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Cover: Pictured are hatch-year (left) and adult female (right) chestnut-sided warblers (*Setophaga pensylvanica*) caught in the same mist-net on 1 July 2015 in south-central Indiana. Chestnut-sided warblers, a rare breeder in the state, require early successional habitat for breeding. Recent creation of such habitat via active forest management in the Yellowwood State Forest (Brown County) has resulted in confirmation of the region's first successful chestnut-sided warbler breeding attempt in 20 years. For more information, see the article in this issue entitled "Confirmation of Successful Chestnut-Sided Warbler Breeding in South-Central Indiana" by Patrick J. Ruhl, John B. Dunning Jr. and Jeffrey K. Riegel. [Photograph by Jeffery K. Riegel]

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Figures 1–4.—Right chelicerae of species of *Centruroides* from Timbuktu. 1. Dorsal view; 2. Prolateral view of moveable finger; 3. *Centruroides* holotype male; 4. *Centruroides* female. Scale = 1.0 mm.

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INFRARED INVESTIGATIONS OF C_{60} AND C_{70} NANOPARTICLE INTERACTIONS WITH THE AMIDE FUNCTIONALITY IN FORMAMIDE

Joe L. Kirsch[1], **E. Brown**[2], **J. Kanter**[2] and **J. Wade**[2]: Department of Chemistry, Butler University, 4600 Sunset, Indianapolis, IN 46260 USA

ABSTRACT. Fourier transform infrared spectroscopy was used to investigate the interactions between C_{60} and C_{70} nanoparticles and the amide functionality in formamide. Infrared spectra were collected for neat formamide, formamide in a toluene environment, formamide in a C_{60} toluene environment, and formamide in a C_{70} toluene environment. A Digilab FTS 7000 infrared spectrometer and a ZnSe circle cell were used for spectral data collection. Challenges were expected and encountered because of solubility compatibilities of the reagents and special ATR spectral collection issues; however, these challenges helped shape the final form of the study and are addressed in the paper. Shifts of the carbonyl, stretching frequency to higher wavenumbers, were observed when formamide was treated with both the C_{60} and C_{70} indicating nanoparticle interactions with its amide functionality. The spectrum of neat formamide showed a carbonyl absorption near 1665 cm^{-1}; the spectrum of formamide in a toluene environment showed a carbonyl absorption near 1669 cm^{-1}; and the spectra of formamide in a toluene environment with C_{60} and C_{70} showed carbonyl absorptions near 1677 cm^{-1}. Spectral subtraction was used to identify the carbonyl absorptions (near 1690 cm^{-1}) for the nanoparticle (C_{60} and C_{70}) - formamide complexes.

Keywords: infrared spectroscopy, nanoparticles, C60 & C70, fullerenes, fullerene/nanoparticle interactions

INTRODUCTION

Significant interest in the interactions of diverse molecular systems with C_{60} and C_{70} nanoparticles has motivated a variety of studies using a number of analysis methods (Holleman et al. 1999; Kyzyma et al. 2008; Jurow et al. 2012; King et al. 2012; Kyrey et al. 2012; Tropin et al. 2013; Bowles et al. 2014). This paper reports the results of an infrared spectroscopy study of the interactions between C_{60} and C_{70} nanoparticles and the important functionality of amide in formamide using toluene as a solvent environment (Aksenova et al. 2013). The initial plan of the investigation was to prepare solutions of formamide with C_{60} and C_{70} nanoparticles and use infrared spectroscopy to seek shifts in the carbonyl absorption of the formamide resulting from its interactions with the nanoparticles. It is well known that C_{60} and C_{70} nanoparticles dissolve in aromatic solvents such as toluene to form colored solutions; C_{60} in toluene is blue, while C_{70} in toluene is red (Ruoff et al. 1993). Certainly, challenges resulting from solubility compatibilities of the relatively polar formamide and the nonpolar toluene solvent needed for the nanoparticle solubility were expected.

Initial spectral collections gave promising results. The infrared spectrum of formamide in a toluene solution saturated with C_{60} showed a shift in the carbonyl absorption to higher wavenumbers compared to formamide in pure toluene. Confirmation experiments, however, generated confusing results by sometimes showing shifts and sometimes showing no shifts. Spectra were collected at different concentrations of the formamide in the toluene C_{60} solvent system in an attempt to address the confusing results, but the experiments yielded the same results of sometimes showing a shift and sometimes showing no shift. Spectra were collected in a circle cell which is an attenuated total reflectance (ATR) device. Some compounds can bind to the ATR crystal rod forming a coating of compound on its surface, and spectral collection showed that formamide is a compound that binds to the ATR rod used in the study. ATR devices also measure the spectrum of the environment near the ATR crystal surface. Possible contributions to the shift and no shift observations in the repeated experiments could be competitive binding of formamide

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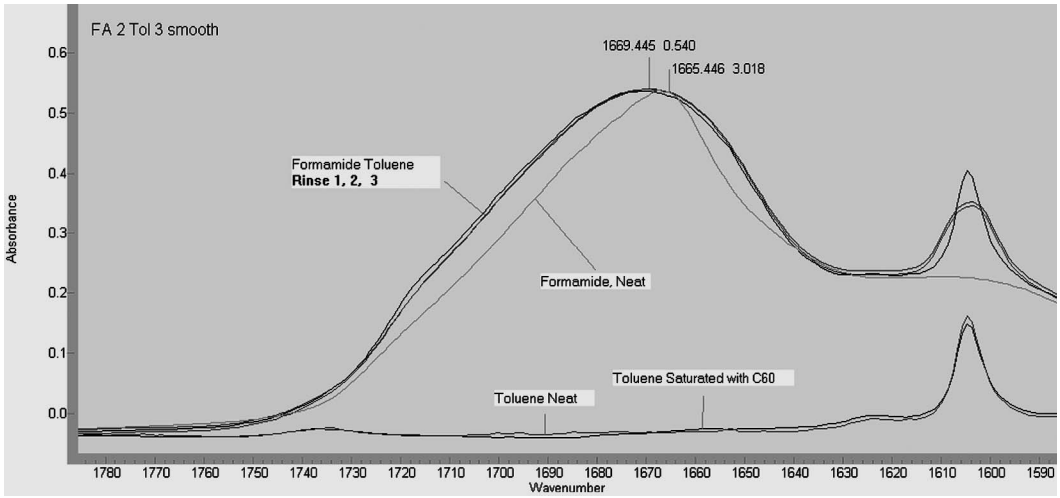

Figure 1.—The spectrum of neat formamide in the circle cell, the spectra of formamide on the circle cell rod after each of three rinses with toluene (the rinse process was over a six hour period of time), the spectrum of toluene, and the spectrum of toluene saturated with nanoparticles (C_{60}). Spectra are auto-scaled to equal intensity along the absorbance axis to clarify shifts or profile changes along the wavenumber axis.

between the ATR rod and nanoparticles along with the thickness of formamide coating on the ATR rod. These notions motivated a new plan of study focusing on nanoparticle interactions with a film of formamide coated on the ATR rod surface.

METHODS AND RESULTS

New experiments with consistent observations.—A Digilab FTS 7000 infrared spectrometer and a circle cell fitted with a ZnSe ATR rod was used to collect spectra for the investigation by averaging 150 scans at a spectral resolution of two wavenumbers. The empty circle cell was used as the background single beam spectrum for spectral collection. The spectrometer was purged with dry air for one hour prior to spectral collection to remove potentially interfering atmospheric water vapor. Figure 1 shows the spectra of neat toluene, toluene saturated with C_{60} nanoparticles, and neat formamide with its carbonyl absorption at 1665 cm^{-1}. The spectra in Fig. 1 show that toluene and toluene saturated with nanoparticles do not exhibit spectral absorptions in the formamide carbonyl spectral region. Next, the neat formamide was emptied from the circle cell, and the circle cell was rinsed with toluene three times with spectra being collected on the toluene in the circle cell during each rinse. Figure 1 shows that formamide remained bound to the ATR rod after each rinse with toluene. The spectra in all Figures are auto-scaled to equal intensity along the Absorbance axis to clarify shifts or profile changes along the wavenumber axis. The rinsing of the circle cell with toluene and the collection of the spectrum of formamide bound to the ATR rod in a toluene environment required about two hours for each spectrum including cell rinsing, spectrometer purge time, and spectral data collection. The multiple rinsing process of the circle cell with toluene and recollections of the spectra of the formamide coated on the ATR rod during each rinse, therefore, were completed over a six hour period of time. Figure 1 shows an observable shift to higher wavenumbers after the first rinse with no further shifts after the second and third rinses. This observation indicates that after two hours of immersion in toluene, a coating of formamide remained bound to the ATR rod and also that the stable coating of formamide was bound to the ATR rod after six hours of immersion in toluene. A reasonable description of the "rinsed circle cell spectra" is formamide bound to or coated on the ATR rod in a toluene environment.

After the cell rinsing process was completed, the toluene in the circle cell was replaced with toluene saturated with C_{60}, and spectra recollected. Spectra of formamide bound to the ATR rod immersed in toluene saturated with C_{60} were collected immediately, three hours,

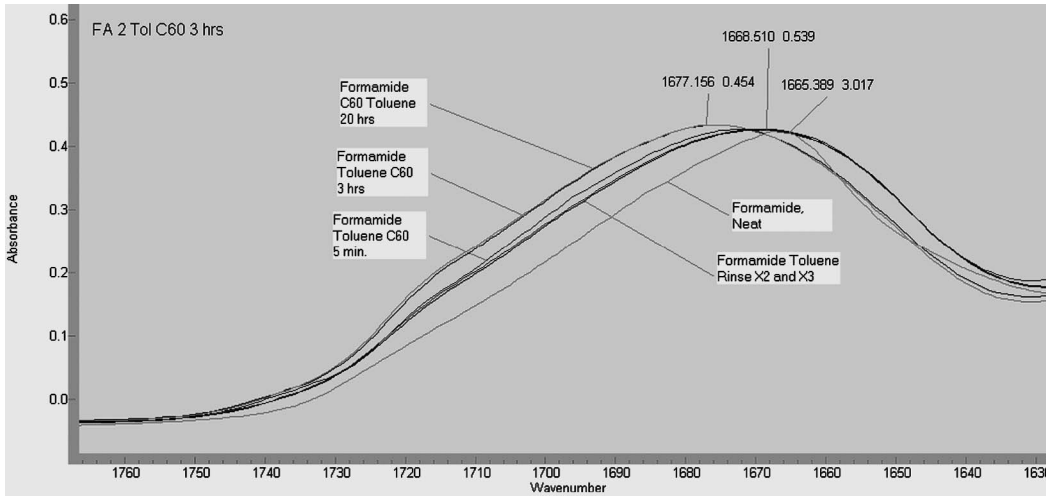

Figure 2.—The spectrum of neat formamide in the circle cell; the spectra of formamide on the circle rod after rinse 2 and 3; and the spectra of formamide on the circle rod in a toluene saturated with C_{60} environment at five minutes, three hours, and twenty hours after the addition of toluene saturated with C_{60}. Spectra are auto-scaled to equal intensity along the absorbance axis to clarify shifts or profile changes along the wavenumber axis.

and twenty hours after the addition of the toluene saturated with C_{60} (Fig. 2). A small but observable shift in the formamide carbonyl absorption to higher wavenumbers occurred immediately after the addition of toluene saturated with C_{60} with a major shift observed three hours after the addition. No further shift in the carbonyl absorption was observed after sitting for twenty hours. The observed shifts immediately, after three hours, and after twenty hours suggest a C_{60} interaction with the formamide coating on the ATR rod in a toluene solvent environment. Similar experiments were performed with formamide and toluene saturated with C_{70}, and shows results similar to the formamide-C_{60} experiments (Fig. 3).

DISCUSSION

The carbonyl infrared absorption in amide functionality is characterized by an absorption peak below 1700 cm^{-1} compared to "normal" carbonyl absorption peaks observed above 1700 cm^{-1}. This observation has been explained by the lone pair electrons on the nitrogen being delocalized into the amide carbon–nitrogen chemical bond generating a minor resonance structure with a carbon–oxygen single bond. This minor resonance structure adds single bond character to the carbonyl bond and contributes to the lower wavenumbers observed for the carbonyl infrared absorption in amide functionality (Avram & Mateescu 1970).

