morphology

The effect of reducing serum concentration from 10% to 1.25% in two-fold serial dilutions was investigated after 24 h of culture at 25 °C to determine if serum reduction decreased cell viability. Only two conditions were used: DMEM plus CO_2, and SFX-Insect Medium minus CO_2 (Figure 6). Treatments were performed in triplicate. Significant increases in cell death were observed when DMEM was used at 2.5% and 1.25% serum, but not at 5% serum compared to 10% (baseline). In contrast, when SFX-Insect Medium was used, no significant increases in cell death were observed at 5%, 2.5% or 1.25% serum compared to 10%. Interestingly, flow cytometric analysis revealed changes in cell morphology following serum reduction. Figure 7 shows that when DMEM was used, there was a significant decrease in the geometric mean of forward light scatter (a measure of cell size, X axis) at serum concentrations of 2.5% serum compared to baseline ($p = 0.0043$). Significant decreases in forward light scatter were also observed when 5% serum ($p = 0.0039$) and 1.25% serum ($p = 0.0019$) were used compared to baseline (data not shown). When SFX-Insect Medium was used, decreases in forward light scatter were not observed, however, there was a significant decrease in the geometric

Table 1. Comparison of cell viability at 24 h and 48 h at 25 °C. Percent increase in cell death above baseline at 24 h and 48 h in DMEM or SFX-Insect Medium, without and with 10% serum in the absence or presence of CO_2 is noted ± standard deviation. Statistical significance comparing 24 h with 48 h is depicted (* = $p \leq 0.05$; ** = $p \leq 0.005$; *** = $p \leq 0.0005$). (DMEM = Dulbecco's Modified Eagle Medium; SFX-IM = SFX-Insect Medium; Sf = serum-free).

Medium	Condition	% ↑ Death 24h	% ↑ Death 48h	Significance
DMEM	Sf -CO2	21.53 (±2.3)	36.75 (±3.24)	*
	+Serum –CO2	17.84 (±2.08)	39.04 (±2.8)	**
	Sf +CO2	21.47 (±2.78)	22.92 (±3.15)	*
	+Serum +CO2	15.32 (±0.12)	22.99 (±1.4)	**
SFX-IM	Sf -CO2	48.11 (±0.80)	50.82 (±0.86)	*
	+Serum –CO2	14.91 (±1.04)	26.15 (±1.14)	**
	Sf +CO2	46.39 (±3.28)	56.41 (±3.02)	***
	+Serum +CO2	15.03 (±0.74)	24.59 (±0.93)	**

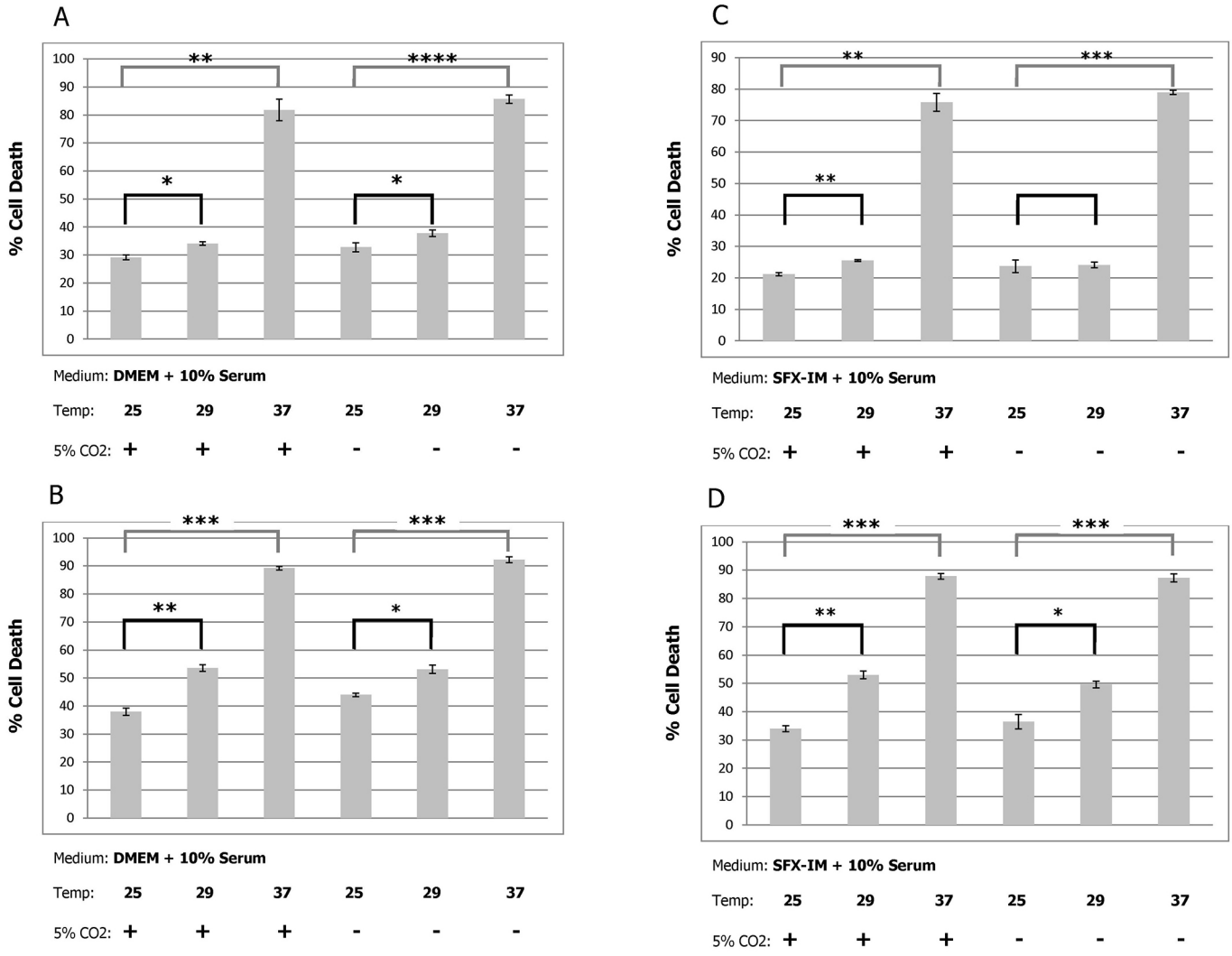

Figure 5. Temperatures exceeding 25 °C compromise cell viability. Two assays were conducted with DMEM (A and B) and SFX-Insect Medium (C and D). In all cases 10% serum was included. Culture conditions are indicated beneath the bar graphs. Percent cell death is shown. Statistical significance between paired treatments is indicated with brackets (black brackets compare 25 °C to 29 °C; gray brackets compare 25 °C to 37 °C). (* = $p \leq 0.05$; ** = $p \leq 0.005$; *** = $p \leq 0.0005$; **** = $p \leq 0.00005$).

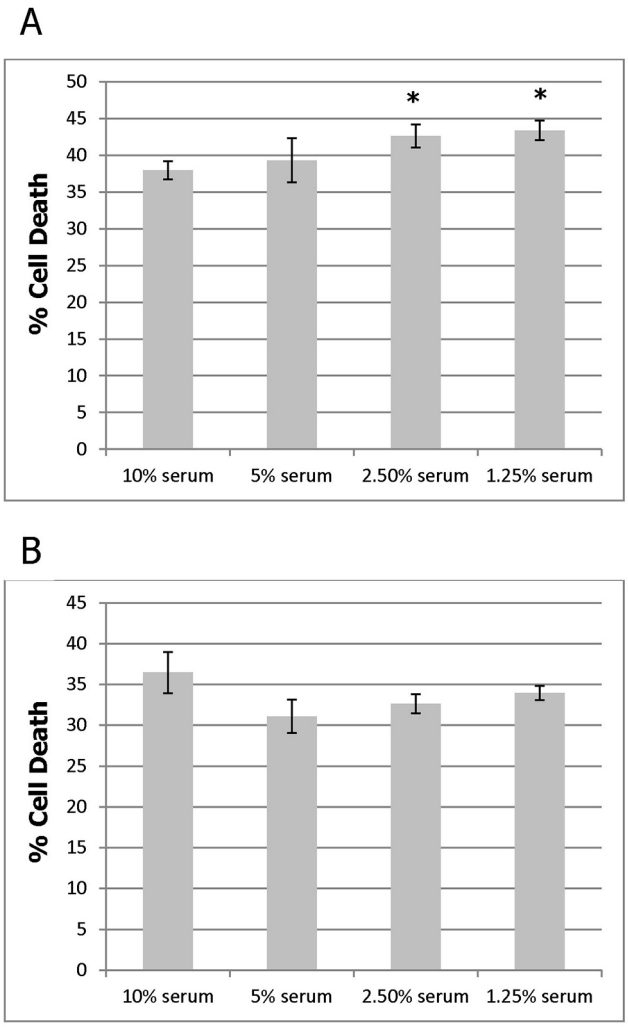

Figure 6. Effect of serum titration on cell viability. Coelomocytes were cultured 24 h in either DMEM + 5% CO_2 (A) or SFX-Insect Medium without CO_2 (B) at serum concentrations as indicated. Assays were conducted at 25 °C. Percent cell death is shown. Statistical significance compared to baseline (10% serum) is indicated. (* = $p \leq 0.05$).

mean of side light scatter (a measure of cell granularity, Y axis) at serum concentrations of 2.5% compared to baseline ($p = 0.0209$). Significant decreases in side light scatter were also observed when 1.25% serum ($p = 0.0452$), but not 5% serum ($p = 0.0561$), were used compared to baseline (data not shown).

Amphotericin B Toxicity

Because fungal contamination is often a complication when attempting to culture coelomocytes long-term, the effects of increasing the concentration of amphotericin B, an antimycotic agent, above baseline (0.25 µg/mL, see Methods) was tested. Figure 8 illustrates that amphotericin B in DMEM at 25 °C causes significant increases in cell death at 2.75 µg/mL, but not at lower concentrations. In SFX-Insect Medium, toxicity was observed at both 2.75 µg/mL and 1.5 µg/mL but not at lower concentrations.

DISCUSSION

Our results demonstrate that coelomocytes of *E. hortensis* exhibit the highest level of cell viability when cultured in DMEM or SFX-Insect Medium supplemented with 10% newborn calf serum at 25 °C. Serum contains a complex array of substances that are important for chelating water-insoluble or labile nutrients, binding to and neutralizing toxic compounds, and transporting nutrients into cells. Serum also provides protease inhibitors, hormones, growth factors, amino acids, proteins, vitamins, lipids, carbohydrates, minerals and trace elements (Arora 2013).

The absence of CO_2 did not significantly compromise viability at 24 h when 10% serum was present whether DMEM or SFX-Insect Medium was used. This observation is most likely attributed to the fact that complete medium is supplemented with 10 mM HEPES (see Materials and Methods), a zwitterion which buffers well in the pH range of 7.2 – 7.4 without the need for a gaseous atmosphere (Shipman 1969). The provision of a HEPES-buffered medium would have been particularly important for stabilizing the pH in DMEM which contains 3.7g/L $NaHCO_3$ (versus 0.35g/L $NaHCO_3$ in SFX-Insect Medium) which requires artificial levels of CO_2 to maintain the required pH (Arora 2013). It is also notable that although the difference for DMEM + serum without or with CO_2 wasn't largely different at 24 h, it was significantly different at 48 h. The same batch of coelomocytes were used for the extended time study. Specifically, in Assay 2 values of 17.84% versus 15.32% were observed for DMEM + serum without or with CO_2, respectively (Figure 2B, gray brackets). These values increased considerably and significantly after 48 h (39.04% versus 22.99%) (Table 1 and Figure 4A, gray brackets). It is interesting to speculate that perhaps the buffering capacity of HEPES in DMEM is adequate at 24 h in the absence of CO_2, but after 48 h its ability to maintain the necessary pH for coelomocyte survival is overwhelmed due to metabolic byproduct accumulation during the cell culture period and the high $NaHCO_3$ concentration in DMEM, thereby necessitating CO_2 as the backup buffering system. This would account for the ~13% reduction in cell death when CO_2 was present.

Temperatures of both 29 °C and 37 °C showed significant increases in cell death compared to 25 °C. This was particularly striking at 37 °C where an increase of more than 50% compared to baseline was noted. These results are consistent with those reported by Tumminello and Fuller-Espie (2013) who used DMEM/10% serum/5% CO_2 medium

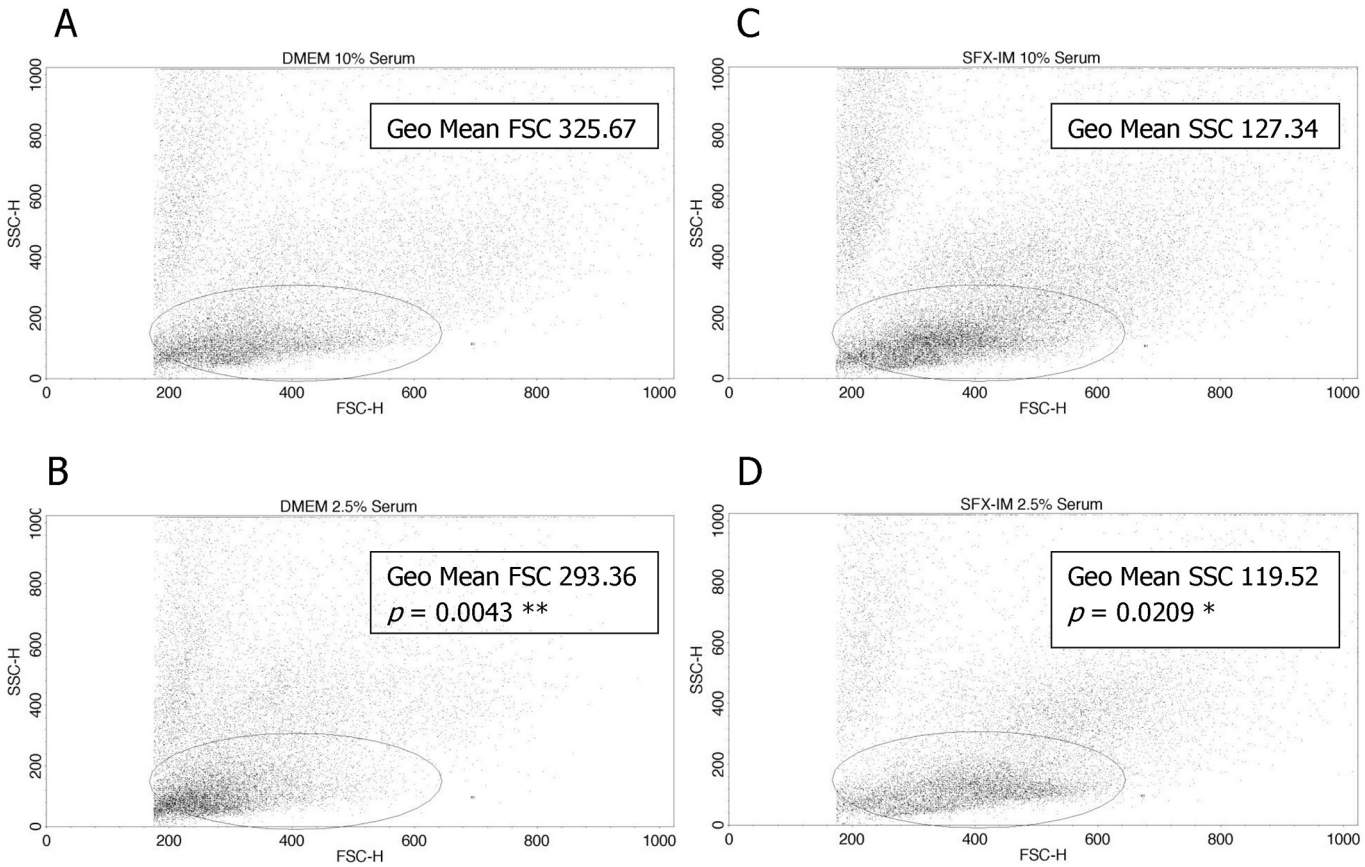

Figure 7. Analysis of light scatter by flow cytometry reveals cell morphology changes during serum deprivation. Coelomocytes were cultured 24 h in either DMEM + 5% CO_2 (A and B) or SFX-Insect Medium without CO_2 (C and D) at 25 °C. Figures are representative of samples run in triplicate. A and C represent 10% serum. B and D represent 2.5% serum. Significant decreases in forward light scatter (FSC) or side light scatter (SSC) were observed for DMEM and SFX-Insect Medium, respectively. p values indicate statistical significance based on average of triplicate data comparing 10% serum (baseline) to 2.5% serum (* = $p \leq 0.05$; ** = $p \leq 0.00$).

and employed a much wider range of temperatures from 4 °C to 44 °C. In that earlier study, which also employed *E. hortensis*, production of reactive oxygen species (ROS) and H2AX histone phosphorylation were also investigated in response to heat stress. That study showed that as temperature increased, both ROS and histone phosphorylation increased above baseline temperatures of 20 °C or 25 °C. The use of SFX-Insect Medium was not included in that study, so our data provides new information regarding the effects of heat stress when culturing coelomocytes in this alternative medium that does not require the presence of 5% CO_2.