The observed shifts to higher wavenumbers of the carbonyl absorption for formamide interacting with the nanoparticles can be explained by the nitrogen lone pair electrons being donated to the nanoparticles resulting in less delocalization of those electrons into the carbon–nitrogen amide bond, less stability of the carbon–oxygen, single-bond resonance structure, and more stability of the carbon–oxygen, double-bond resonance structure. Many studies suggest that C_{60} and C_{70} are good electron acceptors for molecular systems (Charvet et al. 2012; Schubert et al. 2013; Stranius et al. 2014). Certainly, a reasonable explanation is that the lone pair electrons of formamide donate into the antibonding MO's of the delocalized electrons on the surface of the C_{60} and C_{70} molecules (Feng et al. 2008). A resonance structure model of formamide interacting with nanoparticles and the resulting impact of the nanoparticle bonding on the formamide carbonyl infrared absorption is shown in Fig. 4. So, observed shifts of the formamide carbonyl absorption to higher wavenumbers in both toluene–C_{60} and toluene–C_{70} environments are consistent with formamide bonding to the nanoparticles through the lone pairs of nitrogen in the amide functionality of the formamide.

Analysis enhancement and confirmation through spectral subtraction.—The spectra and obser-

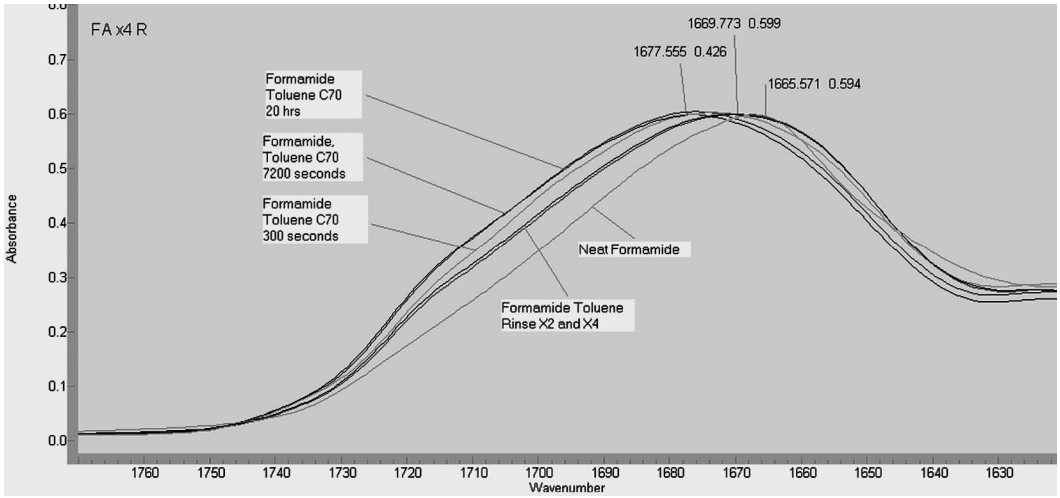

Figure 3.—The spectrum of neat formamide in the circle cell; the spectra of formamide on the circle rod after rinse 2 (three hours) and rinse 4 (six hours); and the spectra of formamide on the circle rod in a toluene saturated with C_{70} environment at five minutes, two hours, and twenty hours after the addition of toluene saturated with C_{70}. Spectra are auto-scaled to equal intensity along the absorbance axis to clarify shifts or profile changes along the wavenumber axis.

vations presented to this point result from "simple" collection of FT IR spectral data. Spectral subtraction has been shown to be a useful tool in the separation of spectral absorptions resulting from mixed absorbing species systems, and it was used in this study to suggest the likely carbonyl absorption of the formamide bound to the nanoparticles. In addition, spectral subtraction was used to address the potential of solvent interference in the spectral analysis (Gillette & Koenig 1984; Honigs et al. 1985; Yang 1994; Siyuan et al. 2010).

The spectra of formamide coated on the ATR rod in toluene saturated with C_{60} (Fig. 2) and in toluene saturated with C_{70} (Fig. 3) are very likely the summations of spectra of an equilibrium mixture of formamide bound and unbound to the nanoparticles. The equilibrium

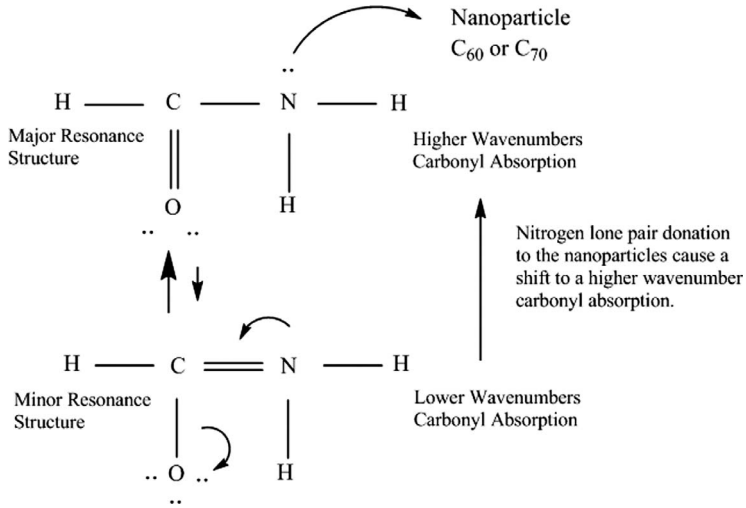

Figure 4.—A resonance structure model suggesting nitrogen, lone pair donation to the nanoparticles (C_{60} and C_{70}) that shifts stability toward the resonance structure with the double bonded carbonyl and supports a shift of the carbonyl absorption to higher wavenumbers.

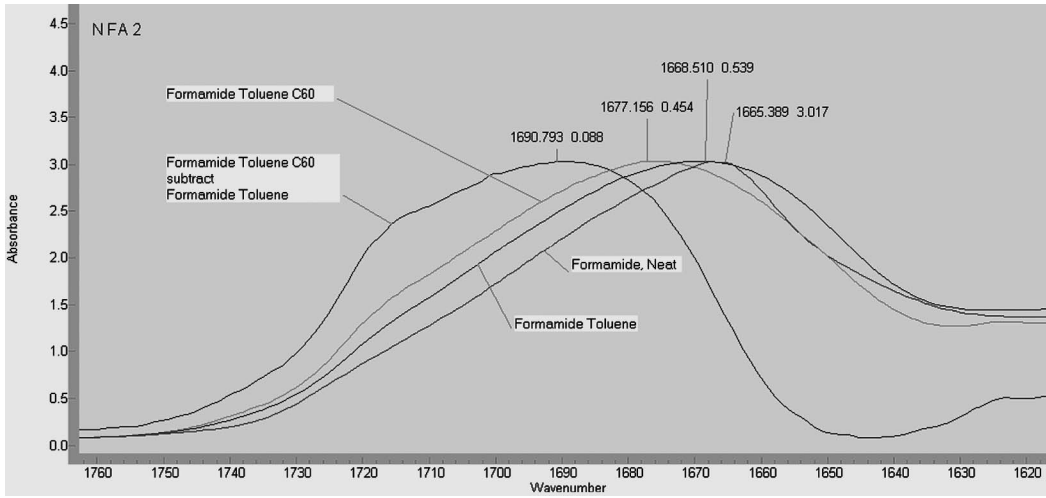

Figure 5.—The spectrum of neat formamide in the circle cell; the spectrum of formamide on the circle rod in a toluene environment; the spectrum formamide on the circle rod in a toluene saturated with C_{60} environment; and the spectrum resulting from spectral subtraction of formamide on the circle cell rod in a toluene saturated with C_{60} environment minus the spectrum of formamide on the circle rod in a toluene environment. The subtraction process yields a carbonyl absorption of formamide interacting with C_{60} in a toluene environment at approximately 1691 cm^{-1}. Spectra are auto-scaled to equal intensity along the absorbance axis to clarify shifts or profile changes along the wavenumber axis.

mixture likely has contributions from chemical bounding character between formamide and nanoparticles, the notion that the interaction takes place on the outer surface of the formamide coating, and the thickness of the formamide coating on the ATR rod. Since the spectra of both the equilibrium mixture along with formamide coated on the ATR rod unbound to

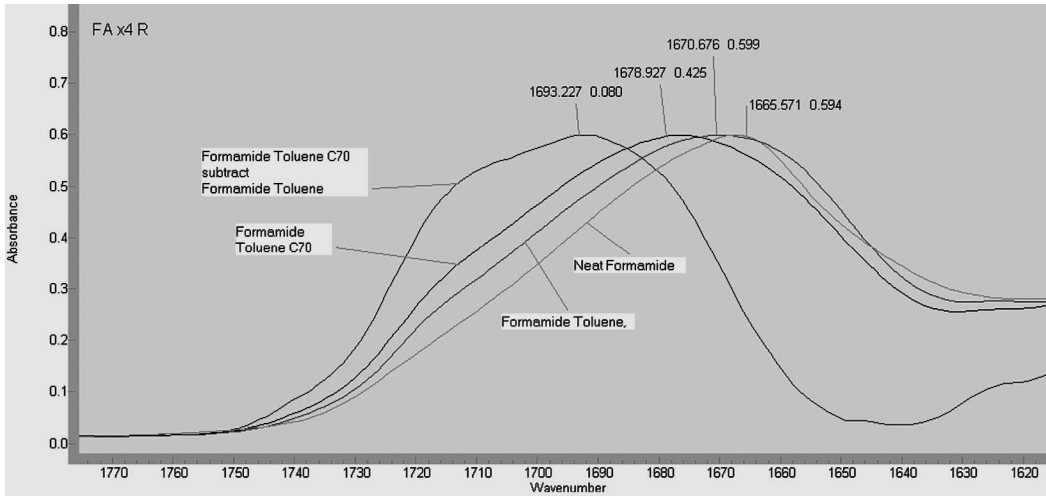

Figure 6.—The spectrum of neat formamide in the circle cell; the spectrum of formamide on the circle rod in a toluene environment; the spectrum of formamide on the circle rod in a toluene saturated with C_{70} environment; and the spectrum resulting from spectral subtraction of formamide on the circle cell rod in a toluene saturated with C_{70} environment minus the spectrum of formamide on the circle rod in a toluene environment. The subtraction process yields a carbonyl absorption of formamide interacting with C_{70} in a toluene environment at approximately 1693 cm^{-1}. Spectra are auto-scaled to equal intensity along the absorbance axis to clarify shifts or profile changes along the wavenumber axis.

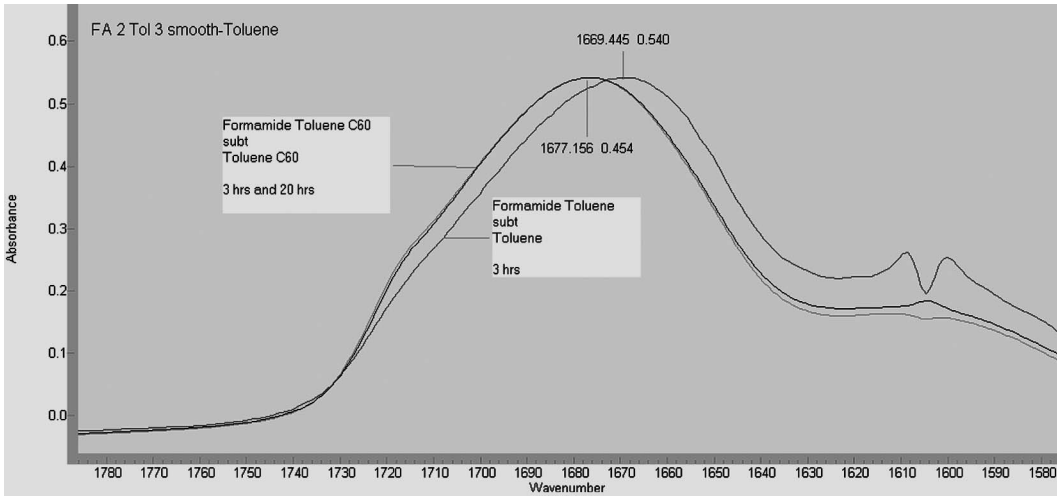

Figure 7.—The spectrum of formamide coated on the circle cell rod in a toluene environment minus the spectrum of toluene in the circle cell and the spectra of formamide coated on the circle cell rod in a toluene with C_{60} environment at three hours and twenty hours minus spectrum of toluene with C_{60} in the circle cell. Spectra are auto-scaled to equal intensity along the absorbance axis to clarify shifts or profile changes along the wavenumber axis.

nanoparticles were measured, the spectra of the formamide coated on the ATR rod bound to the nanoparticles can be obtained by spectral subtraction. Figure 5 shows the result of the spectral subtraction of the spectrum of formamide coated on the ATR rod in a toluene environment saturated with C_{60} minus a subtraction factor times the spectrum of formamide coated on the ATR rod in a toluene environment. Figure 6 shows a similar result for spectral subtraction of the spectrum of formamide coated on the ATR rod in a toluene environment saturated with C_{70} minus a subtraction factor times the spectrum of formamide coated on the ATR rod in a toluene environment.

The subtraction factor that yielded a reasonably good base line leading into and out of the carbonyl absorption for the subtraction process was about 0.6. The spectra resulting from this spectral subtraction process represent the carbonyl absorptions of formamide bound to the ATR rod interacting with C_{60} (Fig. 5) and interacting with C_{70} (Fig. 6). The subtracted spectra show a fairly broad carbonyl absorptions near 1691 cm^{-1} for formamide bound to C_{60} (Fig. 5) and 1693 cm^{-1} for formamide bound to C_{70} (Fig. 6).

Figure 1 shows that the toluene and toluene saturated with C_{60} nanoparticles do not have spectral features that would interfere with spectral interpretation; however, spectral subtraction was used to confirm the lack of interference from the solvent environments. Figure 7 shows the results of the spectral subtraction of the spectrum of formamide coated on the ATR rod in a toluene environment minus a subtraction factor times the spectrum of toluene. Figure 7 also shows the results of spectral subtraction of the spectrum of formamide coated on the ATR rod in a toluene saturated with C_{60} environment minus a subtraction factor times the spectrum of toluene saturated with C_{60}. The subtraction factors yielding good baselines were near 0.9. It is clear from Fig. 7 that the spectra for both formamide coated on the ATR rod interacting with C_{60} and formamide coated on the ATR rod with their respective solvent spectra removed by spectral subtraction show the identical shift observed in Fig. 2, and this analysis supports formamide–nanoparticle interactions in a toluene environment.

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PLASTICITY OF *AESCULUS GLABRA* (HIPPOCASTANACEAE) LEAF TRAITS ALONG SMALL-SCALE LIGHT GRADIENTS WITHIN FOREST STANDS

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ABSTRACT. Phenotypic plasticity in response to environmental heterogeneity is an important adaptive component of plant strategies. This study addresses plasticity of *Aesculus glabra* leaf traits in response to small-scale (within-canopy, within-stand) gradients of light availability in a temperate deciduous forest. Leaf mass per area, stomatal density, stomatal length, water content, leaf life span, and chlorophyll content were measured in two populations in northern Indiana. Light availability was determined through hemispherical canopy photography. Stomatal density, leaf mass per area, and leaf life span were positively correlated with light availability ($r = 0.51$, $p < 0.001$; $r = 0.51$, $p < 0.001$; $r = 0.37$, $p = 0.02$, respectively). Chlorophyll content on an area basis and water content were negatively correlated with light availability ($r = -0.49$, $p = 0.002$; $r = -0.58$, $p < 0.001$, respectively). Most correlations of these leaf characteristics with each other were significant. Chlorophyll content on a mass basis and stomatal length did not correlate with light availability. Leaf life span was longer in branches at the top of the crown than in self-shaded lower branches. Leaf traits in this species show significant plasticity in response to small-scale gradients of light availability. The increase in leaf lifespan with increasing light is atypical, and may be due to poor carbon balance of *A. glabra* under shaded conditions. Since this species leafs out before the canopy does, it is unclear how it perceives and responds appropriately to the full-canopy light environment.

Keywords: *Aesculus glabra*, leaf life span, leaf structure, light gradient, plasticity

INTRODUCTION

Plants, as sessile organisms, must respond adaptively to variations in the environment over a range of temporal and spatial scales. Plasticity, the ability to produce different phenotypes in response to environmental variation, is a key feature of plant adaptation to heterogeneous environments (Schlichting & Pigliucci 1998; Gratani 2014). For example, plasticity has been identified as a significant adaptive property of invasive species (Schweitzer & Larson 1999; Sexton et al. 2002; Herr-Turoff & Zedler 2007). Plasticity allows plant species to occupy a wider range of light environments than they would otherwise be able to (Valladares et al. 2002; Catoni et al. 2015).

Plastic adaptation to the light environment can be accomplished by alteration of such leaf traits as anatomy (Sultan & Bazzaz 1993; Gutschick 1999; Niinemets et al. 1999; Sack et al. 2006; Poorter et al. 2009); photosynthetic apparatus, for example pigment and carboxylating-enzyme content (Dale & Causton 1992; Kull & Niinemets 1998; Rothstein & Zak 2001; Niinemets & Valladares 2004; Poorter et al. 2006); lifespan (Williams et al. 1989; Bongers & Popma 1990; Valladares et al. 2000; Hikosaka 2005; Vincent 2006); and arrangement in the canopy (Poorter & Werger 1999). Spatial scales of adaptation range from global differences among biomes (Wright et al. 2005) to variation within the canopy of individuals (Niinemets and Valladares 2004; Sack et al. 2006).

In this study, I quantified plasticity in leaf traits of the temperate deciduous forest tree *Aesculus glabra* Willd. (Ohio buckeye, Hippocastanaceae; taxonomy follows USDA, NRCS 2014). *Aesculus glabra* is a medium-sized tree of mesic forests of eastern North America (Williams 1990). Its range extends from Maine and Minnesota to Texas and northern Georgia (USDA, NRCS 2014). It had importance values >10% in some pre-settlement mesic forests in the Midwest (Crankshaw et al. 1965; Williams 1990), and currently occurs as a frequent understory species with occasional representation in the canopy (Hicks & Michaelis 2009). *Aesculus glabra* is considered to be shade tolerant but occurs in a range of light environments from

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forest edges and gaps to heavily shaded understory (Williams 1990).

The unusual leafing phenology of *A. glabra* is noteworthy. It leafs out and drops its leaves several weeks earlier than co-occurring tree species such as *Acer saccharum* Marshall, *Carya cordiformis* (Wangenh.) Koch, *Fraxinus americana* L., *Tilia americana* L., and *Celtis occidentalis* L., which are common canopy dominants in the mesic forests where *A. glabra* occurs (Henderson et al. 1993; Augspurger & Bartlett 2003; Augspurger 2004; Augspurger & Reich 2008). It thus makes use of the period of high light availability in the spring in temperate deciduous forest understory (Hicks & Chabot 1985), a strategy similar to that of spring ephemeral herbs (DePamphilis & Neufeld 1989; Henderson et al. 1993; Augspurger & Reich 2008). Since leaves expand before the canopy develops, it was hypothesized that correlations of *A. glabra* leaf characteristics with the full-canopy light environment would be weak. The primary goal of this study was to test for such relationships.

Previous studies of leaf plasticity have typically compared leaf traits in seedlings grown in or mature plants maintained in contrasting environments. The current study examines phenotypic variation in relation to naturally-occurring light gradients in forest stands, a smaller spatial scale than has been typical of earlier studies.

METHODS

Study sites.—Research was conducted in two sites in Wabash County, Indiana, which is located in the Eastern Corn Belt Plains Ecoregion (EPA 2013). Both sites are second-growth, mesic forest fragments east of the town of Liberty Mills. Mean annual temperature at Wabash, about 25 km from the study area, is 9.4° C, and mean annual precipitation is 979 mm (Indiana State Climate Data Archive 2010). Soils in both study areas are Hapludalfs (Ockley and Fox series; Ruesch 1983).

Two sites with large populations of *A. glabra* were chosen. Previous measurements from these sites indicate that mean light levels decline from ca. 60% of values found in the open at the forest edge to ca. 10% of open values in the interior, and that light levels decrease by ca. 95% as the canopy leafs out (Hicks, unpublished; cf. Hicks & Taylor 2015).

The forest at the Taylor site (41°2′33.54″ N, 85°43′45.72″ W, elevation ca. 225-240 masl, 4.3 ha) is dominated by *Celtis occidentalis*, *Acer saccharum*, *Fraxinus americana*, *A. glabra*, *Carya cordiformis*, and *Fagus grandifolia* Ehrh. (Hicks & Taylor 2015). This site was never cleared completely for agricultural use, but was cut over for timber extraction in the 1930's (Taylor 1998). The largest canopy trees were approximately 65 cm in diameter at breast height (DBH). This site is bounded by open agricultural land to the south and the Eel River to the north.

The Flory-Gemmer site (41°2′23.60″ N, 85°41′9.78″ W, elevation ca. 230-240 masl, 9.0 ha) is about 3.5 km east-southeast of Taylor. This site was mostly deforested in the 1960's, but had been allowed to regrow for about 40 years at the time of the study. Canopy trees in the portion used in this study reached maximum sizes of about 30 cm DBH, with dominant species being *A. saccharum*, *C. occidentalis*, and *Prunus serotina* Ehrh. The site is bordered by open agricultural land to the north, east, and south, and by regenerating old field to the west.

Field and laboratory procedures.—*Aesculus glabra* saplings from 1.5 to 2.5 m tall were chosen by randomly locating two transects at each site. This size class was studied because *A. glabra* is a dominant understory species in this size category (Hicks & Taylor 2015), and for easy access to the canopy of study plants. To sample a wide range of light environments, one transect in each site was positioned near the boundary between the forest and adjacent open land, and one in the forest interior, ca. 15 m from the edge. Forty-one saplings were located along the transects, 26 at Taylor and 15 at Flory-Gemmer. However, since complete data were not available for all individuals, sample size varies from 38 to 41. Data from both sites were combined, as there were no significant differences between sites in soil type, light availability, or leaf traits.

Estimates of leaf life span were made for two branches on each sapling. One branch was near the bottom of the canopy and one in an exposed position at the top of the canopy. Branches were selected in the spring of 2006, prior to budbreak. I marked each branch with a loose wire tie, and counted leaves at intervals through the growing season. Leaf counts were made approximately weekly during leafing-out and leaf-fall and at approximately two-week intervals in the middle of the growing season. During leafing-out, leaves were counted as emerged if they had expanded to > 1 cm in total length (lamina

plus petiole). Leaves were counted as missing if they were abscised or if all leaflets had turned yellow or brown. This method gives estimates that are aggregates for branches, rather than for individual leaves.

Leaf lifespan (LLS) was calculated on a per-branch basis. To measure LLS, I estimated the date at which 50% of the leaves on a tagged branch had emerged, and the date at which 50% of the leaves were absent. (This usually required a linear interpolation of the proportion of the leaves present on the sample dates before and after the 50% level was achieved.) LLS for each branch was the number of days between 50% leaf emergence and 50% absence.

The light environment for each lower-canopy branch was quantified with a hemispherical photo technique, which calculates light availability based on all forms of canopy openness, including gaps and stand edges (Rich 1989). Canopy photos were taken with a Nikon Coolpix 4500 camera equipped with a Nikon fisheye adaptor lens (Nikon Inc., Melville, New York, USA). Images were analyzed by the program Gap Light Analyzer (Frazer et al. 1999), calculated for the period 15 April—1 September. Light availability is expressed as the percent of light available at the photo point, relative to an area with no forest canopy or other overhead obstructions, integrated over the specified time period. The light environment for each lower-canopy branch was measured on 14 July 2006, a time when the canopy was fully leafed out. The camera was positioned on a tripod with the lens next to the marked branch. I collected the nearest adjacent branch for destructive sampling at the time when the canopy photos were taken. Samples were taken from two randomly chosen leaves on the sampled branch.

In the lab, disc samples were removed with a 1.27 cm^2 punch. Ten discs were massed, then dried (60° C, 24 hr) and remassed. Mass values were used to estimate leaf water content and leaf mass per area (LMA). Water content was calculated as (loss of mass on drying) / (dry mass). Ten more discs were used to determine total chlorophyll content using N,N-dimethylformamide extraction (Moran & Porath 1980; Inskeep & Bloom 1985). Finally, stomate density and length were determined on cuticle peels from the lower leaf surface. (No stomates occurred on the upper side of the blade.) Peels were made using Archer adhesive (Carolina Biological Supply, Burlington, North Carolina, USA). The peels were observed at 400× with a Nikon Alphaphot-2 microscope (Nikon Inc., Melville, New York, USA) and the number of stomates in a field of known area was counted (three fields per leaflet, ca. 0.7 mm^2 per field). Lengths of guard cells were estimated with a calibrated eyepiece micrometer (three fields per leaflet, 10 stomates per field).

Statistics and calculations.—Statistical tests were performed by SPSS (SPSS 2013). Relationships of leaf characteristics with light were assessed by the Linear Regression procedure. Correlations of leaf traits with each other were determined by the Bivariate Correlation procedure using Pearson coefficients. Differences between upper and lower branches were evaluated with Paired-sample t-tests. A critical value of 0.05 was used to indicate significance in all cases. Use of nonparametric procedures (Kendall's tau and Wilcoxon) did not change the outcome of any statistical test.

Plasticity indices (PI) were calculated, following Valladares et al. (2000), as

$$100 \times [(\text{maximum value of trait}) - (\text{minimum value of trait})] / [\text{maximum value of trait}]$$

Maxima were taken as the trait values at 23% light and minima as values at 3% light levels in the regression analyses presented below; these were the maximum and minimum total light values found in the current study.