Serum deprivation at concentrations 2.5% or lower in DMEM significantly affected increases in cell death, but this effect was not seen with SFX-Insect Medium as detected by PI uptake. Changes in cell morphology, however, were observed when serum concentrations were reduced to 5% or lower for DMEM and to 2.5% or lower for SFX-Insect medium; coelomocytes exhibited significant decreases in forward side scatter in DMEM and side scatter in SFX-Insect Medium. Therefore, measuring changes in the light scatter properties of coelomocytes by flow cytometry, and not relying solely on PI uptake, revealed deleterious effects of serum starvation that would have otherwise been overlooked. Changes in light scatter properties associated with necrotic and apoptotic processes have been well documented (Swat *et al.* 1981; Ormerod *et al.* 1995; Darzynkiewicz and Li 1996).

Although nystatin and amphotericin B are both included in complete media (see Material and Methods), there are sometimes problems with fungal contamination in our lab using assays that extend beyond 24 h incubation. Presumably the source is from the earthworms that, despite all attempts to sterilize the habitats and food of the animals, are not completely microbial free and bacteria and fungi do populate the coelomic cavity. The availability of different types of antimycotic agents is very limiting for cell culture purposes. This prompted a second look at whether the amphotericin B concentration that was being used in the complete media (0.25 µg/mL) could be increased in order to eliminate issues with fungal outgrowth in cultures incubated for extended time periods. Suppliers of antibiotics and antimycotics often

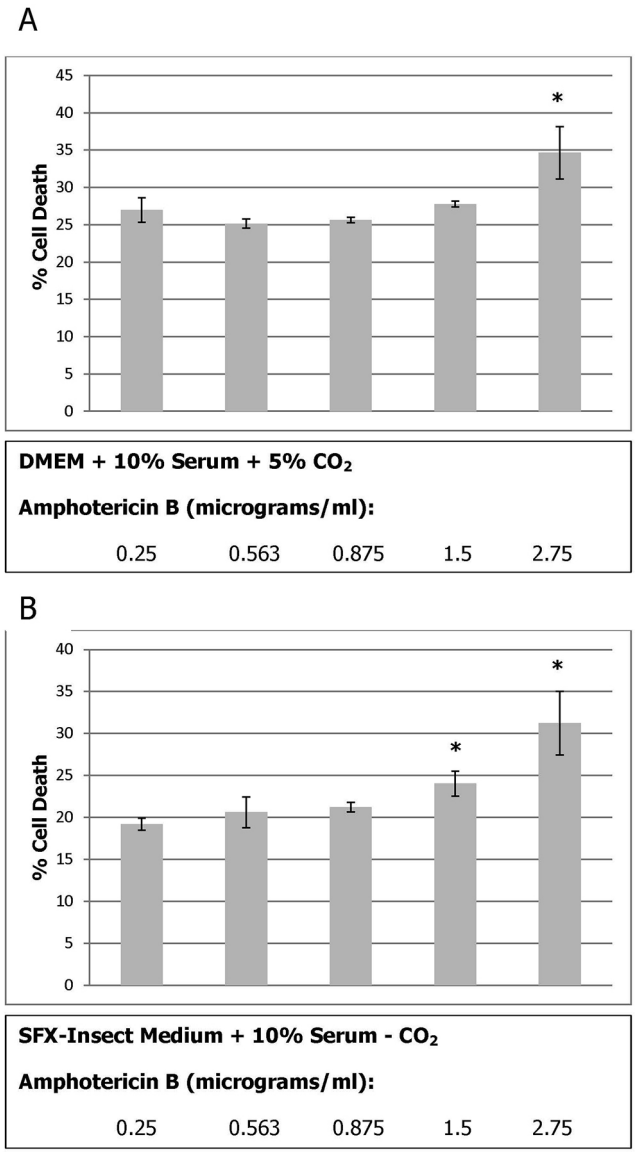

Figure 8. Effects of amphotericin B titration on cell viability. Coelomocytes were cultured 24 h in either DMEM + 5% CO_2 (A) or SFX-Insect Medium without CO_2 (B) at concentrations of amphotericin B as indicated. Assays were conducted at 25 °C. Percent cell death is shown. Statistical significance above baseline (0.25 µg/mL) is indicated. (* = $p \leq 0.05$).

recommend that these drugs, especially amphotericin B, be tested for their toxicity depending on the particular cell line that is being used (e.g. refer to Life Technologies website at http://www.lifetechnologies.com/us/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/use-of-antibiotics-and-antimycotics.html). Working with mouse osteoblast and fibroblast cell lines, Harmsen *et al.* (2011) show clearly the need to establish the level of sensitivity to amphotericin B when conducting *in vitro* investigations. Amphotericin B was toxic to coelomocytes at 2.75 µg/mL in the two experimental conditions studied compared to 0.25 µg/mL (baseline control). These conditions included: 1) DMEM/10% serum plus 5% CO_2 at 25 °C; and 2) SFX-Insect Medium/10% serum minus CO_2 at 25 °C. Toxicity was also observed at 1.5 µg/mL with SFX-Insect Medium, but not with DMEM. Importantly, no significant increase in cell death was observed at 0.875 µ/mL compared to 0.25 µg/mL (baseline control), indicating that the complete medium we use routinely could be further supplemented with a higher concentration of amphotericin B in order to inhibit fungal contaminants when longer culture periods are needed. There were no notable differences in forward or side scatter properties in the amphotericin trials as were observed with the temperature trials (data not shown). Therefore, this study demonstrates clearly that toxic effects are observed with this antimycotic agent when used at high concentrations. This reagent should be used at concentrations that are not toxic when conducting *in vitro* experiments with earthworm coelomocytes. There may be species disparities, e.g. differences in toxicity between *E. fetida* versus *E. hortensis* so researchers should determine appropriate ranges empirically with different species of earthworms.

Although this study employed a relatively sophisticated method to determine cell viability involving instrumentation that most undergraduate teaching labs would not use, it should be mentioned that trypan blue exclusion is an excellent alternative for measuring cell viability (Strober 2001), and this technique could be readily adopted by educators and researchers planning to use earthworm coelomocytes as indicators of toxicity by environmental xenobiotics such as polycyclic aromatic hydrocarbons and heavy metals which have been tested previously for their inhibitory effect on immune responses in *E. fetida* and *E. hortensis* (Patel *et al.* 007; Fuller-Espie *et al.* 2011).

CONCLUSION

In summary, we conclude that the coelomocytes of *E. hortensis* exhibit the highest level of cell viability when cultured with 10% newborn calf serum at 25 °C in both DMEM and SFX-Insect Medium. Lowering this to 5% with SFX-Insect Medium poses not observable detrimental effects at measured by PI-uptake or side light scatter analyses, but decreases in forward light scatter were noted at this concentration with DMEM. We did not test fetal calf serum (FCS) in this study in an effort to reduce costs for educators interested in using earthworms in their cell biology labs, but using FCS should not pose any problems and may actually improve culturing conditions. If DMEM is used, then CO_2 should be provided if planning to culture more than 24 h, but this can be omitted for shorter periods of culture providing HEPES is included in the culture medium. SFX-Insect Medium can be used an alternative to DMEM without CO_2, but a minimum of 5% serum should be included. To

help minimize fungal contamination during longer-term culturing needs, nystatin (50 µg/ml) and amphotericin B (0.875 µg/ml) should be included in the culture medium. This information shows that coelomocytes of earthworms can be cultured easily for research and teaching purposes without the need for a CO_2 incubator which will be helpful for institutions that lack this capacity.

ACKNOWLEDGEMENTS

The authors thank the Pennsylvania Academy of Science for awarding Darcy Harris a high school research grant to help fund this work. Gratitude is also extended to additional funding sources including the Cabrini College Faculty Grants, Initiatives and Sabbaticals Subcommittee for awarding Dr. Sheryl Fuller-Espie a Faculty Development Summer Grant, the Cabrini College Joseph Romano Undergraduate Research Fund for providing a summer undergraduate research stipend to Juliann Jakeman and faculty research stipend to Dr. Fuller-Espie, and the Cabrini College Borneman Undergraduate Scholarship Fund for providing a summer undergraduate research stipend to Jennifer Daly. Additionally, the Cabrini College Science Department Research Fund provided funding for laboratory reagents.

LITERATURE CITED

Arora, M. 2013.Cell culture media: A review. Mater Methods 3:175.

Belmeskine, Hl, Brousseau, P., Haddad, S., Vandelac, L., and Fournier, M. 2011. Effects of polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans on phagocytic response of *Eisenia andrei* coelomocytes. J. Xenobiotics 1:e6, 29-34.

Cook, S.R., Sperratore, M.M., and Fuller-Espie, S.L. 2015. Nitric oxide production in celomocytes of the earthworm *Eisenia hortensis* following bacterial challenge. Inv. Surv. J. 12:46-65.

Cossarizza, A., Cooper, E.L., Suzuki, M.M., Salvioli, S., Capri, M., Gei, G., Quaglino, D. and Franceschi, C. 1996. Earthworm leukocytes that are not phagocytic and cross-react with several human epitopes can kill human tumor cell lines. Exp. Cell Res. 234:174-182.

Darzynkiewicz, Z. and Li, X. 1996. Measurements of cell death by flow cytometry. In: Techniques in Apoptosis: A User's Guide. TG Cotter and SJ Martin, Eds. Portland Press, London. pp. 71-106.

Engelmann, P., Kiss, J., Csöngei, V., Cooper, E.L. and Németh P. 2004. Earthworm leukocytes kill HeLa, HEp-2, PC-12 and PA317 cells *in vitro*. J. Biochem. Biophys. Methods 61:215-227.

Fuller-Espie, S.L. 2010. Using flow cytometry to measure phagocytic uptake in earthworms. J. Microrbiol. Biol. Educ. 11(1): 144-151.

Fuller-Espie, S.L., Nacarelli, T., Blake, E.L., and Bearoff, F.M. 2010. The effect of oxidative stress on phagocytosis and apoptosis in the earthworm *Eisenia hortensis*. Inv. Surv. J. 7:89-106.

Fuller-Espie, S.L. Bearoff, F.M. and Minutillo, M. 2011. Exposure of coelomocytes from the earthworm *Eisenia hortensis* to Cu, Cd, and dimethylbenz[a]anthracene: An *in vitro* study examining reactive oxygen species production and immune response inhibition. Pedobiologia 545:S31-S36.

Gupta, S., Kushwah, T., and Yadav, S. 2014. Earthworm coelomocytes as nanoscavenger of ZnO NPs. Nanoscale Re. Lett. 9:259.

Harmsen, S., McLaren, A.C., Pauken, C. and McLemore R. 2011. Amphotericin B is cytotoxic at locally delivered concentrations. Clin. Orthop. Relat. Res. 469(11):3016-3021.

Hayashi, Y., Engelmann, P., Foldbjerg, R., Szabó, J., Somogyi, I., Pollák, E., Molnár, L., Autrup, H., Sutherland, D.S., Scott-Fordsmand, J. and Heckmann, L.H. 2012. Earthworms and humans *in vitro*: characterizing evolutionarily conserved stress and immune responses to silver nanoparticles. Environ. Sci. Technol. 46(7): 4166-4173.

Howard, D.R. and Miskowski, J.A. 2005. Using a module-based laboratory to incorporate inquiry into a large cell biology course. Cell Biol. Educ. 4(3):249-260.

Irizar, A., Duarte, D., Guilhermino, L., Marigómez, I. and Soto, M. 2014. Optimization of NRU assay in primary cultures of *Eisenia fetida* for metal toxicity assessment. Ecotoxicology 23(7):1326-1335.

Kladt, N., Hanslik, U. and Heinzel, H-G. 2010. Teaching basic neurophysiology using intact earthworms. J. Undergrad. Neurosci. Educ. 9(1):A20-A35.

Melear, C.T. and Lunsford, E. 2007. Worms cultivate our curriculum: A long-term, theme-based unit. Science Activities: Classroom Proj. and Curric. Ideas 44(2):48-54.

Ormerod, M.G., Cheetham, F.P.M., Sun, X-M. 1995. Discrimination of apoptotic thymocytes by forward light scatter. Cytometry 21: 300-304.

Patel, M., Francis, J., Cooper, E.L. and Fuller-Espie, S.L. (2007) Development of a flow cytometric, non-radioactive cytotoxicity assay in *Eisenia fetida*: An *in vitro* system designed to analyze immunosuppression of natural killer-like coelomocytes in response to 7, 12 dimethylbenz[a]anthracene (DMBA). Eur. J. Soil Biol 43:S97-S103.

Shannon, K.M., Gage, G.J., Jankovic, A., Wilson, W.J. and Murzullo, T.C. 2014. Portable conduction velocity experiments using earthworms for the college and high school neuroscience teaching laboratory. Adv. Physiol. Educ. 38(1):62-70.

Shipman, C. 1969. Evaluation of 4-(2-hydroxyethyl)-1-piperazineëthanesulfonic acid (HEPES) as a tissue culture buffer. Proc Soc Exp Biol Med. 130:305-310.

Strober, W. 2001. Trypan blue exclusion test of cell viability. Curr. Protoc. Immunol., May: Appendix 3: Appendix 3B. doi: 10.1002/0471142735.ima03bs21.

Swat, W., Ignatowicz, L., Kisielow, P. 1981. Detection of apoptosis of immature CD4+8+ thymocytes by flow cytometry. J. Immunol. Meth. 137: 79-87.

Tumminello R.A. and Fuller-Espie, S.L. (2013) Heat stress induces ROS production and histone phosphorylation in celomocytes of *Eisenia hortensis*. Inv. Surv. J. 10:50-57.

Yanqin, L., Yan, S., Zhenjun, S., Shijie, L., Chong, W., Yan, L. and Yuhong, G. 2007. Coelomic fluid of the earthworm *Eisenia fetida* induces apoptosis of HeLa cells *in vitro*. Eur. J. Soil Biol. 43:S143-S148.

Journal of the Pennsylvania Academy of Science 89(2): 69-79, 2015

EVALUATION OF CHIRONOMIDAE DIVERSITY IN THE LITTLE PAINT CREEK WATERSHED, PENNSYLVANIA[1]

REBECCA L. WEBB[2], DEANNAH DICK* AND RYAN WILSON*

Department of Biology, University of Pittsburgh at Johnstown, Johnstown, PA 15904

ABSTRACT

The health of a watershed can be assessed by evaluating the benthic macroinvertebrates that are present in the waterways. Benthic chironomid larvae (Diptera: Chironomidae) are often used as a biological indicator of ecological health. The chironomid larvae are particularly tolerant to contaminants, with different taxa exhibiting different levels of tolerance. Thus, evaluation of the abundance and diversity of chironomids within a watershed can be an assessment tool for the biotic health of the watershed. Although several studies of Pennsylvania watersheds have identified the presence of Chironomidae, our study aimed to further understand chironomid diversity by assessing them to the subfamily and genus level. We surveyed eight sites over Little Paint Creek and three tributaries within the Little Paint Creek watershed in southwestern Pennsylvania. By comparing the chironomid abundance and diversity to previously published EPT data for the watershed, our data suggests that water quality of tributary UNT 45234 is better than previously predicted and may be better than that of tributary UNT 45242. The limited number of less tolerant subfamilies of chironomids in the Little Paint Creek samples suggests that this stream may have reduced water quality. We established a baseline of chironomid abundance and diversity within the Little Paint Creek watershed. Our future goal for the project will be to continue to monitor the chironomids at these sites and to compare our findings to areas within the watershed which have been reported to have higher rates of contaminants due to acid mine drainage and Marcellus shale drilling, to determine their effect on aquatic macroinvertebrate diversity. [J PA Acad Sci 89(2): 69-79, 2015]

INTRODUCTION

Many studies assess aquatic organismal diversity to evaluate the quality of the water within a watershed, and to determine the biotic health of the waterways (Burger, 1997; Byrne *et al*., 2013; Fore *et al*., 1996; Hart *et al*., 2014; Jun *et al*., 2012; Lu *et al*., 2013; Meng *et al*., 2009). Benthic macroinvertebrate community diversity, expressed as the Index of Biotic Integrity (IBI), is commonly used to determine the quality of a particular stream. The IBI is used to determine the health of the watershed and is an accepted determinant for designating the usage of streams (Fore *et al*., 1996). The Pennsylvania Department of Environmental Protection assigns levels of stream protection based on many determinants, one of which is the IBI (Pennsylvania and Water Quality Standards, 2012). Higher organism diversity results in a high IBI value, which in turn provides a potentially greater level of protection for the stream (Pennsylvania and Water Quality Standards, 2012). Waterways with the most diversity are protected and labeled as Exceptional Value or High Quality streams.

Another commonly used assessment for stream health is the EPT index, which reports the number of different Ephemeroptera, Plecoptera, and Trichoptera taxa in a particular waterway. However, this index does not include the potentially useful information that could be gained from looking at other taxa, such as benthic Chironomidae. In most studies, benthic chironomid larvae are only identified to the order Diptera and family Chironomidae and are listed as supporting data, along with the reported EPT indices, as the EPT+C index (Abhijna *et al*., 2013; El-Khayat *et al*., 2011; Ferreira *et al*., 2011; Jun *et al*., 2012; Panas *et al*., 2014; Reckner, 2011; Relyea *et al*., 2012). Currently, EPT + Chironomidae index is used along with total taxa richness, diversity and evenness in the rapid bioassessment of a watershed.

However, chironomids, as a group, are generally more tolerant of pollutants in the water than many organisms within the EPT taxa (Abhijna *et al*., 2013; Al-Shami *et al*., 2010; Mousavi *et al*., 2003; Tripole *et al*., 2006). Trichoptera species, for example, have a range of tolerances. However, overall, Trichoptera are more sensitive to environmental disturbances than Chironomidae. An increase in the proportion of Chironomidae relative to EPT should

[1]Accepted for publication May 2015

[2]University of Pittsburgh at Johnstown, Department of Biology, 450 Schoolhouse Road, Johnstown, PA 15904, rwebb@pitt.edu, 814-269-2913

*These authors contributed equally to this paper

be considered as an indicator of environmental stress (Mandaville, 2002). Chironomidae larvae are not typically identified further than family, because it requires extensive analyses of multiple microscopic morphological structures, and are not further distinguished in the IBI. By assessing the diversity and abundance within the Chironomidae family, an evaluation of streams can be conducted to support and verify the EPT data, providing further insight into the health of the water. We suggest that when EPT + Chironomidae index is low that the chironomids should be further identified to subfamily and genus to help understand the health of the stream.

Chironomidae have adapted to virtually every aquatic environment and are important bioindicators of aqueous ecosystem health. These holometabolous organisms spend their embryonic and larval stages in the water and underlying sediment, where they can burrow in and exist for two weeks to several years before emerging from the pupal case on the surface of the water to become a flying insect (Armitage *et al*., 1995). Due to their life cycle, changes in both the water and the sediment can affect the presence of Chironomidae. Globally, different genera of Chironomidae larvae have been found in moderate and extreme temperatures (Bouchard *et al*., 2006; Carrillo *et al*., 2004; Hayford *et al*., 1995), at a range of altitudes (Hayford and Ferrington Jr, 2006; Koshima, 1984), on every continent, and in both clean and contaminated waters (Bode, 1990; Bode *et al*., 1996; Mandaville, 2002). Distinct taxa of chironomids have differing levels of tolerance to pollution; therefore chironomid diversity is positively correlated to water quality (Bode, 1990; Bode *et al*., 1996; Mandaville, 2002). Hilsenhoff generated a tolerance index for the subfamilies of Chironomidae, based on abundance and diversity of chironomids in relationship to the water quality, that can be used to evaluate different watersheds (Hilsenhoff, 1988; Mandaville, 2002). Streams containing chironomid genera most sensitive to pollution are considered of better quality than those which contain only tolerant genera.

Chironomid diversity within the eastern part of the United States has been reported for Ohio, North and South Carolina, Florida, Georgia and New York (Bolton, 2012; Caldwell, 1984; Epler, 2001; Simpson and Bode, 1980). Within the order Diptera, the family Chironomidae includes 11 subfamilies, 339 genera and over 4,000 identified species (Ferrington Jr, 2008). In Ohio, for example, six of the subfamilies and multiple genera and species, totaling 510 Chironomidae taxa, have been identified (Bolton, 2012). Although a checklist of Ohio chironomid larvae has recently been published, to our knowledge there has been no similar statewide survey of chironomids completed in Pennsylvania in clean or contaminated waters (Bolton, 2012). A study in 2011 of the middle Penns Creek Watershed (located in central Pennsylvania) established baseline conditions of the streams within the watershed to assess the stream's health (Panas *et al*., 2014). This study did not identify chironomids beyond family. Additionally, studies conducted on waterways in the Little Paint Creek (LPC) Watershed, in Southwestern Pennsylvania, have only classified chironomids (and other benthic macroinvertebrates) to family (Dillon and Lee, 2002; Reckner, 2011). It is unknown whether the subfamilies and genera distribution patterns within surrounding states extend into Pennsylvania. While we predict that many of the subfamilies and genera are the same as in other states, with similar predominant subfamilies, this has not been evaluated previously. Within the Little Paint Creek Watershed specifically, there are sites of acid mine drainage and Marcellus shale wells (Reckner, 2011). The previous work within the watershed began to evaluate the health of the watershed, by assessing the EPT and fish within the watershed at known historical monitoring sites where little was known about the diversity of the macroinvertebrates (Reckner, 2011).

This study is devoted to cataloguing the abundance and diversity of chironomid larvae classified to subfamily and genus, at clean sites within the Little Paint Creek Watershed, in order to evaluate the relative health of the waterways. Sites with more diverse genera of chironomids and those with more sensitive chironomid subfamilies are considered to be healthier than those with reduced diversity and only more tolerant subfamilies. We assessed the total number and types of subfamilies and genera at each of eight sites, from Little Paint Creek and three of its tributaries. We calculated the Shannon diversity index (H), and genus richness values (DMn), and we used the Hilsenhoff tolerance values, to determine the health of the LPC watershed based on chironomid data. We compare our data to the EPT data published previously in the Little Paint Creek Watershed Conservation Plan (Reckner, 2011). Our study begins to establish a baseline assessment of chironomids from which further studies within the watershed can be compared. By analyzing benthic macroinvertebrates in differing environments, environmental stressors can be further understood.

MATERIAL AND METHODS

Study Sites

The Little Paint Creek (LPC) watershed is located in Cambria and Somerset counties in Southwestern Pennsylvania and consists of 13 square miles. LPC is a third order stream (second order tributary to Paint Creek) (Fig 1A) (Reckner, 2011). Chironomidae larvae were collected from a total of eight clean sample sites located on LPC and three of its tributaries (Fig 1B). One of the sample sites is on LPC at Route 160. One site was chosen on the tributary known as UNT 45242 (also known as Fox's Run). Six sites on the Pitt-Johnstown campus were also selected for sampling:

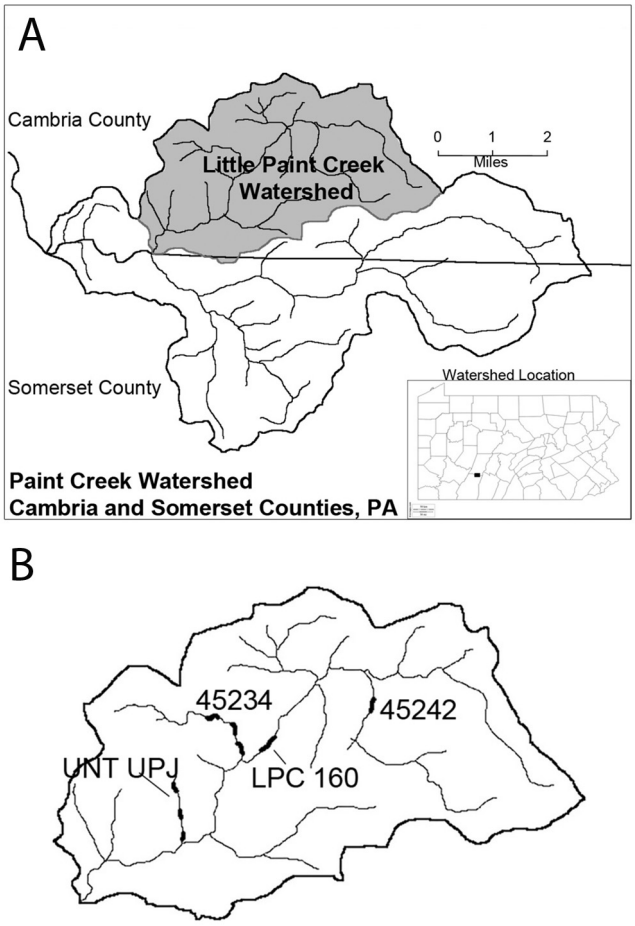

Figure 1. Map of the Little Paint Creek Watershed. LPC watershed is located in Southwest Pennsylvania and is adjacent to the Paint Creek Watershed (Reckner, 2011) (A). Collection sites on four streams in the watershed are marked in black. On the two streams with multiple collection sites, the site furthest from LPC is site 1 (B).

three were chosen on UNT 45234 and three on an Unnamed Tributary at Pitt-Johnstown (UNT UPJ), which is an ephemeral tributary and does not have a stream designation according to USGS data. On these two tributaries, site 1 is the furthest from LPC and the three sites were chosen to be relatively equidistant apart, covering the length of the tributary. All sites chosen for this study were not located in or near areas known to be contaminated due to acid mine drainage or Marcellus Shale drilling (Reckner, 2011). The sites on LPC at route 160 and on UNT 45242, as well as site number 3 on UNT 45234, coincide with the sites chosen for evaluation in the previous study of the watershed. These sites were initially chosen because they were historical monitoring sites on larger tributaries with no published records (Reckner, 2011). The UNT# represents the USGS stream code. Collections were made from May 20 through September 1, 2014, with the vast majority of the collections completed by June 16.

Extensive water chemistry assessment for the Little Paint Creek Watershed was reported in the LPC Coldwater Conservation Plan (2011). The plan reports the pH, alkalinity, and conductivity, as tested every three months for a total of five years (Reckner, 2011).

Chironomidae Sampling

At each site, three 3 m^2 areas, which included two riffles and one pool when available, were sampled by kick net. Samples were sieved, rinsed and transferred to a collection jar. The initial sorting of Chironomidae from the other benthic macroinvertebrates collected was carried out with a dissecting microscope. Larvae were preserved in 95% ethanol. Each larval head capsule was dissected, cleared using 10% KOH for 3 to 24 hours, and mounted in Hoyer's mounting medium or Aqua-Poly/Mount (Polysciences, Inc.) for examination under a compound microscope. Morphological analysis of the head included assessment of the mentum, mandibles and antennae. The structure of the abdominal tubules was observed in the terminal abdominal segments. Head and abdominal sections were used to identify the subfamily and genus for each larva using a dichotomous key of larval morphology (Coffman and Ferrington Jr., 1996), and were independently verified by an expert in the field.

Data Analysis

Chironomid abundance, number of subfamilies and genera, genus richness (using the Menhinick's diversity index, DMn), and genus and subfamily diversity (using the Shannon-Wiener diversity index, H) were calculated for each site and for each tributary. The Menhinick's diversity index is used in ecological studies to represent the number of different genera found within a site. The Shannon diversity index, which is the most common measure of diversity, is used to assess the abundance of each subfamily and genus and whether one is predominant at a site. If the larvae are distributed between the subfamilies, or genera, the H value is higher than if there is an abundance of one genus, or subfamily. The data were also compared between riffle and pools within a single site.

RESULTS

Among the eight sites sampled from the three tributaries and LPC in the Little Paint Creek watershed, over 1,000 Chironomidae larvae were evaluated. Five subfamilies and 31 genera were identified within the watershed (Appendix 1). The most predominant subfamilies across all sites were

Orthocladiinae, Chironominae and Diamesinae, (Fig 2A, B). Across the sites sampled, the subfamilies Tanypodinae and Prodiamesinae were either not present or only found in very limited numbers. Interestingly in all three tributaries, but not in Little Paint Creek, we identified larvae near the unique genus *Rheocricotopus*.

UNT 45234

From tributary UNT 45234, five subfamilies (Chironominae, Diamesinae, Orthocladiinae, Tanypodinae, and Prodiamesinae) and 23 genera were identified (Appendix 1) among over 400 larvae examined (most from site 1 with 323 larvae). On sites 2 and 3, each had 54 larvae collected for analysis. The predominant larvae were within the subfamilies: Chironominae (44%), Orthocladiinae (38%), and Diamesinae (14%) (Fig 2A, B), with the subfamily Shannon index of diversity value of 1.08 (Fig 3A, black), which is the highest among the tributaries and LPC. Among the subfamily Chironominae, the sites within tributaries UNT 45234 and UNT 45242 had the most genera (Appendix 1). Eight genera were identified within UNT 45234 and six genera within UNT 45242.

Of the eight sites sampled within the watershed, site one of UNT 45234 had the most genera (Appendix 1). For the three sites on UNT 45234, the genus richness values (DMn) were 0.95, 0.95, and 1.22 (Fig 3B, black), and the Shannon diversity index values (H) for the genera were 1.73, 2.08, and 2.14 (Fig 3B, black), contributing to a richness value DMn of 1.15 (Fig 3A, black) and a diversity value H of 2.06 (Fig 3A, black) for the tributary.

There is variability among the values for genus richness (DMn), and Shannon diversity index (H) for the genera and the subfamilies, when comparing the three sites on the tributary (Fig 3B). As stated in the Methods section, we collected kick-net samples from three kicks for each site. There was no apparent trend in the data from the riffle samples as compared to the pool samples of the tributary (Appendix 1).