RESULTS

Light availability was significantly correlated with stomatal density ($r = 0.51$, $p < 0.001$), LMA ($r = 0.51$, $p < 0.001$), water content ($r = -0.58$, $p < 0.001$), chlorophyll on a mass basis ($r = -0.49$, $p = 0.002$), and leaf lifespan ($r = 0.37$, $p = 0.02$); data for all significant correlations are shown in Fig. 1. Chlorophyll on a per-area basis and stomatal length were not significantly correlated with light ($r = 0.08$, $p = 0.65$ and $r = 0.10$, $p = 0.53$, respectively). There were significant correlations between most pairs of leaf variables, other than those including chlorophyll on a per-area basis or stomatal length (Table 1).

PI values, indicating the response of leaf traits to the light gradient within the stand, were 0.43 for stomatal density, 0.39 for LMA, 0.11 for water content, 0.45 for chlorophyll per unit mass, and 0.21 for leaf lifespan.

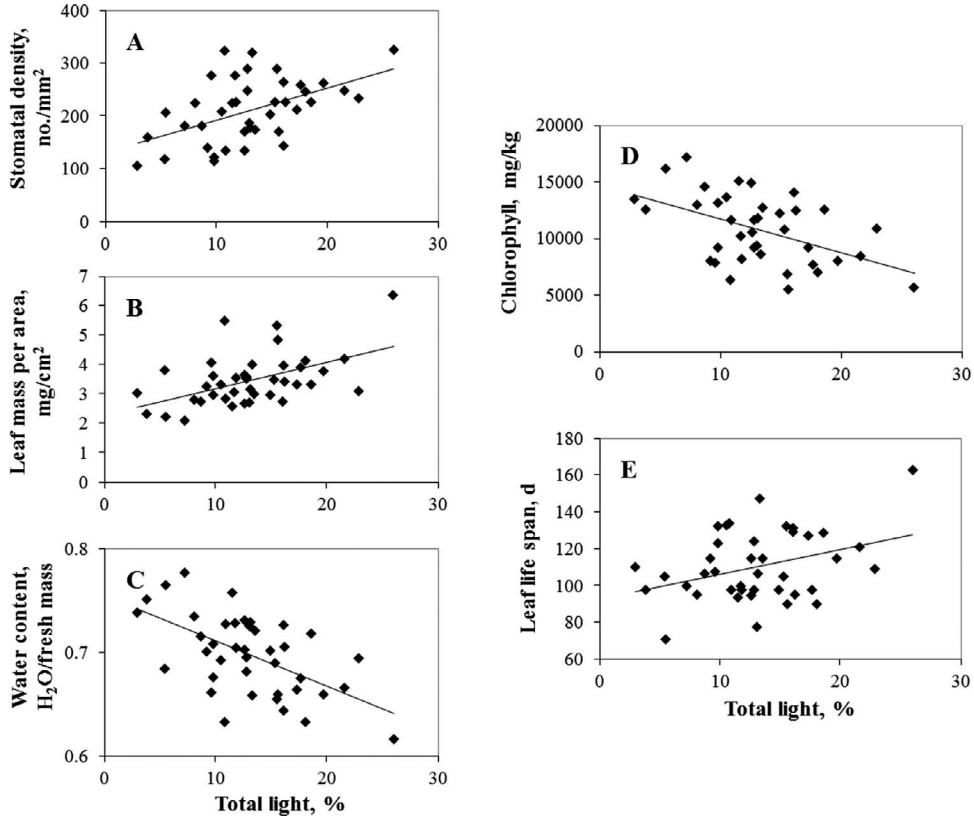

Figure 1.—Scatter plots of leaf characteristics in relation to light for *Aesculus glabra* from two deciduous forest sites near Liberty Mills, IN. N varies from 38 to 41. Regression equations are provided in the form $Y = C + m \times X$, where Y = dependent variable, C = Y intercept, m = slope, and X = independent variable (total light in all cases). A. Stomatal density ($r = 0.51$, $p < 0.001$; $Y = 132 + 6.11 \times X$). B. Leaf mass per area ($r = 0.51$, $p < 0.001$; $Y = 2.31 + 0.090 \times X$). C. Water content ($r = -0.58$, $p < 0.001$, $Y = 0.755 - 0.0040 \times X$). D. Chlorophyll ($r = -0.49$, $p = 0.002$, $Y = 14760 - 300 \times X$). E. Leaf lifespan ($r = 0.37$, $p = 0.02$, $Y = 93.0 + 1.35 \times X$).

Table 1.—Pearson correlation coefficients for relationships among leaf characteristics of *Aesculus glabra* leaves from two deciduous forest sites in Liberty Mills, IN. N varies from 38 to 41. Statistical significance is coded as: n = $p > 0.05$, * = $0.001 < p < 0.05$, ** = $p < 0.001$.

	Water content, gH_2O/gfw	Chlorophyll, mg/kg	Chlorophyll, mg/m^2	Leaf life span, d	Stomatal density, $no./mm^2$	Stomatal length, μm
Leaf mass per area, mg/cm^2	−0.88 **	−0.83 **	−0.15 n	0.52 *	−0.58 **	0.08 n
Water, gH_2O/gfw	–	0.86 **	0.26 n	0.52 *	0.56 **	0.01 n
Chlorophyll, mg/kg		–	0.61 **	−0.28 n	−0.51 *	−0.20 n
Chlorophyll, mg/m^2			–	0.24 n	−0.07 n	−0.28 n
Leaf life span, d				–	0.28 n	−0.28 n

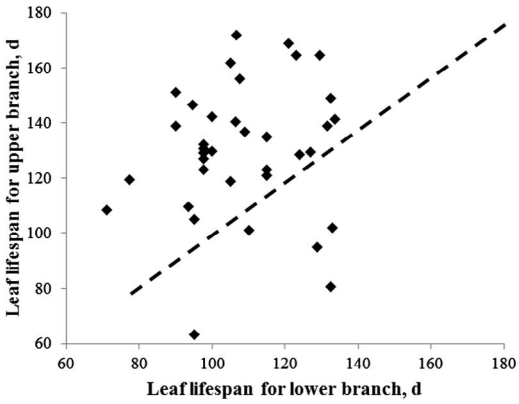

Figure 2.—Scatter plot of leaf lifespan of leaves on upper and lower branches of *Aesculus glabra* from two deciduous forest sites near Liberty Mills, IN. Dashed line indicates equal leaf lifespan in upper vs. lower branches. N = 39.

Leaf lifespan was significantly longer on upper branches than on lower branches of the same plant (Fig. 2; 108 $\pm$ 16 d for lower branches vs. 130 $\pm$ 24 d for upper branches, paired-samples t-test, t = −5.00, $p < 0.001$, n = 39).

DISCUSSION

Aesculus glabra displayed significant plasticity of leaf traits. Stomatal density, LMA, water content, chlorophyll per unit mass, and leaf lifespan were correlated with the light environment in a small-scale spatial gradient from the edge to the interior of a small forest stand. Also, leaf lifespan differed significantly within the canopy of individual plants.

Adjustment of leaf characteristics within a forest stand occurred on a scale of tens of meters, with many traits changing a correlated way. The relationships of light to stomatal density, LMA, water content, and chlorophyll per area found in this study are typical of those observed in a variety of species (cf. Boardman 1977; Gutschick 1999; Niinimets & Valladares 2004). PI values of stomatal density, LMA, chlorophyll on a mass basis, and leaf lifespan fell within the range found by other studies on temperate deciduous trees (Abrams & Kubiskey 1990; Ashton & Berlyn 1994; Lei et al. 1996; Sack et al. 2006; Baltzer & Thomas 2007; Seiwa & Kikuzawa 2011; Wyka et al. 2012; Legner et al. 2014). Data on plasticity of LLS do not appear to be available for temperate trees, but a study of four tropical species found PI values for LLS to range from 0.24 to 0.58 (recalculated from Vincent 2006). This suggests that *A. glabra* has relatively low plasticity for this trait. Stomate length and chlorophyll per area often do not change with changes in the light environment (Abrams & Kubiske 1990; Niinemets et al. 1998; Sack et al. 2006), as found in the current study. Consequently, although *A. glabra* initiates leaf expansion earlier than other woody species in the same environment, plastic variation in its leaf traits is generally of the same magnitude and in the same direction as documented for other temperate forest trees.

Plasticity of leaf traits in response to variation in the light environment, as documented in the current study, is regarded as adaptive. Plasticity increases the ability of leaves in a range of environments to take up carbon and to make a positive contribution to the carbon balance (photosynthetic fixation vs. respiratory loss of carbon) of the whole plant. Leaves in low-light environments typically have low LMA. Such leaves have lower content of structural materials relative to photosynthetic cells. This, combined with their maintenance of a greater leaf surface area per mass of leaf, increases their ability to capture light. High LMA in plants from high-light, open environments allows greater tolerance of stress such as physical damage (Niinemets et al. 1999; Valladares et al. 2002; Poorter et al. 2009; Catoni et al. 2015).

The increased leaf lifespan observed in *A. glabra* in higher light environments is unexpected. Theory predicts that leaves in environments with low resource levels, e.g., the low light levels of the forest understory, should be retained longer (Kikuzawa 1991). This prediction has been supported by observational and experimental data (Williams et al. 1989; Bongers & Popma 1990; Valladares et al. 2000; Hikosaka 2005; Vincent 2006). The reason that *A. glabra* does not fit this generalization may lie in its poor ability to maintain a positive carbon balance in the understory when the canopy has leafed out (Henderson et al. 1993; Augspurger & Reich 2008).

Plasticity in the narrow sense refers to variation in phenotypic characteristics of a particular genotype in relation to environmental variation (Schlichting & Pigliucci 1998). *Aesculus glabra* displays this form of plasticity, as indicated by the difference in LLS between upper- and lower-canopy branches of the same individual. Intra-canopy variation has also been found in other temperate deciduous trees, indicating that this is a frequent strategy for plants whose canopies

span a range of light environments (Niinemets et al. 1999; Niinemets & Valladares 2004; Sack et al. 2006; Wyka et al. 2012; Legner et al. 2014).

Gianoli & Valladares (2012) suggest that a broader view of plasticity, in which "related but not identical genotypes" are "exposed to different environments", is useful in ecological studies. This concept of plasticity also fits the current study, although the individuals studied are of unknown genetic relatedness. The breeding system of *A. glabra* is unknown (Lim et al. 2002), although *A. pavia*, in the same section of the genus *Aesculus* as *A. glabra*, is capable of both outcrossing and selfing (Chanon 2005). The relatively high degree of intra-population relatedness found by Lim et al. (2002) suggests that *A. glabra* populations consist of similar genotypes. Consequently the broad concept of plasticity also applies to phenotypic variation in *A. glabra*'s response to light gradients.

Since *A. glabra* initiates leaf expansion early in the spring, before the forest canopy develops, how does it produce leaves whose characteristics are related to the summer light environment? At least two mechanisms might lead to the observed correlation, namely proximate light cues during *A. glabra* leaf growth, prior to canopy expansion, and carryover effects from previous years.

Proximate effects of light during leaf expansion are well known. Jurik et al. (1979) found that the light environment during leaf expansion affects subsequent development of leaf structure and function. *Aesculus glabra* may respond to shading by the trunks and branches of still leafless neighbors.

Carryover effects from the previous season also are known in deciduous forest trees. Kimura et al. (1998) and Uemura et al. (2000) used shading experiments to show that some shoot and leaf characteristics in Japanese *Fagus* species are affected by light availability during the previous growing season. The *A. glabra* populations studied here were in stands that had not experienced significant changes in structure in the previous year, so leaf characteristics may represent the effect of the previous year's light microenvironment.

Augspurger & Reich (2008) showed that *A. glabra* leaf senescence occurred earlier in plants that were artificially shaded prior to canopy expansion. Effects were observed in the first year of shading, indicating importance of proximate cues. However, the effect was increased by several years of shading, consistent with a carryover effect.

Although *A. glabra* leafs out at a time when the light environment is rather uniform, it still possesses sufficient plasticity to respond appropriately to variation in the light environment at small spatial scales. The ability of *Aesculus glabra* to occupy a wide range of forest environments, from edges to gaps to understory in secondary forest in the current study, to the understory of older forests (Hicks & Michaelis 2009), is consistent with its well-developed plasticity. Currently, and in the near future, understory plants in temperate forests of northeastern North America face challenges from canopy opening due to the death of *Fraxinus* (Hoven et al. 2014). It is likely that *A. glabra* will be able to respond adaptively to gap formation and increased light; however, it is unknown whether this species has sufficient plasticity to outcompete shade-tolerant neighbors and shade-intolerant invaders in the race to the canopy.