UNT UPJ

Within the sites on UNT UPJ, over 400 larvae were assessed, with 88, 90 and 264 larvae from the three sites. An average of 140 larvae per site for UNT UPJ, is similar to that of UNT 45234. The majority (72%) of the larvae were identified as Diamesinae (Fig2A), and of those 92.5% were the genus *Diamesa* (Appendix 1). Sites 1 and 3 had a predominant Diamesinae presence. Of the four subfamilies identified from the tributary, Orthocladiinae was the other subfamily that was prevalent within this tributary, 27% (Fig 2A) and the subfamily Shannon diversity index of the tributary was 0.66 (Fig 3A, light gray). Each of the sites of tributary UNT UPJ had genus richness values, DMn, (0.75, 0.95, 0.55) that were equal to or lower than the genus richness at any of the other sites sampled in the LPC watershed (Fig 3B, light gray). This tributary had the least subfamily diversity, with a Shannon diversity index (H) of 0.65 (Fig

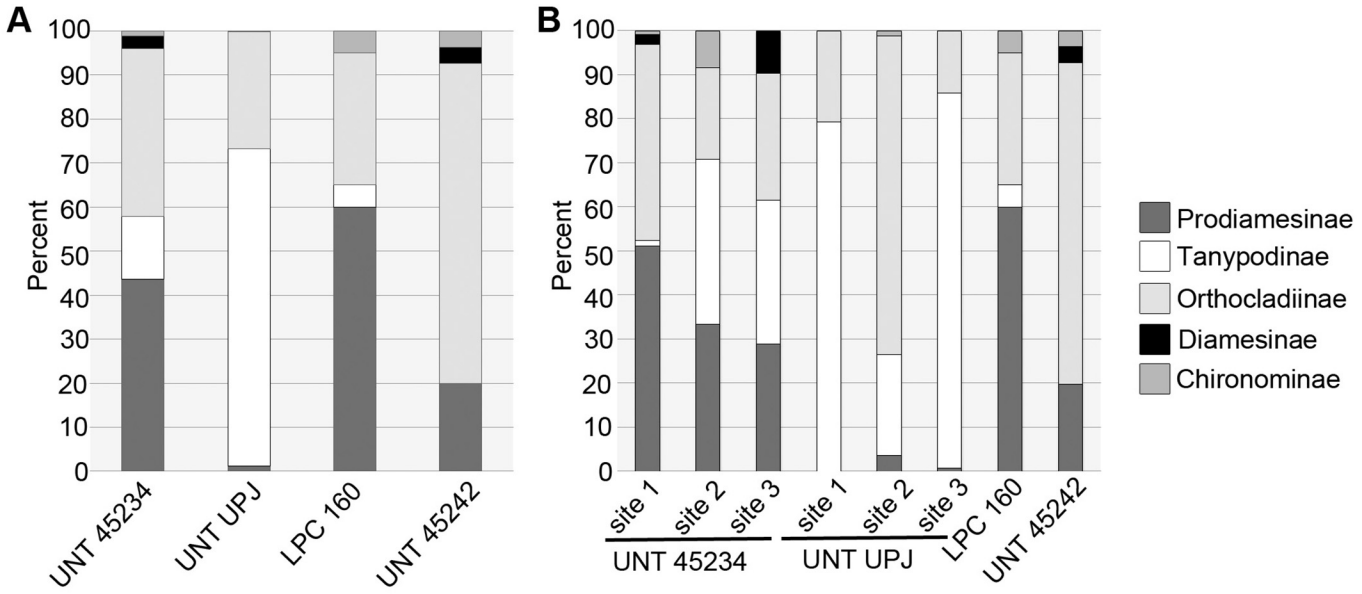

Figure 2. Tributary specific and site specific proportions of each observed Chironomidae subfamily. The average percentage of each subfamily among Little Paint Creek and the three tributaries sampled (A). Variation in subfamily abundance across the eight sites sampled within the LPC watershed (B).

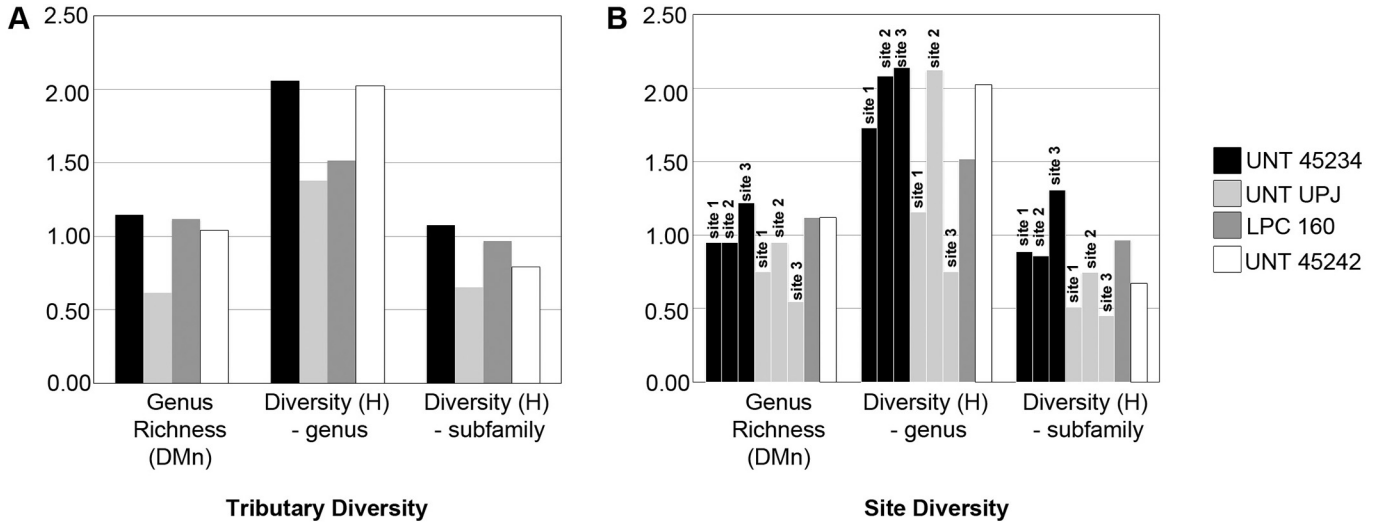

Figure 3. Comparison of Richness (DMn), Genus diversity (H) and Subfamily diversity (H) of Chironomidae sampled in the LPC watershed. The genus richness and genus and subfamily diversity among Little Paint Creek and the three tributaries sampled (A). The genus richness and genus and subfamily diversity across the eight sites sampled within the LPC watershed (B).

3A), however site 2 had one of the highest genera Shannon diversity values of 2.12, comparable to site 3 from UNT 45234 (Fig 3B, light gray).

The multiple sites on UNT UPJ had similar habitats, but had variable genus richness (DMn) values and extensive variability for the genera Shannon diversity index (H). Within each site sampled, there was no apparent difference in overall abundance, type of subfamilies, or type of genera, between kick-net samples from pools or riffles.

LPC 160

The subfamily Chironominae was most abundant at the LPC 160 site, although the site also contained larvae from the subfamilies Diamesinae, Orthocladiinae and Prodiamesinae, 60%, 5%, 30%, and 5% respectively (Fig 2A). The overall abundance of chironomids at this site was less than at any of the other sites, with 20 larvae; however, even though the number of larvae was low, the subfamily Shannon diversity index (H) was 0.97 (Fig 3A,B, dark gray) and the genus richness was 1.12 (Fig 3A,B, dark gray), which are comparable to that of UNT 45234 (Fig 3B, black). The genera Shannon diversity index was 1.52 (Fig 3A,B, dark gray), lower than that of UNT 45234.

UNT 45242

Unlike many of the other sites sampled within the LPC watershed, the subfamily Orthocladiinae was predominant in the site on UNT 45242, comprising approximately 73% of the collection of 156 larvae (Fig 2B). Six genera within the Orthocladiinae subfamily were detected within this tributary (Appendix 1). The diversity of genera within the subfamily Orthocladiinae was more extensive than any other subfamily among the sites sampled within the watershed (Appendix 1). In addition to the six genera in UNT 45242, there were eight identified genera in UNT 45234, eight genera in UNT UPJ, and two genera in LPC 160. As in UNT 45234 and LPC160, Chironominae larvae were among the most abundant subfamilies, 20% (Fig 2A). Unlike the sites on UNT UPJ, there were no Diamesinae larvae within the UNT 45242 site. The tributary had a genus richness of 1.04 (Fig 3A,B, white) and a genera Shannon diversity index of 2.02 (Fig 3A,B, white), values that are comparable to UNT 45234 and UNT UPJ.

Chironomids are present in LPC and all three tributaries tested in this study. The specifics of the number and presence of different genera can be seen in Appendix 1.

DISCUSSION

The LPC Coldwater Conservation plan, published in 2011, evaluated the quality of the water in the LPC watershed and proposed a conservation plan to maintain or improve the water quality for aquatic inhabitants and for recreational

use (Reckner, 2011). Within this study, the authors reported the EPT index for multiple sites within the watershed, and listed the number of Chironomidae. We assessed the chironomid diversity in the Little Paint Creek Watershed by identifying chironomids to subfamily and genus for eight sites on Little Paint Creek and three tributaries, to support and verify the findings of the water quality in conservation plan, to evaluate the water quality of previously untested tributaries, and to establish a baseline understanding of chironomids for use in further studies. The sites chosen for this study were not located in or near areas known to be contaminated due to acid mine drainage or Marcellus Shale drilling (Reckner, 2011), and three of which were sites evaluated in the conservation plan (Reckner, 2011). By comparing the presence of different subfamilies, which vary in their tolerance to pollutants, we were able to determine the relative health of some of the water in the watershed - the presence of more sensitive subfamilies indicating higher quality water. The number of different genera, represented as the genus richness (Menhinick's diversity index, DMn), and the distribution of the larvae among the different genera (Shannon diversity index, H), further support our subfamily analysis, as a decrease in genus diversity is associated with the presence of a stressor in the environment.

UNT 45234

Based on its location within the watershed and previous water chemistry data, UNT 45234 is not expected to contain acid mine drainage or Marcellus Shale contaminants (Reckner, 2011). However, the LPC conservation plan noted that based on sampling at a single site on tributary UNT 45234, there were a limited number of fish species and reduced EPT diversity, uneven distribution or evenness of species, and variability in species richness (number of different species present). These data suggest that UNT 45234 may be impaired as a result of the urbanization of the area and the consequential industrial pollutants in the water, as supported by the water chemistry data (Reckner, 2011). Evidence of human disturbance was observed during our collections, in the form of abandoned tires and pipes near the water.

Through the collection of five subsamples using Surber Sampling, Reckner did not collect any chironomids from UNT 45234 (our site 3) in 2010, and collected 84 larvae when sampled in 2011 (Reckner, 2011). We collected and identified 54 larvae from this same location, which included larvae from five subfamilies and 23 genera (Appendix 1). Collections from site 1 on this tributary resulted in the most chironomid larvae; however the average number of larvae among most of the tributaries sampled (UNT 45234, UNT UPJ and UNT 45242) was within the same approximate range.

While the overall abundance of chironomid larvae was variable among the eight sites along the four tributaries in our study, we found the most larvae and the highest diversity of genera within the family Chironominae within one site in UNT 45234. The presence of Chironominae was expected based on assessment in similar watersheds in the northeastern portion of the United States (Bode, 1990; Bode *et al*., 1996). However, the richness of the Chironomidae genera was approximately equal between the sites on UNT 45234, LPC 160 and UNT 45242. Thus, it appears as though the richness data for the genera of Chironominae do not follow the expected trend of other benthic macroinvertebrates established by the published EPT data for UNT 45234 (Reckner, 2011). The similarity of Chironomidae genera richness between the sites suggests that despite the diminished water quality as assessed by the water chemistry, UNT 45234 water quality is similar to that of other tributaries within LPC watershed.

As demonstrated by the Hilsenhoff tolerance values, Chironomidae subfamilies vary in the degree of tolerance to water pollutants. Tanypodinae and Prodiamesinae are the subfamilies of Chironomidae most tolerant of pollutants in the environment, each with a tolerance value of 7. Orthocladiinae and Chironominae each have tolerance values of 6, Diamesinae tolerance is 2, and Podonominae tolerance is 1 (Bode *et al*., 1996; Hilsenhoff, 1988; Mandaville, 2002). Some of the most tolerant and least tolerant subfamilies, Tanypodinae and Diamesinae, respectively, were identified within the samples from UNT 45234. The presence of some of the least tolerant chironomids within this tributary further suggests that the quality of the water in UNT 45234 may not be as poor as previously thought.

UNT UPJ

UNT 45234 and UNT UPJ had similar averages of 140 chironomid larvae per site. However, UNT UPJ has lower levels of diversity for both subfamilies and genera and a lower genus richness value, as compared to all the other sites evaluated. This is the first published evaluation of any of the benthic macroinvertebrates within this tributary. In accordance with previous data on chironomid distribution, Orthocladiinae larvae were present in the waters where Diamesinae larvae were identified (Bode, 1990). Unlike the other tributaries assessed within the LPC watershed, this tributary had the highest proportion of larvae within the rare, less tolerant subfamily, Diamesinae. The lower values for most of the indices evaluated initially suggests that this tributary has diminished water quality as compared to the other tributaries and to LPC. However, this speculation is confounded by the presence of the less tolerant Diamesinae. Additional evaluation of other locations on the tributary, as well as EPT and water chemistry data, will be needed to gain a full understanding of the water quality for UNT UPJ.

LPC 160

We evaluated the same site on Little Paint Creek, the third order stream, as was assessed in the LPC conservation plan (Reckner, 2011). Like the high levels of species richness for EPT measured by Reckner, we also found a high level of genus richness for chironomids at this site. However, unlike the more evenly distributed EPT communities, using the Shannon diversity index, we found lower genus distribution as compared to UNT 45234 and UNT 45242. In addition, there were disproportionately more of the tolerant subfamilies, Chironominae and Orthocladiinae. However, there were a few Diamesinae larvae identified in our study. Our findings of reduced genus distribution and more tolerant subfamilies indicate that this site on LPC may be of slightly lesser quality than originally predicted by the EPT data (Reckner, 2011).

UNT 45242

In contrast to UNT 45234, UNT 45242 has been listed as a High Quality stream as a function of the macroinvertebrate diversity and abundance assessment, with a higher proportion of EPT than the relative proportion of Chironomidae (Reckner, 2011). Only two chironomids were identified in samples taken in 2010, while 14 were identified in 2011 (Reckner, 2011). In 2014, we assessed 156 larvae, with genus richness similar to that from tributary UNT 45234 and LPC 160, and Shannon diversity index for genera similar to that of UNT 45234, indicating similar diversity in the number of genera and an evenness in the abundance of the different genera. The total number of larvae within the site on UNT 45242 is similar on average to the number of larvae from single sites on UNT 45234 and UNT UPJ. These data suggest that upon evaluation of the chironomids, the water quality within UNT 45234 and UNT 45242 is relatively comparable. Interestingly, mostly highly tolerant subfamilies of chironomids, Orthocladiinae and Chironominae, were identified in UNT 45242. The lack of Diamesinae, less tolerant larvae, in UNT 45242, indicates that UNT 45242 may have slightly poorer water quality than UNT 45234. This conflicts with the EPT assessment from 2011, which suggested that the water quality in UNT 45242 was better than UNT 45234.

We have now generated a catalog of chironomids that were present during monitoring within the Little Paint Creek watershed. In North America, the subfamilies Chironominae, Orthocladiinae, and Tanypodinae are the most prevalent (Coffman and Ferrington Jr., 1996; Williams and Feltmate, 1992). The more rare subfamilies Diamesinae, Prodiamesinae, and Podonominae are normally found in environments in which Orthocladiinae are also found (Bode, 1990). Like previous studies in other states, we found Chironominae, Orthocladiinae, and Tanypodinae subfamilies to be the most predominant.

The presence of chironomid larvae, more and less tolerant subfamilies, and a diverse collection of genera, along with the data from the LPC watershed conservation plan, suggest that Little Paint Creek and the tributaries tested within the LPC watershed have good water quality (Reckner, 2011). Our data suggest that evaluation of the chironomids within the water allows for further assessment of water quality. At some sites sampled, the chironomid assessment indicated that the water quality within the watershed was different than what was originally concluded. Because of differences between the conclusions made from the IBI and EPT indices and our chironomid data, we suggest that both the EPT and chironomid diversity data be used to evaluate water quality.

To gain better insight into the overall health of the watershed, we would like to analyze the abundance and diversity of chironomid larvae, along with an evaluation of the EPT in tributaries that have reported poor water quality, in close proximity to acid mine drainage and Marcellus shale wells. These data will allow us to determine if the sites of acid mine drainage and Marcellus shale drilling have impacted the quality of the water within the watershed. Our current data create a baseline for the abundance and diversity of Chironomidae with the LPC watershed. Long-term monitoring will be necessary to determine the impact of pollutants on the health of the LPC watershed.

ACKNOWLEDGEMENTS

This work was supported by the Alice Waters Thomas Fund for undergraduate research in the University of Pittsburgh at Johnstown natural areas. We would like to thank K.T. Lee and S. Patrick for assistance with collections and Barbara Hayford for her expert opinion on identifications.

LITERATURE CITED

Abhijna, U.G., Ratheesh, R., and Kumar, A.B. (2013). Distribution and diversity of aquatic insects of Vellayani lake in Kerala. Journal of environmental biology / Academy of Environmental Biology, India 34, 605-611.

Al-Shami, S.A., Rawi, C.S., HassanAhmad, A., and Nor, S.A. (2010). Distribution of chironomidae (Insecta: Diptera) in polluted rivers of the Juru River Basin, Penang, Malaysia. Journal of environmental sciences 22, 1718-1727.

Armitage, P.D., Cranston, P.S., and Pinder, L.C.V. (1995). The Chironomidae: biology and ecology of nonbiting midges (London; New York: Chapman and Hall).

Bode, R.W. (1990). Chapter 14 Chironomidae. In The aquatic macroinvertebrates of eastern North America, B.A. Peckarsky, ed. (Ithaca, NY: Cornell University Press).

Bode, R.W., Novak, M.A., and Abele, L.E. (1996). Quality Assurance Work Plan for Biological Stream Monitoring in New York State (Albany, NY: NYS Department of Environmental Conservation).

Bolton, M.J. (2012). Ohio EPA Supplemental Keys to the Larval Chironomidae (Diptera) of Ohio and Ohio Chironomidae Checklist, Ohio Environmental Protection Agency, and D.o. Surface Water, eds. (Columbus, OH).

Bouchard, R.W., Jr., Carrillo, M.A., and Ferrington, L.C., Jr. (2006). Lower lethal temperature for adult male Diamesa mendotae Muttkowski (Diptera: Chironomidae), a winter-emerging aquatic insect. Aquatic Insects 28, 57-66.

Burger, J. (1997). Methods for and approaches to evaluating susceptibility of ecological systems to hazardous chemicals. Environmental health perspectives 105 Suppl 4, 843-848.

Byrne, P., Reid, I., and Wood, P.J. (2013). Changes in macroinvertebrate community structure provide evidence of neutral mine drainage impacts. Environmental science Processes & impacts 15, 393-404.

Caldwell, B.A. (1984). Two new species and records of other chironomids from Georgia (Diptera: Chironomidae) with some observations on ecology. Georgia Journal of Science 41, 81-96.

Carrillo, M.A., Cannon, C.A., and Ferrington Jr, L.C. (2004). Effect of sex and age on supercooling point of winter-active *Diamesa mendotae* Muttkowski (Diptera: Chironomidae). Aquatic Insects. 26, 243-251.

Coffman, W.P., and Ferrington Jr., L.C. (1996) Chapter Chironomidae. In An introduction to the Aquatic Insects of North America, Merritt, R.W., and Cummins, K.W., ed. (Univ. of California, Berkeley: Kendall/Hunt Publishing Company).

Dillon, B.A., and Lee, K.T. (2002). Effects of Abandoned Mine Drainage on Crayfish Distribution Along a pH Gradient. In Modern Approaches to the Study of Crustacea, E.A. Escobar-Briones, F. , ed.

El-Khayat, H.M., Mahmoud, K.M., Mostafa, B.B., Tantawy, A.A., El-Deeb, F.A., Ragb, F.M., Ismail, N.M., El-Said, K.M., and Taleb, H.M. (2011). Habitat characteristics for different freshwater snail species as determined biologically through macroinvertebrate information. Journal of the Egyptian Society of Parasitology 41, 651-664.

Epler, J.H. (2001). Identification manual for the larval Chironomidae (Diptera) of North and South Carolina. A guide to the taxonomy of the midges of the southeastern United States, including Florida. (Special Publication SJ2001-SP13 North Carolina Department of Environmental and Natural Resources, Raleigh, NC, and St. John's River Water Management District, Palatka, FL), pp. 526.

Ferreira, W.R., Paiva, L.T., and Callisto, M. (2011). Development of a benthic multimetric index for biomonitoring of a neotropical watershed. Brazilian journal of biology = Revista brasleira de biologia 71, 15-25.

Ferrington Jr, L.C. (2008). Global diversity of non-biting midges (Chironomidae; Insecta-Diptera) in freshwater. Hydrobiologia 595, 447-455.

Fore, L.S., Karr, J.R., and Wisseman, R.W. (1996). Assessing invertebrate responses to human activities: evaluating alternative approaches. Journal of the North American Benthological Society. 15, 212-231.

Hart, L.A., Bowker, M.B., Tarboton, W., and Downs, C.T. (2014). Species composition, distribution and habitat types of Odonata in the iSimangaliso Wetland Park, KwaZulu-Natal, South Africa and the associated conservation implications. PloS one 9, e92588.

Hayford, B., and Ferrington Jr, L.C. (2006). Distribution of Chironomidae (Diptera) in Lake Hovsgol, Mongolia. . In The Geology, Biodiversity, Ecology of Lake Hovsgol (Mongolia), C.E.S. Goulden, T.; Gelhaus, J.; Boldgiv, B., ed. (Leiden, The Netherlands: Backhuys Publisher), pp. 433-452.

Hayford, B.L., Sublette, J.E., and Herrmann, S.J. (1995). Distribution of chironomids (Diptera: Chironomidae) and ceratopogonids (Diptera: Ceratopogonidae) along a Colorado thermal spring effluent. Journal of the Kansas Entomological Society (Special Publication 1) 68, 77-92.

Hilsenhoff, W.L. (1988). Rapid field assessment of organic pollution with a family-level biotic index. J N Am Benthol Soc 7, 65-68.

Jun, Y.C., Won, D.H., Lee, S.H., Kong, D.S., and Hwang, S.J. (2012). A multimetric benthic macroinvertebrate index for the assessment of stream biotic integrity in Korea. International journal of environmental research and public health 9, 3599-3628.

Koshima, S. (1984). A novel cold-tolerant insect found in a Himalayan glacier. Nature 310, 225-227.

Lu, D.Q., Zhang, Y., Cai, D.S., Liu, S.R., Chen, Y.H., and Wang, B.X. (2013). Choice of macroinvertebrate metrics for constructing a benthic-index of biotic integrity based on the disturbance gradients in the Qinjiang River Basin. Huan jing ke xue= Huanjing kexue / [bian ji, Zhongguo ke xue yuan huan jing ke xue wei yuan hui "Huan jing ke xue" bian ji wei yuan hui] 34, 137-144.

Mandaville, S.M. (2002). Benthic Macroinvertebrates in Freshwaters: Taxa Tolerance Values, Metrics, and Protocols (Soil & Water Conservation Society of Metro Halifax).

Meng, W., Zhang, N., Zhang, Y., and Zheng, B. (2009). Integrated assessment of river health based on water quality, aquatic life and physical habitat. Journal of environmental sciences 21, 1017-1027.

Mousavi, S.K., Primicerio, R., and Amundsen, P.A. (2003). Diversity and structure of Chironomidae (Diptera) communities along a gradient of heavy metal contamination in a subarctic watercourse. The Science of the total environment 307, 93-110.

Panas, J., Niles, J., Silknetter, S., and Bilger, M. (2014). Bioassessment of benthic macroinvertebrates of the Middle Penns Creek, Pennsylvania Watershed. Journal of the Pennsylvania Academy of Science 88, 57-62.

Pennsylvania, D.E.P., and Water Quality Standards, D.o. (2012). A Benthic Macroinvertebrate Index of Biotic Integrity for wadeable freestone riffle-run streams in Pennsylvania (Harrisburg, PA).

Reckner, M. (2011). Little Paint Creek Coldwater Conservation Plan (Paint Creek Regional Watershed Association).

Relyea, C.D., Minshall, G.W., and Danehy, R.J. (2012). Development and validation of an aquatic Fine Sediment Biotic Index. Environmental management 49, 242-252.

Ross, R.M., Long, E.S., and Dropkin, D.S. (2008). Response of macroinvertebrate communities to remediation-simulating conditions in Pennsylvania streams influenced by acid mine drainage. Environmental monitoring and assessment 145, 323-338.

Simpson, K.W., and Bode, R.W. (1980). Common larvae of Chironomidae (Diptera) from New York State streams and rivers with particular reference to the fauna of artificial substrates. New York Museum Bulletin 439, 1-105.

Tripole, S., Gonzalez, P., Vallania, A., Garbagnati, M., and Mallea, M. (2006). Evaluation of the impact of acid mine drainage on the chemistry and the macrobenthos in the Carolina stream (San Luis, Argentina). Environmental monitoring and assessment 114, 377-389.

Williams, D.D., and Feltmate, B.W. (1992). Aquatic Insects (CAB International).

Appendix 1: Total taxa identified in each of the sites sampled in the LPC watershed.

	Stream	UNT 45234												UNT UPJ						LPC 160			UNT 45242		
	Site Number	1			2			3			1			2			3			1			1		
	Kick Number (Pool/ Riffle)	1P	2R	3R	1R	2R	3P	1P	2R	3R	1R	2P	3R	1R	2P	3R	1R	2R	3R	1P	2R	3R	1P	2R	3R
Subfamily	Genus																								
Chironominae	*Paratanytarsus*	33	38	41		1	4	3	10	1						1				2	4			4	1
	Cladopelma	1																							
	Endochironomus	3																					1		2
	Asheum						2																		
	Stelechomyia	1																							
	Paratendipes	2	21	16																3	3		3	3	
	Microchironomus	3																							
	Rheotanytarsus																								1
	Phaenospectra																						1		
	Polypedilun								1					1				2					2	2	1
	Unknown	2	1		1									1									5		1
Diamesinae	*Boreoheptagyia*																								
	Sympotthastia		1		1		1				10														
	Potthastia gaedii group										1														
	Diamesa		2	1	1				8	3	1	11	46	13	3		134	75	12			1			
	Unknown				1	2	3			6				1		2	1								
Orthocladiinae	*Parametriocnemus*	7	59	51			1	1	5			3	4	12	2			14	4	4	1		1	37	26
	Eukiefferiella	1	3	3			3						1	3		1		1							
	Parachaetocladius	1																							2
	Cricotopus	1																							1
	near Rheocricotopus								2					2		2		5	2						1
	Orthocladius			2					3					20	1			3	1						
	Symposiocladius																							1	2
	Parorthocladius																	1							
	Bryophaenocladius					1																			
	Corynoneura																		1						
	Brillia											2	7	1				1	1		1				

Appendix 1: Continued from previous page.

	Stream	UNT 45234												UNT UPJ								LPC 160			UNT 45242		
	Site Number	1			2			3			1			2			3			1			1				
	Kick Number (Pool/ Riffle)	1P	2R	3R	1R	2R	3P	1P	2R	3R	1R	2P	3R	1R	2P	3R	1R	2R	3R	1P	2R	3R	1P	2R	3R
Subfamily	Genus																										
	Pseudorthocladius	1								1						1											1
	Unknown	10	2						3				1	7		8		3					2	20	5		
Tanypodinae	*Natarsia*	3	3																								
	Telopelopia	1																									
	Alotanypus							3	1															3			
	Procladius								1														1				
	Unknown																						1				
Prodiamesinae	*Prodiamesa*	2																									
	Monodiamesa																										
	Unknown	1					2								1					1			5				
Unknown		6	1						1	1			1	6		1	2	1						15	5		
Total Larvae Per Kick		79	131	114	4	4	16	7	35	12	12	16	60	67	7	16	137	106	21	10	9	1	22	85	49		
Total number of Genera per kick		14	7	6	2	2	5	3	8	3	3	3	4	7	3	4	1	8	6	3	4	1	6	6	10		
Total Larvae Per Site			324			24			54			88			90			264			20			156			
Total Subfamilies per site			5			4			4			2			4			3			4			4			
Total number of genera per site			17			7			9			4			9			9			5			14			
Genus Richness (D) per site			0.94			1.43			1.22			0.264			0.95			0.55			1.12			1.12			
Total Larvae per tributary						402									442						20			156			
Total number of genera per tributary						23									13						5			14			

Journal of the Pennsylvania Academy of Science 89(2): 80-87, 2015

RE-ANALYSIS OF BREEDING BIRD DENSITY IN EASTERN PENNSYLVANIA WOODLOTS[1]

RALPH G. MANCKE[2,4] & THOMAS A. GAVIN[3]

[2]800 Trenton Road, Apartment 357, Langhorne, Pennsylvania, 19047 USA
[3]Department of Natural Resources, Cornell University, Ithaca, New York, 14853 USA

ABSTRACT

Previously, we developed statistical models for the densities of 36 breeding bird species occurring in 176 woodlots in eastern Pennsylvania, USA. Here, we present a re-analysis of the same data after eliminating many highly correlated variables ($|r| > 0.5$), decreasing our number of candidate models from thousands to 12, and correcting a statistical error that we had made previously. Our distance-to-edge variable was a measure of depth in the woodlot that depended on distances to 4 woodlot edges. We could not use our area variable because of its very high correlation with our distance-to-edge variable. Our isolation variable was not highly correlated with either our distance-to-edge or area variable. Two other key variables related to buildings at the woodlot edges. As previously, our models were nonlinear, and we used bootstrapped data sets to help ascertain the best model for each species. We determined that 3 species were forest-interior species, and 2 of these also responded negatively to increasing isolation. We found 3 edge species, plus 2 modified edge species that occurred only at suburban or urban edges; both of the latter are known to nest on buildings. Our results agreed well but were conservative compared to an extensive literature. However, they did not agree well with our previous analysis. In particular, we were not able to confirm our previous conclusion that buildings near the edges of woodlots were important to many bird species in eastern Pennsylvania, USA. [J PA Acad Sci 89(2): 80-87, 2015]

INTRODUCTION

Previously, we developed statistical models for densities of 36 breeding bird species occurring in 176 woodlots in eastern Pennsylvania, USA (Mancke and Gavin 2000). We focused especially on the effect of "depth" in the woodlot, where depth was a modified distance-to-edge variable that incorporated the shortest distance to edge and also distances to edges in 3 other directions. We also investigated different types of edges, including measures of the numbers of buildings near edges. Furthermore, we explored the effects of woodlot isolation and some local variables such as slope, aspect, elevation, openness of canopy, and forest age. We did not use woodlot area as a variable. Our models were nonlinear, and we used bootstrapped data sets to help ascertain the best model for each species. After our study was published, we gradually realized that our analysis was flawed. First, some of our variables were "highly correlated" (defined here to mean $|r| > 0.5$). Second, our model selection process was difficult and poorly done, primarily due to too many candidate models, which numbered in the thousands. And third, we made a statistical error to be explained below. Here, we made the appropriate modifications and re-analyzed our original data, and important changes in conclusions ensued.

METHODS

We used the same data as before (see Table 2 of Mancke and Gavin (2000)). One of us (RM) censused birds in 387 sample plots (circles of 50 m radius) in 176 woodlots in eastern Pennsylvania, USA. Woodlots were in four locations in Berks and Lehigh Counties that varied from a rural to urban context and in distance to a woodlot larger than 5000 ha. We chose woodlots that were neither open woods (too open for a full canopy), nor second-growth woods (too young for a full canopy), nor floodplain woods, in order not to complicate our analysis further. Sample plots were situated randomly in the woodlots, except not within 50 m of an edge. We had two kinds of predictor variables – landscape variables, which involved distances or areas extending beyond the sample plots, and local variables, which were measured completely within the sample plots. Predictor variables are discussed below. The response variable was $\ln(1 + \underline{p})$, where $\underline{p}$ was the number of individuals of the species (except fledglings) detected in the sample plot. Both $\underline{p}$ and $\ln(1 + \underline{p})$ were measures of bird density, because the sample plot had a fixed area.