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INFLUENCE OF LOW DENSITY GARLIC MUSTARD PRESENCE AND HARDWOOD LEAF LITTER COMPOSITION ON LITTER DWELLING ARTHROPOD DIVERSITY

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ABSTRACT. Garlic mustard (*Alliaria petiolata* (M. Bieb.) Cavara & Grande) is a non-native plant that commonly invades hardwood forest understory plant communities. Such invasions have the potential of restructuring forest communities and influencing community function. Litter dwelling arthropods were collected from areas with and without garlic mustard, and were identified to family. Forest characteristics, including canopy cover, forest basal area, litter depth, and soil moisture, were also measured. Plot locations with and without garlic mustard did not differ in the forest characteristics. However, arthropod richness was significantly reduced in areas with garlic mustard compared to areas without. Arthropod richness and diversity were positively related to leaf litter species diversity. In nonmetric multidimensional scaling ordination, mature garlic mustard density influenced a few arthropod taxonomic groups. However, it is likely that forest characteristics that facilitate the intensity of garlic mustard colonization (i.e., canopy cover, moisture) may be part of that influence. Additionally, leaf litter species richness provided a strong relationship with the majority of taxonomic groups. While garlic mustard presence may have a minor influence on the litter dwelling arthropod community, leaf litter richness and diversity play a major role in defining the arthropod community diversity and individual taxonomic group abundances. Management to control garlic mustard in forests may have little impact on leaf litter dwelling arthropods, especially if the litter layer remains intact.

Keywords: *Alliaria petiolata*, diversity, garlic mustard, litter dwelling arthropods, Tullgren-Berlese trap

INTRODUCTION

Addition of non-native plants to forest understory communities not only alters the composition of the community, but also alters community function (Gordon 1998; Maskell et al. 2006). Environmental characteristics (e.g., pH, fertility, light, moisture) are often different between common habitats with and without non-native plant species (Maskell et al. 2006). Much of the time, alterations to a community structure or function are subtle without fully reconstructing the native plant community (Mandryk & Wein 2006). In addition to the plant community, changes to arthropod communities by adding an exotic species may be variable, but may provide further insight into the importance of such plant additions (Marshall & Buckley 2009; Simao et al. 2010).

Garlic mustard (*Alliaria petiolata* (M. Bieb.) Cavara & Grande [Brassicaceae]) is a Eurasian plant species introduced to North America prior to 1868 (Nuzzo 1993). As a monocarpic, obligate biennial, garlic mustard seeds germinate in early spring and plants subsequently overwinter as leaf rosettes (Cavers et al. 1979). An erect stem is produced during the following spring, flowering in late spring, and seeds are dispersed during mid- to late-summer (Cavers et al. 1979; Anderson et al. 1996). Garlic mustard success in forest understories likely stems from its ability to grow under a wide range of light conditions and to further acclimate to the current condition in which it is growing (Cavers et al. 1979, Anderson & Dhillion 1991). Additionally, self-pollination ensures a single individual within a forest can easily begin the establishment of a population (Anderson et al. 1996). Self-pollination serves non-native forest invaders by allowing them to colonize rapidly as a result of micro-site disturbances, as seen in other forest understory species (Oswalt & Oswalt 2007; Marshall & Buckley 2008).

Major management concerns regarding garlic mustard invasion center on the restructuring of understory plant communities. Numerous studies

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have investigated the environmental and plant community changes that occur following garlic mustard invasion and management activities to remove the species (e.g., McCarthy 1997; Hochstedler et al. 2007; Stinson et al. 2007; Rodgers et al. 2008). One possible mechanism for altering plant communities is the potential ability for garlic mustard to out-compete some neighboring species (Meekins & McCarthy 1999). A second possible mechanism may be related to the reduction in arbuscular and ecto-mycorrhizal fungi, which would influence competitive abilities (Roberts & Anderson 2001; Stinson et al. 2006; Wolfe et al. 2008). However, Lankau (2011) found recovery and potential resistance of soil microbial communities in response to garlic mustard invasion. Overall management of garlic mustard may be as simple as reformed and proper deer management, demonstrated as a complex interaction of deer overabundance facilitating garlic mustard success (Kalisz et al. 2014).

Arthropod communities respond to nonnative plant colonization with alteration to trophic and physical structure (Marshall & Buckley 2009; deHart & Strand 2012). deHart & Strand (2012) found shifts in predator feeding behavior, likely due to shifts in prey sources due to garlic mustard invasion. However, Dávalos & Blossey (2004) found no change in predatory ground beetle richness or abundance with colonization by garlic mustard. While certain sites may exhibit decreases in arthropod abundances due to garlic mustard invasion, other sites exhibit no relationship (Dávalos & Blossey 2004). In contrast, springtail abundance does correlate positively with garlic mustard invasion (Alerding & Hunter 2013).

Leaf litter absence or disturbance typically has a direct impact on arthropod communities in forests (Sayer 2005). With decreased litterfall, abundances in arthropods also decrease (David et al. 1991). This is likely related to leaf litter providing buffers against temperature and moisture changes, as well as food sources (both the litter itself and prey) (David et al. 1991). Additionally, the physical structure influences predator-prey interactions, increasing numbers of already abundant predators and increasing numbers of most prey (Bultman & Uetz 1984). Abundances of soil and litter arthropods are greater in older leaf litter comprised of several tree species compared to single species litter or younger leaf litter of several species (Kaneko & Salamanca 1999; Hansen 2000).

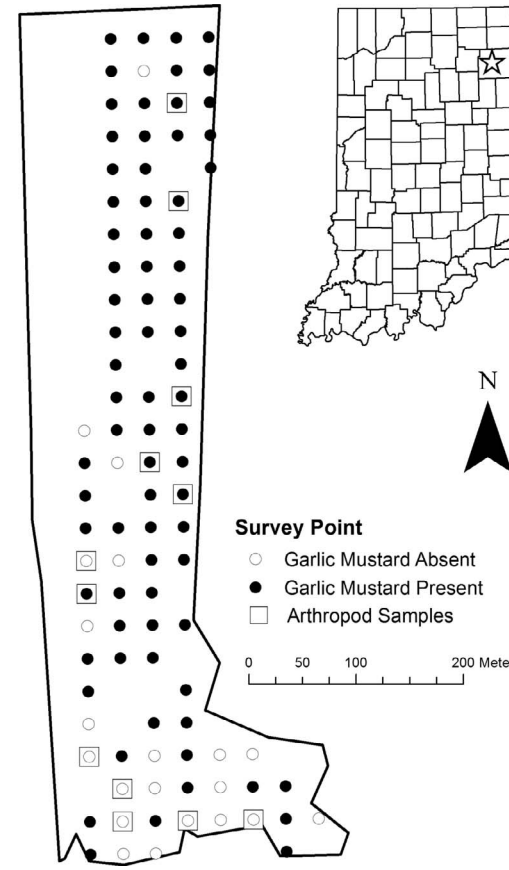

Figure 1.—Forest location (star) in Indiana and survey plot locations within forest.

We were interested in investigating arthropod community responses to established low density garlic mustard populations, as well as other forest characteristics. The objectives of this study were to quantify differences in leaf litter arthropod community abundance and diversity in areas with and without garlic mustard, and to test relationships between arthropod community diversity and leaf litter composition.

METHODS

A systematic grid of points (30 m spacing) was used to locate areas within a mature, second growth hardwood forest (41° 7' 20" N, 85° 8' 14" W; 13.7 ha; Fig. 1) in Fort Wayne, Allen County, Indiana, with and without garlic mustard. The overstory of this forest is dominated by *Acer saccharum*, *Tilia americana*, *Ulmus americana*, and *U. rubra*, with *Prunus serotina, Quercus rubra,* and *Q. velutina* being common

(Arvola et al. 2014). Across all plant strata, this forest is less diverse than others in the region and the understory is less dense overall (Arvola et al. 2014). The forest occurs in the Auburn Morainal Complex physiographic division and dominated by Blount-Morley silt loam soils (Franzmeier et al. 2004). At each grid point, all garlic mustard individuals within a 5 m radius circular plot were counted as mature (flowering/producing seed) or immature (leaf rosette) during July 2012. At each point, forest characteristics of percent canopy cover was measured with a concave spherical densiometer using standard techniques (Lemmon 1956); forest basal area was measured with a 10-factor prism using standard techniques (Avery & Burkhart 2002); litter depth was measured to the nearest 0.5 cm; and percent volumetric soil moisture was measured with a 12 cm Field Scout TDR probe (Spectrum Technologies, Plainfield, IL). The relationship between leaf litter moisture and soil moisture is variable and highly dependent on litter age (Nelson 2001).

Six points each with and without garlic mustard were randomly selected from the grid survey. Leaf litter was collected (down to mineral soil surface) within 1 m^2 quadrats centered at the selected grid points during September 2012. Arthropods were sorted from the litter samples using Tullgren-Berlese funnel traps (Southwood & Henderson 2000) with a 25 watt incandescent bulb as the light and heat source, stored in 70 percent ethanol at 0 °C until identified, and identified to the finest taxonomic level (typically family) using Triplehorn & Johnson (2005). Taxonomic nomenclature followed ITIS (2015). After arthropods were sorted from litter (approximately 72 hours), all intact leaves were identified to species using Barnes & Wagner (2004) and Jackson (2004), and counted. Arthropod family richness (count) and diversity (Shannon index) were calculated for each plot, as well as leaf species richness and diversity.

Arthropod and leaf richness and diversity, as well as forest measures, were compared between areas with and without garlic mustard using a Student's t-test. Relationships between leaf richness and diversity, and arthropod richness and diversity were identified using simple linear regression. Nonmetric multidimensional scaling (NMDS) ordination was used to compare arthropod taxonomic group abundance dissimilarities and relate those to environmental factors (R^2 cuttoff = 0.2). Statistical analysis was conducted using base package of R (version 3.1.1, The R Foundation for Statistical Computing) and vegan package for ordination (metaMDS function), vector correlations (envfit function), and species accumulation curves with rarefaction method (specaccum function) (version 2.2-1, Oksanen et al. 2015).

RESULTS

A total of 98 grid points were surveyed, with 77 of those having garlic mustard present (Fig. 1). The six randomly selected points with garlic mustard had a mean of 45.6 (SD $\pm$ 30.8) mature and 10.2 (SD $\pm$ 5.7) immature individuals, which did not differ from the other grid points with garlic mustard ($t = 0.21$, $df = 75$, $p = 0.838$; $t = 0.07$, $df = 75$, $p = 0.944$; respectively). Additionally, forest characteristics of canopy cover, basal area, litter depth, and soil moisture, did not differ between plots with garlic mustard selected for arthropod sampling and those not selected ($t = -0.43$, $df = 75$, $p = 0.668$; $t = -0.30$, $df = 75$, $p = 0.766$; $t = -0.94$, $df = 75$, $p = 0.352$; $t = -0.58$, $df = 75$, $p = 0.561$; respectively). Similarly, these four environmental variables did not differ between plots without garlic mustard selected for arthropod sampling and those not selected ($t = -0.74$, $df = 19$, $p = 0.470$; $t = -0.437$, $df = 19$, $p = 0.667$; $t = 1.22$, $df = 21$, $p = 0.236$; $t = 0.217$, $df = 19$, $p = 0.831$; respectively). Species accumulation curves for both arthropods and leaf litter both exhibited negative exponential functions (Fig. 2).

Further analysis includes only plots selected for arthropod sampling. Forest structure (canopy cover and basal area) and soil moisture did not significantly differ between plots with and without garlic mustard (Table 1). However, litter depth was significantly greater in plots with garlic mustard present (Table 1). Of the diversity measures, only arthropod richness was significantly different between plots with and without garlic mustard (Table 1). Additionally, arthropod abundance (count of individuals) was not significantly different between plots with and without garlic mustard ($t_{(2),10} = 1.17$, $p = 0.269$; Table 2). Similarly, leaf abundance was not significantly different between plots with and without garlic mustard ($t_{(2),10} = 0.59$, $p = 0.569$; Table 3). We pooled the plots for simple linear regression analysis. Leaf species richness had no significant influence on arthropod family richness or diversity (Fig. 3 A, B). However,

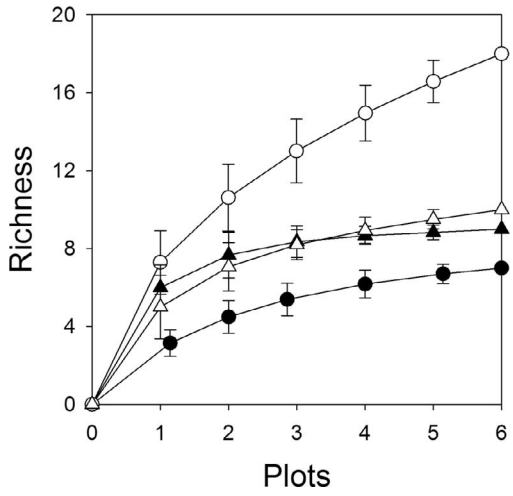

Figure 2.—Species accumulation curves for arthropods and leaf litter sampling (with standard deviation). Circles represent arthropods; triangles represent leaf litter. Open symbols indicate garlic mustard absence; closed symbols indicate garlic mustard presence.

arthropod richness and diversity were positively related with leaf diversity (Fig. 3 C, D). Arthropod abundance was not significantly related to leaf litter richness or diversity.