Our candidate models were *a priori* models based on our

[1]Accepted for publication May 2015
[4]Corresponding author: email: rgmancke@hotmail.com, phone: 215-478-6551

field experience, knowledge of the literature, and personal judgment. The first candidate model was for a forest species with uniform density in all the sample plots: $\ln(1 + p) = B$, where B was a constant parameter. Candidate model 2 was for a forest-interior species, i.e., a forest species that avoided the woodlot edges: $\ln(1 + p) = B*\exp(-K_1/D^2)$, where D was depth, measured in meters, B and K_1 were constant parameters, and K_1, measured in m^2, was positive. This model is an S-shaped curve that increases from zero at small depths to B at large depths. D was defined for a sample plot by the empirical equation: $1/D^2 = 1/d_1^2 + 1/d_2^2 + 1/d_3^2 + 1/d_4^2$, where d_1 was the shortest distance from the center of the sample plot to the woodlot edge, and d_2 to d_4 were distances to edges in three other directions. If d_2, d_3, and d_4 were very large relative to d_1, their terms dropped out and $D = d_1$. If d_2, d_3, and d_4 were smaller, $D < d_1$, until $D = d_1/2$ where $d_1 = d_2 = d_3 = d_4$. In effect, the sample plot was more edge-like if more edges were nearby. D ranged from 5.8 to 1620 m. Candidate model 3 was for a forest species with uniform density throughout each individual woodlot, but density decreased with increasing isolation, defined as the shortest distance, in kilometers, from the woodlot to a woodlot larger than 5000 ha (and previously called dbw). The model was $\ln(1 + p) = B*\exp(\text{isolation}/I)$, where B and I were constant parameters, and I, measured in km, was positive. Variable isolation ranged from 0 to 40 km.

Our basic distance-to-edge predictor variable for a sample plot was $1/D^2$, which we used above in model 2 for a forest-interior species. We developed other models for modified forest-interior species that avoided some, but not all, of the woodlot edges. For example, consider a possible forest species that avoided only suburban or urban edges. Consider also, for example, a sample plot where the only suburban or urban edge was at the end of line d_3. In this case, the only pertinent term in the equation for $1/D^2$ was $1/d_3^2$. Let us re-write $1/d_3^2$ as $[(1/d_3^2)/(1/d_1^2 + 1/d_2^2 + 1/d_3^2 + 1/d_4^2)]*(1/D^2)$. The ratio in brackets had good qualities: 1) it was a simple fraction that varied from 0 to 1 depending on how many of the 4 edges were suburban or urban edges; and 2) it had no units and was independent of scale. We called this ratio suburb (previously it was typesu). Term suburb/D^2 was a modified distance-to-edge predictor variable for the sample plot. We could now write candidate model 4 for a modified forest-interior species that avoided only the suburban or urban edges: $\ln(1 + p) = B*\exp(-K_2*\text{suburb}/D^2)$, where B and K_2 were constant parameters, and K_2, measured in m^2, was positive. For a species that avoided only edges that were not suburban or urban, the appropriate variable was $(1\text{-suburb})/D^2$.

In Table 2 of Mancke and Gavin (2000), there are 10 fractions like suburb, from which we could create 10 pairs of modified distance-to-edge predictor variables such as suburb/D^2 and $(1\ \text{suburb})/D^2$. We rejected 6 of these 20 variables that occur only once or zero times in Table 3 of Mancke and Gavin (2000). Then we noted that 11 of the remaining 14 variables correlated highly with $1/D^2$ (i.e., $|r| > 0.5$, except 0.492 in one case). Two other variables correlated highly only with each other. If we were to create a model like model 4 for each of these variables, the regression coefficient for the variable in a fitted model would be affected by the correlated independent variables not included in the model (Neter *et al*. 1990, 411). In effect, we would not be able to tell which variable the species was responding to. These variables were all constructed similarly, and all would be used similarly to investigate the effects of different possible biotic interactions (predators, brood parasites, competitors, and food resources) at the different types of woodlot edges (Ambuel and Temple 1983). Therefore, we used the same rule, based on $|r| > 0.5$, to eliminate one of each pair of these highly correlated variables. For each of the 11 high correlations mentioned above, we retained $1/D^2$ and eliminated our new variable. We trusted the historical importance of our basic distance-to-edge variable relative to a new, untested variable. For the other 2 highly correlated variables, we retained the one that occurs more often in Table 3 of Mancke and Gavin (2000). We were left with variables suburb/D^2 and $\text{none2}/D^2$, which latter was a measure of how many of the 4 edge points had no buildings within 200 m (none2 was previously called Ic200). We have already used suburb/D^2 in model 4. We did not create an analogous model with $\text{none2}/D^2$ because no forest species would be expected to avoid only edges with no buildings within 200 m. This variable will be important below. The range of suburb/D^2 was 0 to 0.012 m^{-2}, and the range of $\text{none2}/D^2$ was 0 to 0.011 m^{-2}.

Edge birds were also present in our sample plots. Candidate model 5 was an edge bird model for a species present only at forest edges: $\ln(1 + p) = A*[1 - \exp(-K_{1A}/D^2)]$, where A and K_{1A} were constant parameters, and K_{1A}, measured in m^2, was positive. For a species present only at suburban or urban edges, candidate model 6 was a modified edge bird model: $\ln(1 + p) = A*[1 - \exp(-K_{2A}*\text{suburb}/D^2)]$. For a species present only at edge points with no buildings within 200 m, candidate model 7 was another modified edge bird model: $\ln(1 + p) = A*[1 - \exp(-K_{3A}*\text{none}^2/D^2)]$. K_{2A} and K_{3A}, both measured in m^2, were constant, positive parameters. Species could also respond to more than one landscape variable, so we created candidate combination models 8-12 by multiplying models 2 and 4-7 by $\exp(-\text{isolation}/I)$. Note that model 3 is the same as model 1 times $\exp(-\text{isolation}/I)$. Therefore our 12 candidate models were symmetrical in this respect. There were no other candidate combination models because we used only one distance-to-edge variable per model.

We also wanted to incorporate local variables into our models – measures of slope, aspect, elevation, openness of canopy, and forest age defined in Table 2 of Mancke and Gavin (2000). For example, consider variable Islope, which = 1 if the slope in the sample plot was 30% or greater, and = 0 otherwise. We wanted to create 12 new candidate models by multiplying each of the above 12 landscape models by [B(or A) + E*Islope], where E was a constant parameter

that could be positive or negative – i.e., we believed that the effect of each local variable would be multiplicative rather than additive. However, we would be effectively introducing new product variables – Islope/D^2, Islope*isolation, Islope*suburb/D^2, and Islope*none2/D^2). When we investigated correlations between these new variables and the previous variables, we found several high correlations. The same was true for the other 4 local variables. We concluded that we could not incorporate local variables into our models as we had wanted.

In summary, we had 4 landscape variables and no local variables. The highest correlation was between $1/D^2$ and suburb/D^2 ($r = 0.32$). We had 12 candidate models, compared to thousands in Mancke and Gavin (2000). This decrease helped us greatly in determining the best model for each species (see below).

Next, we wanted the best-fitting, "final" model for each species, and we wanted it to be robust to the kinds of data variations that are inherent in field work. We accomplished this using bootstrapped data sets (Hjorth 1994), which we created using SPSS (SPSS for Windows, Version 16.0, Chicago, SPSS Inc., through IBM SPSS Statistics for Windows, Version 22.0, Armonk, New York, IBM Corp.) by randomly choosing 387 sample plots with replacement after each choice. Bootstrapped data sets introduce nothing extraneous; data are varied using the data itself (Hjorth 1994) (see Mancke and Gavin (2000) for more discussion about this). For each species, we fitted the 12 candidate models to each of 50 bootstrapped data sets, creating 600 fitted models. We used different bootstrapped data sets for each species. Dr. H. Lawrence Hotchkiss, a computer consultant at the University of Delaware, wrote an SPSS script that collected all of the important information from the 600 fitted models into one table. We then rejected every fitted model that did not satisfy two requirements: 1) the *P* value of the best-fit value of every parameter associated with a variable (i.e., every parameter except B or A) was less than 0.05; and 2) the best-fit value of every parameter in an exponent was positive. The 50 bootstrapped data sets were more than the 10 that we had used previously. This was feasible due to our many fewer candidate models.

We also corrected a statistical error that we had made previously. Many of our models had K/D^2 in the exponents. In Mancke and Gavin (2000), we had written these with T^2 in place of K, where T, measured in meters, had been a constant parameter. When fitting these models, we had forgotten that the actual parameter to be fitted was T^2, and we had asked SPSS to fit for the best value of T, not T^2. There had been no problem with the fitted value of T; it was exactly equal to the square root of the fitted value of K. The problem had been that the *P* value of the fitted value of T had been too small. Therefore, because we required the fitted parameters to have *P* values less than 0.05, we had incorrectly judged some fitted models to be acceptable. In our re-analysis, we corrected this problem by using un-squared parameters in these exponents. (One might expect that any change in *P* value would be too small to be important. However, when, for example, we re-fitted the 24 models in Table 3 of Mancke and Gavin (2000) with K in place T^2, we found that 14 of the 24 K values had *P* values > 0.05, compared to only one T value.)

Let us return to the portion of the 600 fitted models that acceptably passed our two requirements. For each of the 50 bootstrapped data sets, we identified the fitted model with the highest r^2 as the "winning" fitted model. We then counted how many times each candidate model was represented in the 50 winning fitted models. The final model for the species was selected as the candidate model with the most winning fitted models. Because of our bootstrapped data sets, our final models were robust to typical field data variability, and were therefore the most reliable models that we could obtain. In contrast to this straightforward selection process, the analogous process in Mancke and Gavin (2000) had been inexpert, somewhat ambiguous, and even a bit subjective, primarily because of the extremely large number of candidate models. Finally, we fitted our 36 final models to the data for each species to create Table 1 of statistical models that replaced Table 3 of Mancke and Gavin (2000).

RESULTS

We found 3 forest-interior species, i.e., forest species that avoided woodlot edges – red-eyed vireo (*Vireo olivaceus*), ovenbird (*Seiurus aurocapilla*), and scarlet tanager (*Piranga olivacea*) (American Ornithologists' Union 1998) (see Table 1). Two of these also responded negatively to woodlot isolation – ovenbird and scarlet tanager. We found 3 edge species – American robin (*Turdus migratorius*), gray catbird (*Dumetella carolinensis*), and northern cardinal (*Cardinalis cardinalis*), and 2 modified edge species found at suburban or urban edges – European starling (*Sturnus vulgaris*) and house sparrow (*Passer domesticus*). No additional species responded to woodlot isolation. There were no modified forest-interior species that avoided only suburban or urban edges, and there were no modified edge species that required the absence of buildings within 200 m. For the remaining 28 species, the final models were the constant model, B (see Table 1).

DISCUSSION

We did not use variable area (woodlot area in hectares) in either of our analyses. The potential biological effects of distance to edge and area are very different. Forest species can be affected by distance to edge if predators, brood parasites, or competitors enter the woodlots from the edges, or if the amount of food varies near the edges (Ambuel and Temple 1983). Area can be important if the theory of

Table 1. Revised statistical models for woodlot densities of 36 species of breeding birds in eastern Pennsylvania, USA

Species	Final models	Best-fit values of parameters (P values)	r^2
Forest-interior species and species responding negatively to isolation (3 species)			
red-eyed vireo (*Vireo olivaceus*)	$B*\exp(-K_1/D^2)$	B=0.166, K_1=603 (0.033)	0.036
ovenbird (*Seiurus aurocapilla*)	$B*\exp(-K_1/D^2)*\exp(-isolation/I)$	B=0.524, K_1=2165 (0.000), I=44.2 (0.000)	0.287
scarlet tanager (*Piranga olivacea*)	$B*\exp(-K_1/D^2)*\exp(-isolation/I)$	B=0.196, K_1=318 (0.044), I=36.5 (0.009)	0.055
Edge species (5 species)			
American robin (*Turdus migratorius*)	$A*[1 - \exp(-K_{1A}/D^2)]$	A=0.396, K_{1A}=459 (0.005)	0.129
gray catbird (*Dumetella carolinensis*)	$A*[1 - \exp(-K_{1A}/D^2)]$	A=0.759, K_{1A}=1233 (0.000)	0.357
European starling (*Sturnus vulgaris*)	$A*[1 - \exp(-K_{2A}*suburb/D^2)]$	A=0.331, K_{2A}=406 (0.021)	0.189
northern cardinal (*Cardinalis cardinalis*)	$A*[1 - \exp(-K_{1A}/D^2)]$	A=0.320, K_{1A}=1972 (0.003)	0.120
house sparrow (*Passer domesticus*)	$A*[1 - \exp(-K_{2A}*suburb/D^2)]$	A=0.331, K_{2A}=1186 (0.005)	0.215
Species not responding to landscape variables (28 species)			
red-bellied woodpecker (*Melanerpes carolinus*) (B=0.062), downy woodpecker (*Picoides pubescens*) (B=0.060), northern flicker (*Colaptes auratus*) (B=0.033), eastern wood-pewee (*Contopus virens*) (B=0.039), great crested flycatcher (*Myiarchus crinitus*) (B=0.059), blue jay (*Cyanocitta cristata*) (B=0.212), American crow (*Corvus brachyrhynchos*) (B=0.126), black-capped chickadee (*Poecile atricapillus*) (B=0.029), tufted titmouse (*Baeolophus bicolor*) (B=0.154), white-breasted nuthatch (*Sitta carolinensis*) (B=0.032), house wren (*Troglodytes aedon*) (B=0.066), wood thrush (*Hylocichla mustelina*) (B=0.183), cedar waxwing (B*ombycilla cedrorum*) (B=0.032), worm-eating warbler (*Helmitheros vermivorum*) (B=0.019), black-and-white warbler (*Mniotilta varia*) (B=0.032), common yellowthroat (*Geothlypis trichas*) (B=0.037), American redstart (*Setophaga ruticilla*) (B=0.015), eastern towhee (*Pipilo erythrophthalmus*) (B=0.038), field sparrow (*Spizella pusilla*) (B=0.013), song sparrow (*Melospiza melodia*) (B=0.038), rose-breasted grosbeak (*Pheucticus ludovicianus*) (B=0.036), indigo bunting (*Passerina cyanea*) (B=0.068), red-winged blackbird (*Agelaius phoeniceus*) (B=0.017), common grackle (*Quiscalus quiscula*) (B=0.147), brown-headed cowbird (*Molothrus ater*) (B=0.056), Baltimore oriole (*Icterus galbula*) (B=0.066), house finch (*Haemorhous mexicanus*) (B=0.015), American goldfinch (*Spinus tristis*) (B=0.014)			

MacArthur and Wilson (1963) applies, or because of the basic need for territory (Forman *et al*. 1976). The two variables are often highly correlated, because large distances to edges can occur only in large woodlots, while only small distances to edges can occur in small woodlots. Our distance-to-edge predictor variable, $1/D^2$, was very highly correlated with the appropriate area variable, 1/area (r = 0.90). Based on this, for our 3 forest-interior species, if we were to replace K_1/D^2 in Table 1 with J/area, where J, measured in ha, was a constant, positive parameter, we would expect equivalently good fits. This was exactly the case. The replacement models with J/area had equally significant parameters and comparable r^2 values. Therefore, we were not able to distinguish between the effects of depth or area in our re-analysis, nor in our previous analysis in Mancke and Gavin (2000), as pointed out by Parker *et al*. (2005).