NMDS ordination of arthropod taxonomic group abundances resulted in a stress of 0.105 with three dimensions using an alternative Gower dissimilarity, which includes weights to exclude double zeros (i.e., joint absences between taxonomic groups; Anderson et al. 2006). Additionally, joint vectors displayed over the NMDS plot provide a visual representation of the influence environmental factors have on arthropod taxonomic groups (Fig. 4). Presences of vectors represent significant correlations, while direction and length of the vectors represent direction of influence and intensity of relationships, respectively. Armadillidiidae (woodlice) and Chrysomelidae (leaf beetles) were positively influenced by mature garlic mustard densities, while Araneidae (spiders) and Passandridae (flat bark beetles) were negatively influenced. However, leaf litter species richness had a strong influence on most of the arthropod taxonomic groups, positive for some and negative for others (Fig. 4). Using tree species as the environmental factor for joint vectors, none of the tree species were correlated with the arthropod taxa and failed to meet the cutoff to be included in the figure.

DISCUSSION

Garlic mustard is a common understory invader of hardwood forests and has been the focus of extensive management (Stinson et al. 2007). Our study investigated the relationships between garlic mustard presence and leaf litter dwelling arthropods. While garlic mustard presence did result in limited decreased arthropod richness, leaf litter diversity had a clear, strong influence on the richness and diversity of arthropods. Additionally, leaf litter richness had a direct impact on the abundance of most of the arthropod taxonomic groups. Our interpretation of these results is that garlic mustard had a minor role in determining the litter dwelling arthropod community, while the litter layer "community" structure (i.e., diversity, richness) had a major role in defining the arthropod community. Likely the role of garlic mustard in this forest is less important because of the density in our sample locations (55.8 total individuals per plot = 0.7 individuals per m^2). Nuzzo (1999) demonstrated that garlic mustard becomes a fixture in forest communities and varies in density and cover annually. However, when disturbances occur in forests, garlic mustard quickly increases in number (Nuzzo 1999). Our study site is likely in the sustaining population stage of garlic mustard occurrence and the low density nature of this plant in the forest understory may

Table 1.—Comparisons of mean canopy cover, basal area, litter depth, soil moisture, arthropod richness and diversity, and leaf richness and diversity between plots with and without garlic mustard (standard error). Asterisk (*) indicates significant one-tailed t-test.

Garlic mustard	Canopy cover (%)	Basal area (m^2/ha)	Litter depth (cm)	Soil moisture (%)	Arthropod		Leaf litter	
					Richness	Diversity	Richness	Diversity
Present	88.5 (1.9)	29.1 (2.5)	4.2 (0.4)	27.3 (6.6)	2.3 (0.4)	0.71 (0.17)	6.0 (0.3)	0.47 (0.15)
Absent	89.4 (1.3)	31.8 (3.2)	2.0 (0.8)	27.2 (6.4)	5.0 (1.1)	1.06 (0.28)	5.0 (0.7)	0.92 (0.23)
$t_{(2),10}$	0.38	0.67	−2.38	−0.02	2.27	1.06	−1.29	1.64
p-value	0.712	0.521	0.019*	0.987	0.046*	0.312	0.226	0.135

Table 2.—Arthropod taxa total abundances with frequency in parentheses (number of plots) in areas with garlic mustard present and absent.

Class	Order	Family	Present	Absent
Arachnida	Araneae	Araneidae	3 (2)	4 (2)
	Oribatida		0	4 (2)
	Pseudoscropiones		0	9 (5)
Chilopoda			0	2 (2)
Diplopoda			0	1
Insecta	Coleoptera	Apionidae	0	2 (1)
		Chrysomelidae	4 (4)	7 (4)
		Curculionidae	0	1
		Nitidulidae	0	2 (1)
		Passandridae	2 (1)	0
		Staphylinidae	1	1
	Hemiptera	Aphididae	1	0
		Aradidae	0	1
		Lygaeidae	0	1
		Miridae	3 (1)	0
		Nabidae	0	1
	Hymenoptera	Formicidae	0	348 (2)
		Ichneumonidae	0	1
	Psocodoea	Trogiidae	0	5 (1)
Malacostraca	Isopoda	Armadillidiidae	7 (4)	19 (3)
		Total	21 (6)	410 (6)

have minimal influence on the overall arthropod community.

Our plots randomly selected for arthropod sampling were not different in forest structure compared to the population of plots with and without garlic mustard. The only differing forest characteristic was litter depth (greater in garlic mustard plots). This result is similar to Bartuszevige et al. (2007), who found garlic mustard seedling survival was greater in undisturbed litter and also had a clear inverse relationship with reductions in litter depth. Variability in forest structure (i.e., our measures of canopy cover, basal area, litter depth) could influence arthropod abundances and diversity (Jeffries et al. 2006). However, with those forest structure characteristics remaining relatively similar between treatments, we interpret changes in arthropod diversity, richness, and taxonomic group abundances to be influenced more so by the leaf litter layer composition and less by the presence and absence of garlic mustard.

Table 3.—Leaf litter taxa total abundances with frequency in parentheses (number of plots) in areas with garlic mustard present and absent.

Family	Species	Present	Absent
Betulaceae	*Ostrya virginiana*	15 (3)	35 (5)
Fagaceae	*Fagus grandifolia*	24 (5)	45 (4)
	Quercus alba	21 (3)	8 (4)
	Quercus palustris	76 (6)	112 (6)
	Quercus velutina	18 (5)	16 (3)
Lauraceae	*Sassafras albidum*	1	0
Magnoliaceae	*Liriodendron tulipifera*	0	5 (1)
Rosaceae	*Prunus serotina*	0	2 (1)
Salicaceae	*Populus deltoides*	9 (3)	2 (2)
Sapindaceae	*Acer rubrum*	8 (4)	11 (3)
	Acer saccharum	19 (6)	1
	Total	191 (6)	237 (6)

There was a significant difference in arthropod richness between areas with and without garlic mustard, with garlic mustard presence reducing richness. However, since this difference did not extend to arthropod diversity, we argue that garlic mustard is then a minor influence on the arthropod community. Because richness and Shannon's diversity index are positively correlated (Stirling & Wilsey 2001), we would expect to see the influence of garlic mustard in both arthropod richness and diversity if it were a strong or major influence. We did find this strong or major influence on arthropod richness and diversity with leaf litter diversity. Increases in leaf litter diversity significantly increased arthropod community richness and diversity. While not compared statistically, it would be difficult to argue the leaf litter richness and

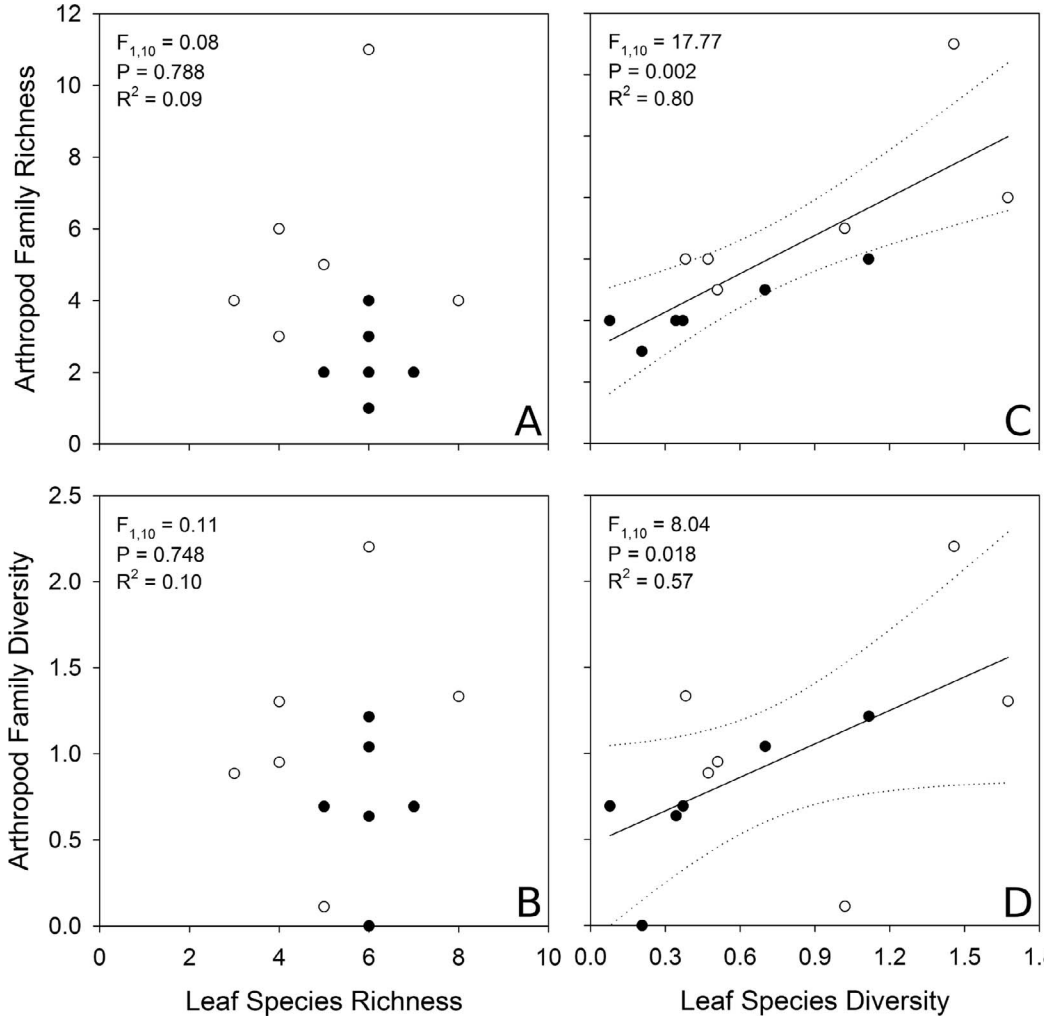

Figure 3.—Simple linear regression relationships between arthropod richness (A, C) and diversity (B, D) and leaf litter richness (A, B) and diversity (C, D) for pooled plots with (closed circles) and without (open circles) garlic mustard. Dotted lines indicate 95% confidence intervals.

diversity (Table 1) differ from overstory richness and diversity, as the mean overstory richness and diversity for this forest has been reported as 4.7 and 0.99, respectively (Arvola et al. 2014). With this similarity in the litter and overstory, garlic mustard likely had no influence on the overstory trees producing litter. In terms of composition, leaf litter in plots with garlic mustard were 37% similar to the overstory composition, while plots without garlic mustard were 48% similar, using data presented in Arvola et al. (2014). Many of the overstory trees reported by Arvola et al. (2014) that differed from our leaf litter were single individuals and not widespread dominating species. While garlic mustard green rosettes may accelerate leaf litter decomposition (Rodgers et al. 2008), the density of garlic mustard in our study forest is likely too low to dramatically change decomposition rates.

Most arthropod groups were not heavily influenced by garlic mustard density, aligning with the vector origin in the NMDS plot. The strong positive influence by garlic mustard density on Armadillidiidae, potentially classified as alkaliphiles (van Straalen & Verhoef 1997), is likely related to an increase in soil and litter pH in garlic mustard colonized areas (Rodgers et al. 2008). Conversely, Araneidae and Passandridae may

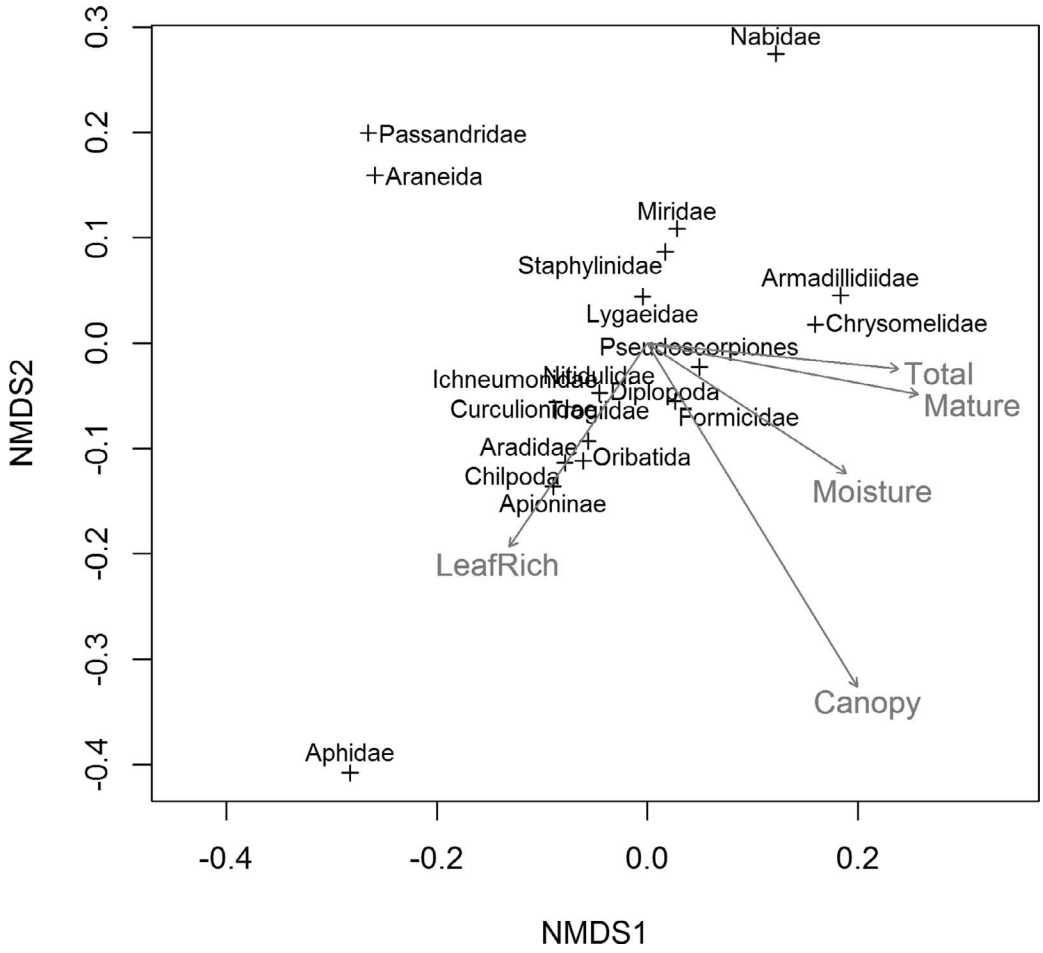

Figure 4.—Nonmetric multidimensional scaling (NMDS) ordination of arthropod taxonomic groups captured. Joint vector direction represents positive influence on groups and length represents intensity of influence. Vectors include percent canopy cover (Canopy), leaf litter species richness (LeafRich), percent volumetric soil moisture (Moisture), count of mature garlic mustard plants (Mature), and count of mature and immature garlic mustard plants (Total).

have been more influenced by canopy cover, soil moisture, or other spatial characteristics not measured in this study (e.g., course woody debris), as evidenced by the alignment and length of the vectors (Sanderson et al. 1995). Leaf litter species richness seems to have had the strongest influence on the most taxonomic groups. While leaf litter species diversity provided significant linear regression equations for arthropod diversity, litter richness influenced individual groups more than litter diversity. The alignment of taxonomic groups with the vector direction visually represents this relationship. Groups such as Aphididae (positive) and Nabidae (negative) had the most dramatic relationships with leaf litter richness. Because leaf litter encompasses a broad range of habitat characteristics (e.g., pH, temperature, moisture, nutrients, shelter), leaf litter species richness would influence many of those and result in arthropod community organization (Bultman & Uetz 1984; Burghouts et al. 1992). It should be noted that NMDS uses the dissimilarity calculations for the entire arthropod community sampled and correlations of forest characteristics in relation to the community. This leads to minor interpretation issues of the two presentations of data (Fig. 4, Table 2). However, for the entire community, leaf litter layer richness influences the taxonomic groups strongly, as evidenced by the direction and length of the vector.

Garlic mustard presence, albeit at low density, in a hardwood forest had minimal influence on litter dwelling arthropods. While arthropod richness was significantly reduced in areas with garlic mustard, abundance and diversity of captured arthropods were not different. Additionally, other forest characteristics, such as canopy cover and soil moisture, that facilitate garlic mustard colonization intensity, may have had more influence on arthropods. There can be positive and negative influence by canopy cover and soil moisture depending on the arthropod family (Greenberg & Forrest 2003). Finally, leaf litter species diversity and leaf litter species richness may have the most important roles in determining arthropod diversity and individual taxonomic group abundances, respectively. While management of garlic mustard may be important for other communities, the leaf litter dwelling arthropods may not be affected. Further research on density dependent impacts is necessary in order to define an acceptable density for management decisions. While low density garlic mustard may have limited impacts, those low density populations may have the greatest seed production per individual facilitating rapid population responses to disturbance (Nuzzo 1999; Pardini et al. 2009). Since litter dwelling arthropods may not be impacted in any great way by garlic mustard, recovery time of such communities following management and restoration may be minimal.

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LAMPREYS OF THE ST. JOSEPH RIVER DRAINAGE IN NORTHERN INDIANA, WITH AN EMPHASIS ON THE CHESTNUT LAMPREY (*ICHTHYOMYZON CASTANEUS*)

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ABSTRACT. This study was initiated in response to concern about parasitism by lampreys on trout in the Little Elkhart River of the St. Joseph River drainage in northern Indiana. Identification of 229 lampreys collected in the St. Joseph River drainage during 1998–2012 revealed 52 American brook lampreys (*Lethenteron appendix*), one northern brook lamprey (*Ichthyomyzon fossor*), 130 adult chestnut lampreys (*I. castaneus*), five possible adult silver lampreys (*I. unicuspis*), and 41 *Ichthyomyzon* ammocoetes. The brook lampreys are non-parasitic and do not feed as adults, so most if not all parasitism on fish in this system is due to chestnut lampreys. Electrofishing surveys in the Little Elkhart River in August 2013 indicated that attached chestnut lampreys and lamprey marks were most common on the larger fishes [trout (Salmonidae), suckers (Catostomidae), and carp (Cyprinidae)] at each of three sites. This is consistent with the known tendency for parasitic lampreys to select larger hosts. Trout in the Little Elkhart River may be more vulnerable to chestnut lamprey attacks because they are relatively large compared to alternative hosts such as suckers. Plots of chestnut lamprey total length versus date of capture revealed substantial variability on any given date. This may be due to variability among individual streams and individual years and may also result from variability among individual lampreys in when they initiate and terminate parasitic feeding.

Keywords: *Ichthyomyzon*, *Lethenteron*, Little Elkhart River, Petromyzontidae, Saint Joseph River

INTRODUCTION

The chestnut lamprey (*Ichthyomyzon castaneus*) is a parasitic species that occurs throughout much of the central United States. Although Cochran (2014) recently compared its biology in the St. Croix River drainage of northwestern Wisconsin with that in tributaries to Lake Michigan in western Michigan, relatively little information is available on its distribution and life history in Indiana. This study was initiated in response to concern about parasitism by lampreys on trout in the Little Elkhart River of the St. Joseph River drainage in northern Indiana. In addition to information on the chestnut lamprey, information on the occurrence and distribution of other lamprey species in the drainage was obtained.

METHODS

Preserved lampreys analyzed during this study were collected during the period 1998–2012 by the City of Elkhart Public Works and Utilities during routine fish surveys in the Saint Joseph River mainstem and its tributaries (Fig. 1). For each specimen total preserved length, as well as whether or not the lamprey was in the ammocoetes or adult (i.e., transformed) stage was recorded. The presence or absence of a divided dorsal fin was used to distinguish between *Lethenteron* and *Ichthyomyzon*. Trunk myomeres were counted from the myomere partly containing the most posterior gill opening to the last myomere with posterior angle positioned above the cloaca (Hubbs & Trautman 1937). After myomere counts for all specimens were completed, they were repeated without reference to the original counts. If a lamprey was an adult *Ichthyomyzon*, bicuspid circumoral teeth were counted (Table 1).

New field data on lampreys and potential hosts in the Little Elkhart River were collected with a stream electrofisher on 17 August 2013. Three reaches were sampled, each for approximately 300 m in an upstream direction. Each lamprey captured was anesthetized, processed as described previously for the preserved

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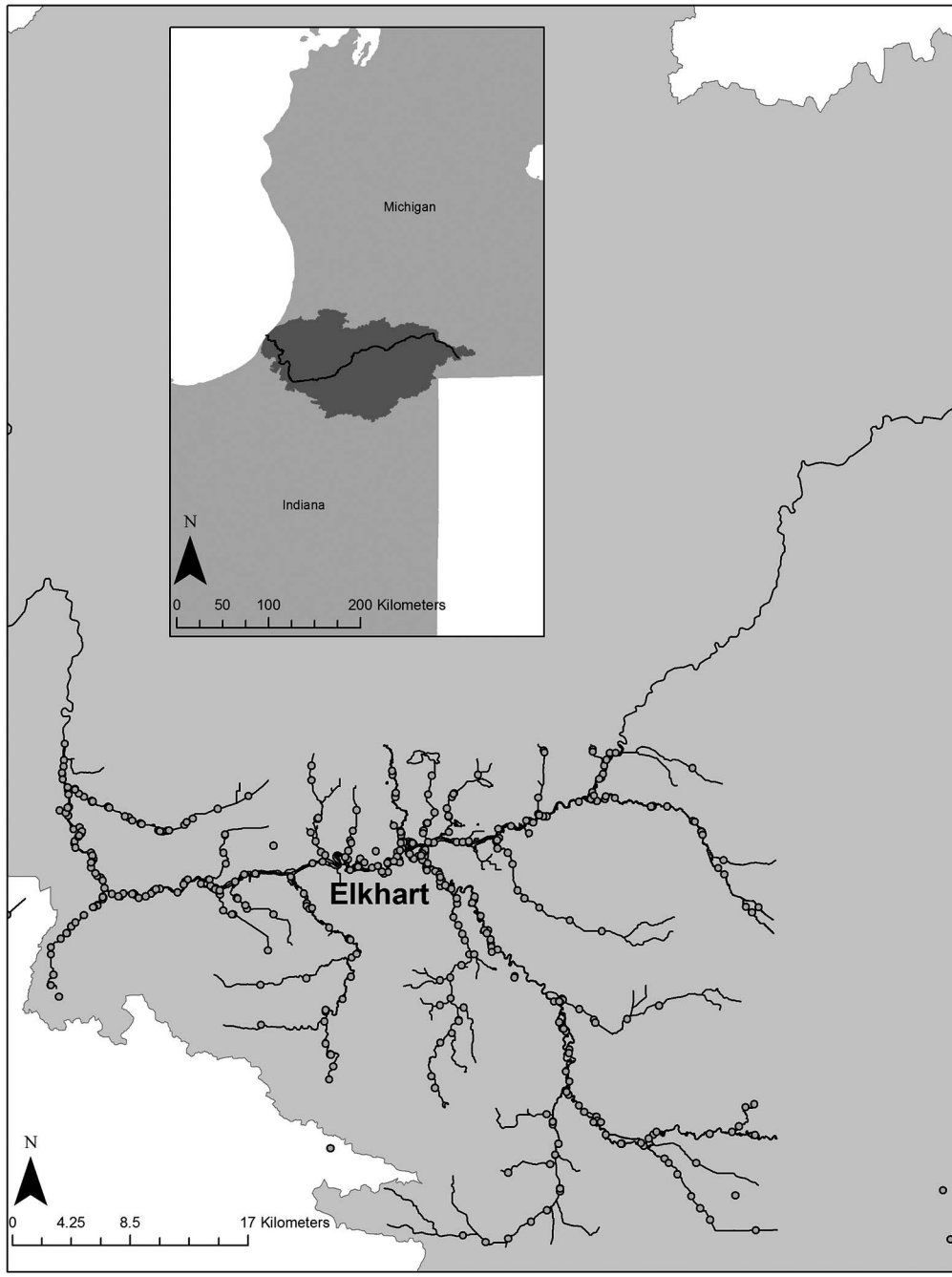

Figure 1.—The St. Joseph River drainage in Michigan and Indiana (inset), including sites in the St. Joseph River drainage where lampreys were collected by the City of Elkhart Public Works and Utilities during the period 1998–2012.

lampreys, and released at the site of its capture. All potential hosts were identified to species, measured for total length, and examined for lamprey marks.

RESULTS AND DISCUSSION

Lamprey fauna of the Saint Joseph River drainage.—The lamprey fauna of the St. Joseph River drainage is diverse. The 229 lampreys

Table 1.—Characters and counts used to distinguish lamprey species.

Species	Trunk myomeres	Circumoral teeth	Dorsal fin	Lateral line pores
American Brook Lamprey	64-75	Unicuspid	Separate	None
Northern Brook Lamprey	47-56	Unicuspid	Connected	None
Silver Lamprey	47-55	Unicuspid	Connected	Black
Chestnut Lamprey	49-56	Bicuspid	Connected	Black

collected during 1998–2012 included 52 American brook lampreys (*Lethenteron appendix*), one adult northern brook lamprey (*Ichthyomyzon fossor*), 130 adult chestnut lampreys (*I. castaneus*), five putative adult silver lampreys (*I. unicuspis*), and 41 *Ichthyomyzon* ammocoetes (Table 2).