Our third basic landscape variable, isolation (the shortest distance, in km, from the woodlot to a woodlot > 5000 ha), can be important if the theory of MacArthur and Wilson (1963) applies, or if predators increase with increasing isolation (Donovan *et al*. 1997). Variable isolation was the only one of the 3 basic landscape variables that was not highly correlated with the other 2; its correlation with $1/D^2$ was r = 0.11, and with 1/area, it was r = 0.09. Nor was isolation highly correlated with any of the other variables, including those rejected or eliminated above. The highest correlation was between isolation and elevation (r = -0.32).

We searched the literature for studies of the effects of area (A), distance to edge (D), or isolation (I) on density or occurrence of our 36 species. We further asked which of these studies is designed such that at least one of its A, D, or I variables is not highly correlated with the other 2 (i.e., $|r| < 0.5$). We found 24 such studies. The responses of the 36 species to the un-correlated variables in these papers are listed in Table 2. We compared our I results directly with Table 2. We found negative I effects for ovenbird and scarlet tanager (see Table 1), but not for the other 34 species. We had no model to test for a possible positive I effect, as listed in Table 2 for brown-headed cowbird (*Molothrus ater*). The 3 positive, 3 negative, and 30 zero D effects that we found (see Table 1) were actually "A or D" effects, as explained above. Therefore, they must be compared with the combined

Table 2. Responses of density or occurrence of our 36 bird species to area (A), distance-to-edge (D), or isolation (I) variables in studies designed such that at least 1 of these 3 variables is not highly correlated with the other two (i.e., $|r| < 0.5$). Responses to the un-correlated variables are listed here.

Species	Response to			References*
	A	D	I	
Forest-interior species and species responding negatively to isolation in our study (3 species)				
red-eyed vireo	0	0	–	7
		+, +, 0, 0, 0, 0, 0, 0, 0, 0, 0, –		13, 17, 2, 9, 10, 15, 16, 18, 21, 22, 24, 1
			0	19
ovenbird		+, +, +, +, +, +, +, +, 0, 0, 0, 0, 0, 0, 0		9, 10, 13, 16, 20, 21, 23, 24, 1, 2, 3, 8, 14, 15, 18
			–	19
scarlet tanager	+			11
		+, +, 0, 0, 0, 0, 0, 0		9, 24, 1, 2, 10, 15, 16, 21
Edge species in our study (5 species)				
American robin		0, 0, 0, –, –, –, –		2, 10, 18, 9, 13, 21, 24
gray catbird		0, 0, –		2, 24, 9
northern cardinal		0, 0, 0, –, –, –, –		17, 22, 24, 1, 2, 13, 16
European starling		–, –, –		9, 13, 24
house sparrow		–		9
Species not responding to landscape variables in our study (28 species)				
red-bellied woodpecker		+, 0, 0, 0, 0		13, 1, 16, 17, 24
downy woodpecker		+, 0, 0, 0, 0, 0, 0		13, 2, 9, 16, 18, 21, 24
northern flicker		0, 0, 0, 0, 0, 0, –		1, 2, 9, 18, 21, 24, 13
eastern wood-pewee		0, 0, 0, 0, –, –		1, 9, 13, 24, 2, 22
great crested flycatcher		0, 0, 0, 0, –		2, 9, 13, 24, 22
blue jay		0, 0, 0, 0, 0, 0, 0, 0, 0, –		1, 2, 9, 16, 17, 18, 21, 22, 24, 13
American crow		+, 0, –, –, –		1, 2, 9, 13, 24
black-capped chickadee		0, 0, 0, 0, 0		2, 9, 10, 18, 21
tufted titmouse		+, 0, 0, 0, 0, 0		13, 1, 16, 17, 22, 24
white-breasted nuthatch		+, +, 0, 0, 0, 0, –		17, 24, 2, 9, 16, 21, 1
house wren		0, 0, –		2, 13, 24
wood thrush		+, 0, 0, 0, 0, 0, –		13, 1, 2, 9, 16, 24, 10
		0	0	6
			–	19
cedar waxwing		0, 0, –, –		2, 18, 9, 21
worm-eating warbler		+, +		23, 24
black-and-white warbler		+, +, +, 0, 0, 0, 0, –		9, 18, 24, 1, 2, 10, 22, 21
common yellowthroat		0, 0, 0, –, –		2, 21, 24, 9, 13
American redstart		+, +, 0, 0, 0, 0, –		9, 24, 2, 10, 15, 18, 21
eastern towhee		0, 0, –, –		2, 24, 1, 16
field sparrow		–, –, –		1, 13, 24
song sparrow		0, –, –, –		2, 1, 9, 24

(Continued on next page)

(Continued from previous page)

Species	Response to			References*
	A	D	I	
rose-breasted grosbeak		0, 0, 0, 0, 0		2, 9, 10, 18, 21
			–	19
indigo bunting		0, 0, –, –, –		2, 13, 9, 22, 24
red-winged blackbird		–, –, –		2, 9, 17
common grackle		0, –, –		2, 9, 24
brown-headed cowbird		0	+	5
		0, –, –, –, –, –, –		2, 1, 4, 9, 12, 22, 24
Baltimore oriole		0, –, –		2, 9, 24
house finch				
American goldfinch		–, –, –, –, –		1, 2, 9, 13, 24

* 1 (Anderson *et al.* 1977), 2 (Austen *et al.* 2001), 3 (Betts *et al.* 2006), 4 (Brittingham and Temple 1983), 5 (Donovan *et al.* 1997), 6 (Driscoll and Donovan 2004), 7 (Dunford *et al.* 2002), 8 (Flaspohler *et al.* 2001), 9 (Freemark and Merriam 1986), 10 (Germaine *et al.* 1997), 11 (Hames *et al.* 2001), 12 Howell *et al.* 2007), 13 (Johnston 1947), 14-15 (King *et al.* 1996, 1997), 16 (Kroodsma 1984), 17 (Lay 1938), 18 (Morneau *et al.* 1999), 19 (Nol *et al.* 2005), 20-21 (Ortega and Capen 1999, 2002), 22 (Strelke and Dickson 1980), 23 (Wenny *et al.* 1993), 24 (Whitcomb *et al.* 1981)

A and D columns in Table 2. (Our study was unique in this respect; none of the analyses in the 24 papers referenced in Table 2 resulted in "A or D" effects.) Overall, our non-zero D and I effects compared well with Table 2 but appeared to be conservative. All 6 species for which we found non-zero effects were among the 11 most common species in our study. This suggests that we would need more data to fully test the other 25 species.

Finally, we wanted to compare our previous results in Mancke and Gavin (2000) with our re-analysis here. Two of the 3 species found to be forest-interior species above (red-eyed vireo and ovenbird) had also been categorized previously as forest-interior species – i.e., species with the multiplicative term $\exp(-\underline{T}^2/\underline{D}^2)$ in their models in Table 3 of Mancke and Gavin (2000). One of the 2 species that responded negatively to variable isolation above (ovenbird) had also previously responded negatively to variable dbw, the previous name for isolation. Five species found to be edge species above had been included in the 18 species categorized previously as edge species – i.e., species with the term $[1 - \exp(-\underline{T}^2/\underline{D}^2)]$ in their models in Table 3 of Mancke and Gavin (2000). This difference was caused primarily by the corrected statistics error discussed above. Finally, the 2 species found above to be positively affected by buildings at the woodlot edges – European starling and house sparrow, both of which are known to nest on buildings (Ehrlich *et al.* 1988) – were among the 21 species categorized previously as being affected by buildings at the woodlot edges – 10 positively and 11 negatively – as detailed in Table 4 of Mancke and Gavin (2000). This large difference resulted primarily from our elimination of all but 2 of the many building-related variables used previously in Mancke and Gavin (2000). If buildings at the edges of woodlots are important to more forest bird species in eastern Pennsylvania, USA, it will take an experimental design better than ours to uncover it.

We have emphasized proper identification of A, D, and I effects in this paper. For example, consider species that may respond negatively to I (isolation). If an entire landscape of forest fragments becomes too isolated from source areas for such species, then they could disappear from that entire landscape. Another example is the proper understanding of A (area) versus D (distance-to-edge) effects. For species that respond positively to D rather than A, i.e., forest-interior species, the warning of Temple (1986) still applies today: "…management practices that preserved large areas of forest, but permitted those areas to have elongated rather than compact shapes, indented rather than entire unbroken perimeters, or inclusions of open habitat within the fragment rather than a solid forest stand, would not benefit forest birds sensitive to fragmentation." Improved experimental designs continue to be needed to correctly identify these important A, D, and I effects.

ACKNOWLEDGEMENTS

We thank J. A. Royle, J.-P. Savard, and several anonymous referees for comments that greatly helped us. We appreciate help with statistics from J. B. C. Harris and C. E. McCulloch. We note that the indispensable SPSS script written by Dr. H. Lawrence Hotchkiss was done on his own initiative before we recognized its importance.

LITERATURE CITED

Ambuel, B., and S. A. Temple. 1983. Area-dependent changes in the bird communities and vegetation of southern Wisconsin forests. Ecology 64:1057-1068.

American Ornithologists' Union. 1998. Check-list of North American Birds, 7th edition. American Ornithologists' Union, Washington, D.C.; plus supplements up to and including the fifty-fifth supplement, 2014, Auk 131:CSi-CSxv.

Anderson, S. H., K. Mann, and H. H. Shugart, Jr. 1977. The effect of transmission-line corridors on bird populations. Am Midl Nat 97:216-221.

Austen, M. J. W., C. M. Francis, D. M. Burke, and M. S. W. Bradstreet. 2001. Landscape context and fragmentation effects on forest birds in southern Ontario. Condor 103:701-714.

Betts, M. G., G. J. Forbes, A. W. Diamond, and P. D. Taylor. 2006. Independent effects of fragmentation on forest songbirds: an organism-based approach. Ecol Appl 16:1076-1089.

Brittingham, M. C., and S. A. Temple. 1983. Have cowbirds caused forest songbirds to decline? Bioscience 33:31-35.

Donovan, T. M., P. W. Jones, E. M. Annand, and F. R. Thompson III. 1997. Variation in local-scale edge effects: mechanisms and landscape context. Ecology 78:2064-2075.

Driscoll, M. J. L., and T. M. Donovan. 2004. Landscape context moderates edge effects: nesting success of wood thrushes in central New York. Conserv Biol 18:1330-1338.

Dunford, W., D. M. Burke, and E. Nol. 2002. Assessing edge avoidance and area sensitivity of red-eyed vireos in southcentral Ontario. Wilson Bull 114:79-86.

Ehrlich, P. R., D. S. Dobkin, and D. Wheye. 1988. The Birder's Handbook: a Field Guide to the Natural History of North American Birds. Simon and Schuster. New York. xxx + 785 pp.

Flaspohler, D. J., S. A. Temple, and R. N. Rosenfield. 2001. Effects of forest edges on ovenbird demography in a managed forest landscape. Conserv Biol 15:173-183.

Forman, R. T. T., A. E. Galli, and C. F. Leck. 1976. Forest size and avian diversity in New Jersey woodlots with some land use implications. Oecologia 26:1-8.

Freemark, K. E., and H. G. Merriam. 1986. Importance of area and habitat heterogeneity to bird assemblages in temperate forest fragments. Biol Conserv 36:115-141.

Germaine, S. S., S. H. Vessey, and D. E. Capen. 1997. Effects of small forest openings on the breeding bird community in a Vermont hardwood forest. Condor 99:708-718.

Hames, R. S., K. V. Rosenberg, J. D. Lowe, and A. A. Dhondt. 2001. Site reoccupation in fragmented landscapes: testing predictions of metapopulation theory. J Anim Ecol 70:182-190.

Hjorth, J. S. U. 1994. Computer Intensive Statistical Methods: Validation Model Selection and Bootstrap. Chapman & Hall. New York. x + 267 pp.

Howell, C. A., W. D. Dijak, and F. R. Thompson III. 2007. Landscape context and selection for forest edge by breeding brown-headed cowbirds. Landsc Ecol 22:273-284.

Johnston, V. R. 1947. Breeding birds of the forest edge in Illinois. Condor 49:45-53.

King, D. I., C. R. Griffin, and R. M. DeGraaf. 1996. Effects of clearcutting on habitat use and reproductive success of the ovenbird in forested landscapes. Conserv Biol 10:1380-1386.

King, D. I., C. R. Griffin, and R. M. DeGraaf. 1997. Effect of clearcut borders on distribution and abundance of forest birds in northern New Hampshire. Wilson Bull 109:239-245.

Kroodsma, R. L. 1984. Effect of edge on breeding forest bird species. Wilson Bull 96:426-436.

Lay, D. W. 1938. How valuable are woodland clearings to birdlife? Wilson Bull 50:254-256.

MacArthur, R. H., and E. O. Wilson. 1963. An equilibrium theory of insular zoogeography. Evolution 17:373-387.

Mancke, R. G., and T. A. Gavin. 2000. Breeding bird density in woodlots: effects of depth and buildings at the edges. Ecol Appl 10:598-611.

Morneau, F., G. J. Doucet, M. Giguère, and M. Laperle. 1999. Breeding bird species richness associated with a powerline right-of-way in a northern mixed forest landscape. Can Field Nat 113:598-604.

Neter, J., W. Wasserman, and M. H. Kutner. 1990. Applied Linear Statistical Models: Regression, Analysis of Variance, and Experimental Designs. Third Edition. Richard D. Irwin, Inc. Burr Ridge, Illinois. xvi + 1181 pp.

Nol, E., C. M. Francis, and D. M. Burke. 2005. Using distance from putative source woodlots to predict occurrence of forest birds in putative sinks. Conserv Biol 19:836-844.

Ortega, Y. K., and D. E. Capen. 1999. Effects of forest roads on habitat quality for ovenbirds in a forested landscape. Auk 116:937-946.

Ortega, Y. K., and D. E. Capen. 2002. Roads as edges: effects on birds in forested landscapes. For Sci 48:381-390.

Parker, T. H., B. M. Stansberry, C. D. Becker, and P. S. Gipson. 2005. Edge and area effects on the occurrence of migrant forest songbirds. Conserv Biol 19:1157-1167.

Strelke, W. K., and J. G. Dickson. 1980. Effect of forest clear-cut edge on breeding birds in east Texas. J Wildl Manag 44:559-567.

Temple, S. A. 1986. Predicting impacts of habitat fragmentation on forest birds: a comparison of two models. Pages 301-304 in Wildlife 2000: Modeling Habitat Relationships of Terrestrial Vertebrates (edited by J. Verner, M. L. Morrison and C. J. Ralph). University of Wisconsin Press. Madison, Wisconsin.

Wenny, D. G., R. L. Clawson, J. Faaborg, and S. L. Sheriff. 1993. Population density, habitat selection and minimum area requirements of three forest-interior warblers in central Missouri. Condor 95:968-979.

Whitcomb, R. F., C. S. Robbins, J. F. Lynch, B. L. Whitcomb, M. K. Klimkiewicz, and D. Bystrak. 1981. Effects of forest fragmentation on avifauna of the eastern deciduous forest. Pages 125-205 in Forest Island Dynamics in Man-Dominated Landscapes (edited by R. L. Burgess and D. M. Sharpe). Springer-Verlag. New York.

Journal of the Pennsylvania Academy of Science 89(2): 88-91, 2015

AN INEXPENSIVE AND MOBILE SEE-THROUGH TUNNEL FOR COLLECTING BIRD FLIGHT PERFORMANCE DATA IN THE FIELD[1]

CLAY E. CORBIN[2,4], KENNETH E. PALLIS[2], BRANDAN L. GRAY[3]

[2]*Biology, Bloomsburg University, Bloomsburg, PA USA 17815*
[3]*Biology, Ohio University, Athens OH USA 45701*

ABSTRACT

Flight performance data for flying animals are valuable for estimating energetic budgets and may be necessary for testing hypotheses of optimal foraging, predator-prey response, and physiological demand. However, measuring the flight performance of birds, particularly in the field, may be expensive and time consuming. Additionally, depending on the level of biological scale or the particular research question, apparatuses such as laboratory-bound wind tunnels may be inappropriate. We offer a see-through tent as an inexpensive alternative for certain situations where the goal is simply to constrain the flight path of a bird for videography. Data on acceleration and morphology were sampled from birds in an old-field habitat in central Pennsylvania, USA and demonstrate the utility of the apparatus. [J PA Acad Sci 89(2): 88-91, 2015]

INTRODUCTION

Recent ecological and evolutionary literature suggests data linking ecology, behavior, and morphology are at a premium (Yong and Moore 1994, Corbin 2008, Corbin *et al.* 2013). Typically, gathering ecomorphological performance data on birds is expensive and time consuming,often involving laboratory-bound wind tunnels or treadmills, and long-hours of animal training (Geyer *et al.* 2013, Maina and Jimoh 2013). These apparatuses are beneficial in that they provide sophisticated systems that facilitate the testing of flight physiology and biomechanics hypotheses in highly controllable settings. Also, they are helping to solve conservation issues such as window-kills (e.g. the flight tunnel at Carnegie Museum's Powdermill Avian Research Center), and wind-farm placement (de Lucas *et al.* 2012). However, depending upon the research goals, these systems may be excessive, inappropriate, and/or impractical. For instance, transporting a wind tunnel into the field is impractical and using such a device to test ecomorphological hypotheses at a community scale would require the coordinated effort of many researchers and laboratories. While some ecologists have begun adapting laboratory materials for field use (see Warrick 1998, Matyjasiak 2013), alternative ideas are always valuable. We present a relatively inexpensive and mobile method for collecting flight performance data in the field.

As part of an ongoing study on avian wing-beat frequency, wing morphology, and flight acceleration (KEP and CEC in preparation), we needed to capture, measure, and mark (USFWS aluminum leg bands) birds in the field and film their release. To acquire standardized data on acceleration, we needed to direct the birds' escape along a relatively fixed path while limiting the presence of obstacles. Initially, we built a convoluted PVC and plastic-mesh rectanguloid contraption. Because portability was a top priority, we used thin tubes of PVC and bonded only certain joints. This structure sagged, became disarticulated easily, and stank of toxic fumes. Inspired by children (of CEC), we suggest an alternative, simpler design to collect flight acceleration data on birds. To demonstrate, we captured, and filmed the release of several birds in old-field habitat on property owned by Bloomsburg University of Pennsylvania in Bloomsburg, PA, USA (~41°01'N, 76°45W).

MATERIALS AND METHODS

The field flight tunnel consists of an "Institutional See-Thru 9 ft Tunnel" from Pacific Play Tents, Inc. (model #20517, length = 274 cm, diameter = 56 cm, Fig. 1), fully stretched and pinned to the ground using tent stakes or sticks, or positioned so that it would not roll. This play tunnel is constructed of a polyester weave with a coil of spring steel embedded and padded along its length. A cinching sleeve cover attached at one end (stock) functions to hold the tunnel in a collapsed state while not in use. Additionally, when partially cinched, this cover helps to prevent the backward escape by a bird during flight trials. To encourage flight

[1]Accepted for publication December 2015.
[4]Corresponding author. email: ccorbin@bloomu.edu, telephone: 570-389-4134

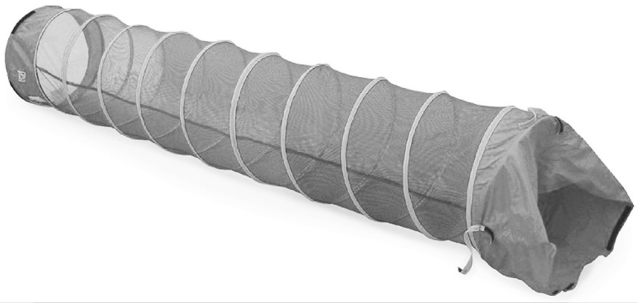

Figure 1. The Institutional See-Thru 9 ft Tunnel" from Pacific Play Tents, Inc. Tunnel, model #20517, size is 274 cm length x 56 cm diameter.

down the tunnel, the opposite open end may (depending on species and habitat) be placed near vegetation.

Aspects of acceleration and wing-beat frequency may be affected by the launch. Birds can be released from a small stand, a hanging perch, or even a small board on the floor of the tunnel. We suggest the release of birds be standardized for each study. In our work with relatively small birds (e.g. passerines, woodpeckers, etc.), we found consistent results if birds are placed wings-folded (i.e. in bander's grip), head towards opposite end with feet and belly touching a board on the floor immediately inside the tunnel, and then released. This ensures birds are all in the same relative position and also standardizes the downward (gravitational) component of acceleration. For our purposes, an ideal event consists of a launch and flight through the tunnel with no contact with inside walls.

Collecting data such as wing-beat frequency and acceleration are facilitated with the use of the tunnel. To demonstrate, we captured, measured, and then filmed the release of several birds in old-field habitat on property owned by Bloomsburg University in Bloomsburg, PA, USA (~41°01'N, 76°45W). The dominant vegetation consists of thick stands of ragweed (*Ambrosia*), goldenrod (*Solidago*), milkweed (*Asclepias*), rose (*Rosa*), and olive (*Elaeagnus*). To catch birds, we employed two loop-interdigitated 12 m x 2.5 m mist nets (30 mm mesh) stretched along a mowed path about 3 m wide during the spring and fall of 2012. Birds were weighed (nearest 0.5 g) with Pesola® spring scales, positioned inside the tunnel as described above, and released.

The lateral views of the releases were recorded with a tripod-mounted Casio EX-ZR100 at 240 fps, positioned externally 2.5 m away and 90° to the side of the tunnel. Films were imported into Logger Pro 3 (2012) to calculate average acceleration. Frame by frame analysis of change in x and y directions is possible, but for simplification and demonstration purposes, we assume net y-forces (lift and gravity) are near zero ($F_{lift} \approx F_{gravity}$), or at least minimized due to the constraints of the tunnel roof, and net acceleration in the x-direction is constant. Hence, the maximum speed (x-direction) of the bird in each video was divided by the time to reach that speed (frame rate = 240 fps) to get average acceleration during that event. Institutional (Bloomsburg University) IACUC approval was obtained prior to data collection.

RESULTS AND DISCUSSION

Twenty-two birds of various species (see Table 1 and Figure 2) were captured and released through the tunnel. In almost all cases, birds launched and flew directly out the other end (changes in y-axis were minor). Generally, it helped to hold the bird within the tunnel for a few seconds prior to release. We presume this allowed the bird to see the vegetation at the other end of the tunnel (Snyder 1946). Additionally, this time was used to adjust recording equipment if necessary.

This tunnel provides a highly mobile means to constrain the flight path of birds upon release and facilitate their videography. In instances where birds are being captured and banded, we feel filming the releases and posting the data to a database (e.g. user defined fields in Bandit, Laurent *et al.* 2011) could potentially improve our understanding of wing morphology and functional ecology with a small increase to project budget and minimal extra effort. For example, Table 1 and Figure 2 are provided to help visualize the sort of data which can be facilitated by adding a tunnel and camera to banding efforts.

The system also is relatively inexpensive. Cost depends heavily on the particular recording system used. While a regular handheld stop- or wristwatch will work in many instances, actually filming birds increases the utility of the event. We used a Casio EX-ZR100 (~US$350-$400), because of high frame-rate capabilities and relatively large pixel number (12.1 MP). However, less expensive cameras are available and suitable. Collecting data such as wingbeat frequency and kinematics would require a camera with high frame-rate capabilities (e.g. the present camera), while collecting data on flight trajectory or acceleration would not. The institutional version of the play tunnel is approximately US$100. While this may seem expensive, lower quality versions may not have padded steel wiring or durable fabrics ideal for field conditions. Mobility in this system is excellent. The tunnel weighs 4 kg, and the company provides a sturdy fabric carry case that can also accommodate banding supplies and nets.

We have a few notes on system caveats and suggestions for improvement. We did not attempt to immediately recapture birds after release. This may be desirable for purposes of repeatability and/or experimentation. Simply adding a mist net at the end of the tunnel for recapture would be a potential strategy. However, we are unsure if a second or third trial would yield similar results due to capture sensitivity and/or stress (see Davis 2005). Additionally, internal lighting may present a problem, particularly in systems where high speed filming is required. The "see-thru" mesh portion of

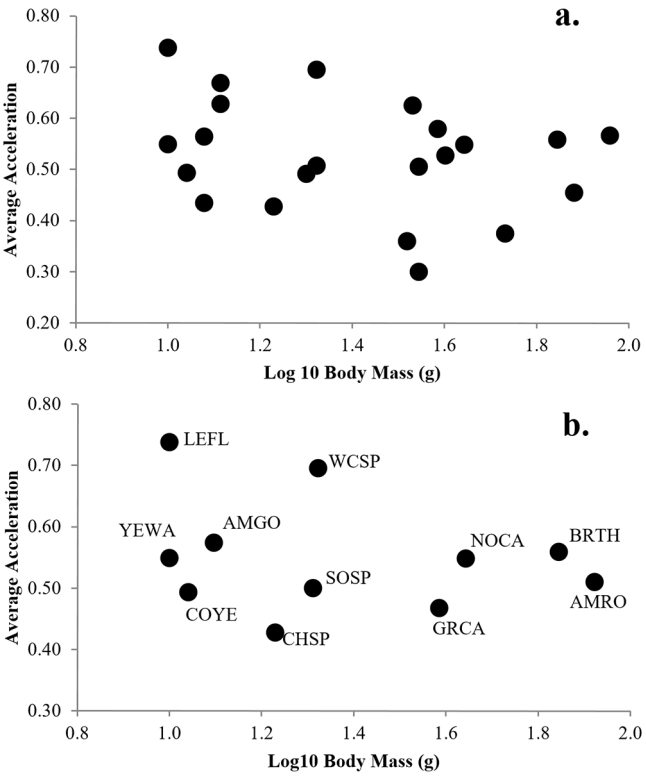

Figure 2a. Acceleration and log10 transformed mass for 22 passerine birds, and b. re-plotted using species averages.

our tunnel is a dark blue color. Lighting may be increased with battery or solar powered rope or ribbon lights that are readily available in hardware stores and online (~ US$15 - $40). Also, a strip (or strips) of light colored material (e.g. a white sheet) could be affixed to the inside of the far wall of the tunnel to increase the contrast between the bird and the background. The efficacy of such accessories needs to be tested. Synchronized cameras, albeit costly, would allow for 3-dimensional data collection and turning maneuverability. Warrick *et al.* (2002) elegantly demonstrate an additional camera simultaneously triangulating the 3-d location of a flying bird and acting as an obstacle to initiate a turning maneuver. The tunnel could be adapted easily to include such a setup.

Finally, with small adaptations, this system would easily suit projects using other flying (e.g. odonates, bats, etc.) or even running (e.g. lizards) species; similar systems have been used for a long time in dog training. Since the tunnel is not rigid, it could be modified to constrain angular, curvilinear, and/or vertical flight paths, or vertical take-offs and landings. Multiple tunnels could be added and configured to record maze-navigating maneuverability (Warrick 1998, Warrick and Dial 1998, Matyjasiak 2013).

In conclusion, we present a simple, inexpensive, and highly portable system for capturing some types of flight performance data in the field. This system assembles in seconds, it is lightweight, durable, washable, harmless to birds, and easily added to the release points of banding stations. Performance data on volant organisms are at a premium (see Norberg 1990, and Provine 1994). Furthermore, performance data collected from utilization of such a system would be a welcome addition to data sets collected by bird banders and researchers.

Table 1. Morphological and performance data of birds flying through the field flight tunnel. Species 4-letter codes used in figure 2 and latin binomials are listed below the table*.

Species (sample size)	Acceleration (m/s^2)	Log10 Mass (g)
American Goldfinch (4)	4.596	1.1
American Robin (2)	6.722	1.96
Brown Thrasher (1)	4.476	1.85
Chipping Sparrow (1)	3.423	1.23
Common Yellowthroat (1)	3.947	1.04
Gray Catbird (7)	3.741	1.59
Northern Cardinal (1)	4.389	1.64
Song Sparrow (2)	3.999	1.31
White-crowned Sparrow (1)	6.95	1.32
Yellow Warbler (1)	4.394	1.00

*AMGO, American Goldfinch (*Spinus tristis*); AMRO, American Robin (*Turdus migratorius*); BRTH, Brown Thrasher (*Toxostoma rufum*); CHSP, Chipping Sparrow (*Spizella passerina*); COYE, Common Yellowthroat (*Geothlypis trichas*); GRCA, Gray Catbird (*Dumatella carolinensis*); NOCA, Northern Cardinal (*Cardinalis cardinalis*); SOSP, Song Sparrow (*Melospiza melodia*); WCSP, White-crowned Sparrow (*Zonotrichia leucophrys*); YEWA, Yellow Warbler (*Setophaga petechia*).

ACKNOWLEDGEMENTS

This study was supported by a Research and Disciplinary Award to all authors from Bloomsburg University's Office of Sponsored Research and to KEP by Bloomsburg University's Honors Program. The manuscript was greatly improved by the comments and suggestions of three anonymous reviewers. The authors have no competing interests to declare.

LITERATURE CITED

Corbin, C. E. 2008. Foraging ecomorphology within North American flycatchers and a test of concordance with southern African species. J. Ornith. 149:83-95.

Corbin, C. E., Lowenberger, L. K. and Dorkosky, R. P. 2013. The skeleton flight apparatus of North American bluebirds (*Sialia*): phylogenetic thrushes or functional flycatchers? J. Morph. 274:909-917.

Davis, A.K. 2005. Effect of handling time and repeated sampling on avian white blood cell counts. J. Field Ornith. 76:334-338.

Geyer, T., Sarradj, E. and Fritsche, C. 2013. Silent owl flight: comparative acoustic wind tunnel measurements on prepared wings. Acta Acustica/Acustica 99:139-153.

Laurent, K., Malordova, M., Singh, J. and J. McFaul, J. 2011. Bandit user manual. Version 3.9, 2011-01-21. http://www.pwrc.usgs.gov/bbl/resources/bandit.

Logger Pro. 2012. Version 3. Vernier Software and Technology, LLC. Beaverton, OR. USA.

de Lucas, M., Ferrer, M. and Janss, G. F. E. 2012. Using wind tunnels to predict bird mortality in wind farms: The case of griffon vultures. PLoS ONE 7:e48092. doi:10.1371/journal.pone.0048092.

Maina, J. N. and Jimoh, S. A. 2013. Structural failures of the blood-gas barrier and the epithelial-epithelial cell connections in the different vascular regions of the lung of the domestic fowl, *Gallus gallus* variant domesticus, at rest and during exercise. Biology Open. doi:10.1242/bio.20133608.

Matyjasiak, P. 2013. Timing and arrival from spring migration is associated with flight performance in the migratory barn swallow. Behav. Ecol. Sociobiol. 67:91-100.

Norberg, U.M. 1990. Vertebrate flight: mechanics, physiology, morphology, ecology and evolution. Springer-Verlag.

Provine, R.R. 1994. Pre- and post-natal development of wing-flapping and flight in birds: embryological, comparative and evolutionary perspectives. In Davies M.N.O, and P.R. Green (eds.) Perception and Motor Control in Birds: an Ecological Perspective. Springer-Verlag.

Snyder, L. L. 1946. "Tunnel fliers" and window fatalities. Condor 48:278.

Warrick, D. R. 1998. The turning- and linear-maneuvering performance of birds: the cost of efficiency for coursing insectivores. Can. J. Zool. 76:1063-1079.

Warrik, D. R., Bundle, M. W., and Dial, K. P. 2002. Bird maneuvering flight: blurred bodies, clear heads. Integ. Comp. Biol. 42:141-148.

Warrik, D. R. and Dial, K. P. 1998. Kinematic, aerodynamic, and anatomical mechanisms in the slow, maneuvering flight of pigeons. J. Exp. Biol. 201:655-672.

Yong,W. and Moore, F. R. 1994. Flight morphology, energetic condition, and the stopover biology of migrating thrushes. Auk 111:683-692.