Lethenteron appendix: The American brook lamprey, a non-parasitic species, is considered to be common in Indiana and occurs in both the northern and southern portions of the state (Simon 2011). Simon (2011) did not include the St. Joseph River drainage within its Indiana distribution, but it was reported from this system by Marenchin & Sever (1981) while Wesley & Duffy (1999) implied that it was widely distributed. All American brook lampreys identified during this study were ammocoetes. No adults would have been present during the time of year collections were made since adult American brook lampreys spawn and die during the spring (Cochran et al. 2012).

Ichthyomyzon fossor: The single northern brook lamprey was collected in Christiana Creek. The northern brook lamprey is a non-parasitic species considered by Simon (2011) to be rare to occasional in occurrence in Indiana. He did not include the St. Joseph River drainage within its Indiana range. Earlier reports (Marenchin & Sever 1981, and references therein), however, suggested that ammocoetes collected in Christiana Creek and Turkey Creek might be northern brook lampreys. In addition Wesley & Duffy (1999) depicted a distribution that included several tributaries to the St. Joseph River.

Ichthyomyzon unicuspis: A few parasitic-phase *Ichthyomyzon* were provisionally identified as silver lampreys rather than chestnut lampreys because they lacked bicuspid circumoral teeth. Simon (2011) considered the silver lamprey to be occasional to common in occurrence in Indiana but did not include the St. Joseph River drainage as part of its Indiana range. Although earlier workers considered silver lampreys to be absent from the eastern shoreline of Lake Michigan (Hubbs & Trautman 1937; Morman 1979), Schuldt et al. (1987) enumerated small numbers of specimens in the Muskegon and St. Joseph Rivers. These specimens would have been collected downstream from any barrier dams and may have completed their parasitic phase in Lake Michigan. By contrast,

Table 2.—Lampreys collected and preserved by biologists of the City of Elkhart Public Works and Utilities during 1998–2012.

Stream	American brook lamprey	Northern brook lamprey	Silver lamprey	Adult chestnut lamprey	*Ichthyomyzon* ammocoetes
Saint Joseph River	6	-	3	59	9
Elkhart River	19	-	1	34	6
Little Elkhart River	6	-	-	13	7
Pine Creek	4	-	-	2	-
Yellow Creek	-	-	1	8	-
Christiana Creek	-	1	-	7	15
Cobus Creek	7	-	-	1	-
Puterbaugh Creek	8	-	-	-	-
Rock Run Creek	1	-	-	2	-
Rowe Eden	-	-	-	-	1
Turkey Creek	-	-	-	-	3
Trout Creek	1	-	-	2	-
Baugo Creek	-	-	-	2	-

Wesley & Duffy (1999) mapped two apparently disjunct populations upstream from barrier dams in the St. Joseph River drainage, one in the vicinity of Elkhart in Indiana waters (the source area for the specimens examined during the current study) and one upstream from Union City in Michigan.

Ichthyomyzon castaneus: The chestnut lamprey was the most abundant lamprey identified during this study. It was first reported from the St. Joseph River drainage in Indiana by Sever & Mould (1982). Simon (2011) considered this parasitic species to be common in Indiana and included the St. Joseph River drainage within its mapped distribution. Wesley & Duffy (1999) mapped a broad distribution within the drainage. Nevertheless, the specimens we examined extend this distribution to include Christiana Creek, Cobus Creek, and the Little Elkhart River from Middlebury downstream to the dam at Bonneyville Mills.

Taxonomic questions: Trunk myomere counts have been used to distinguish species of lampreys (Table 1). However, counts were more variable than expected. The range in counts for all adult *Ichthyomyzon* was 46–61, with modes at 49 and 53. The count for the adult *I. fossor* was 53, and the counts for the putative *I. unicuspis* were 46, 48, 53, 55, and 56 (mean = 51.6). Hubbs & Trautman (1937) reported ranges of 49–56 (mean = 52.6) for *I. castaneus*, 47–55 (mean = 50.5) for *I. unicuspis*, and 47–56 (mean = 50.9) for *I. fossor* (data summarized by Docker 2009). We hypothesize that hybridization among *Ichthyomyzon* species has occurred in this system, resulting in some lampreys that combine the presence of bicuspid circumoral teeth with relatively low trunk myomere counts; this possibility may be enhanced when one species outnumbers the others by a great margin. In addition, we suggest that the gene pool of *Ichthyomyzon castaneus* in the St. Joseph River drainage may have incorporated alleles from the Ohio lamprey (*I. bdellium*), a parasitic species with higher myomere counts (range: 53–62, mean = 56.9; Hubbs & Trautman 1937). Ohio lampreys occur in the Tippecanoe River drainage (Ohio River basin), which borders the St. Joseph River drainage to the south (Simon 2011), and they may have used minor postglacial connections to pass between the two drainages (Gerking 1947; Burr & Page 1986).

An additional question concerns whether parasitic species and their non-parasitic derivatives are in fact distinct species (Docker 2009). The non-parasitic northern brook lamprey is considered to be the derivative of the parasitic silver lamprey and conventionally treated as a separate species. However, Docker et al. (2012) found evidence for gene flow between the two forms and, based upon limited genetic analysis, suggested that they may represent ecotypes of a single species. If northern brook and silver lampreys are indeed one species that might explain the occasional collection of silver lampreys in the St. Joseph River drainage above barrier dams that would prevent upstream dispersal from Lake Michigan.

Biology of the chestnut lamprey.—Electrofishing surveys in the Little Elkhart River in August 2013 indicated that attached chestnut lampreys and lamprey marks were common on the larger fishes at each of three sites, including trout (Salmonidae), suckers (Catostomidae), and carp (Cyprinidae). At the most upstream site, Riverbend Park in Middlebury, where trout are stocked in a catch-and-release program, lamprey marks were observed on brown trout and white suckers (Fig. 2), and we received anecdotal accounts of rainbow trout attacked within 24 hours of being stocked (J. Phillips, pers. comm.). At the intermediate site, the County Road 35 crossing, one chestnut lamprey (188 mm, 11.4 g) attached to a brown trout was collected (Fig. 3). The most downstream site was the impounded area above the dam at Bonneyville County Park, where we collected a chestnut lamprey (201 mm, 16.0 g) on a common carp and observed lamprey marks on another carp and possibly a white sucker (Fig. 4). Chestnut lamprey attachments or marks tended to be on the largest hosts at each site, a pattern consistent with other parasitic lampreys (Cochran 1985; Swink 1991). In addition to chestnut lamprey, *Ichthyomyzon* ammocoetes were observed at all three sites, and one larval *L. appendix* was collected at the County Road 35 crossing.

A plot of chestnut lamprey total length versus date of capture revealed substantial variability on any given date (Fig. 5). A similar plot was obtained by Cochran (2014) for chestnut lampreys from the St. Croix River drainage in Wisconsin. This variability may be due in part to differences among individual streams and individual years, but it may also result from variability among individual lampreys in when they initiate and terminate parasitic feeding

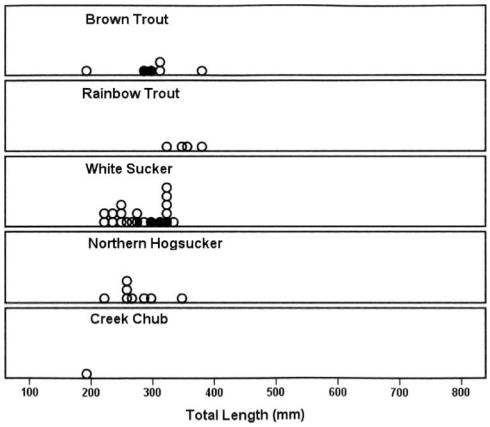

Figure 2.—Total length of fish sampled in the Little Elkhart River at Riverbend Park. Individuals bearing marks caused by lampreys are indicated by solid circles.

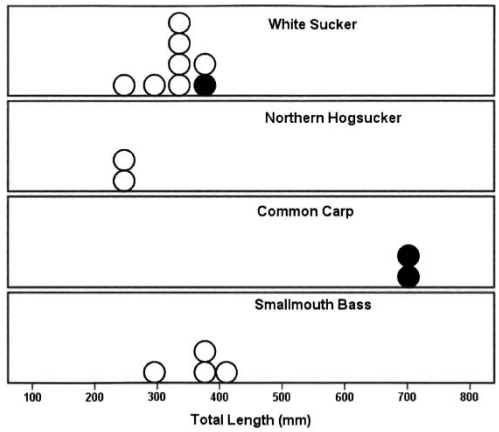

Figure 4.—Total length of fish sampled in the Little Elkhart River at Bonneyville Mills. Individuals bearing marks caused by lampreys are indicated by solid circles.

(Cochran 2014). It is possible that some newly transformed lampreys begin parasitic feeding in the fall, whereas other may begin feeding the following spring. Swink & Johnson (2014), however, found no difference in sizes achieved by sea lampreys (*Petromyzon marinus*) that migrated downstream to Lake Huron in the fall and those that migrated down in the spring, even though the former presumably fed parasitically for a longer period.

Cochran (2014) noted that much of what is known about the chestnut lamprey is based on data collected in two regions, the St. Croix River drainage of Wisconsin and Minnesota and the tributaries to Lake Michigan in western Michigan (especially the Manistee River). In the St. Croix River drainage, redhorse (*Moxostoma* spp.) are relatively abundant and may buffer the impact of chestnut lampreys on trout and other gamefish, whereas in the Manistee River, fewer large catostomids were available as alternatives during the time of annual plantings of thousands of catchable-sized trout. Our results for the Saint Joseph River drainage in Indiana are, as expected, more consistent with the Manistee River. Because trout in the Little Elkhart River are stocked at relatively large size in a system where catostomids are relatively small, they

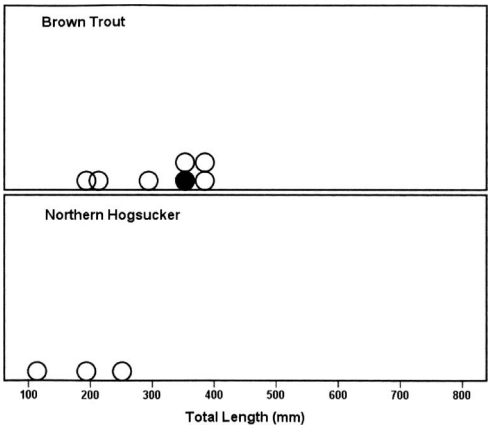

Figure 3.—Total length of fish sampled in the Little Elkhart River above the County Road 35 crossing. Individuals bearing marks caused by lampreys are indicated by solid circles.

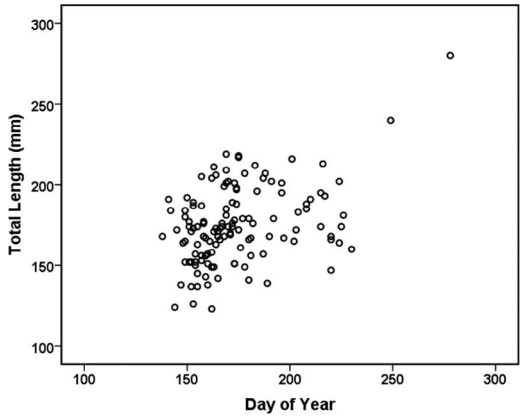

Figure 5.—Total length (mm) of preserved adult (i.e., parasitic-phase) chestnut lampreys from the St. Joseph River drainage versus date of capture (day of year).

may be more vulnerable to lamprey attack (Cochran 2014).

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INFLUENCE OF SCENT AND SEASON ON SHERMAN LIVE TRAP CAPTURES OF *PEROMYSCUS*

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ABSTRACT. Identifying the ideal method to capture small mammals, and the influence of seasonal (monthly) variation on capture rates, is important for maximizing efficiency and time. This study tested the prediction that *Peromyscus leucopus* scent collected in the lab and placed in cleaned (experimental) traps would attract conspecifics with similar or higher frequency than regular clean traps or dirty traps containing the residual scent of previously captured conspecifics. There was no significant difference in capture rates of *P. leucopus* among clean, dirty, or experimental traps. However, dirty traps did have increased sexual bias, with a greater frequency of male captures. Additionally, July had higher capture rates of female *P. leucopus* than September and June, whereas males showed no significant seasonal variation. These findings document the potential influences and results of trap type and season on small mammal capture rates, and provide valuable considerations and recommendations for management practices and future studies using scented, live-capture traps.

Keywords: disease mitigation, live traps, mouse, *Peromyscus*, sexual bias, temporal, trapping

INTRODUCTION

Live traps are one of the most common methods for acquiring live, free-ranging small mammals. Techniques for maximizing small mammal captures have been highly desired in the fields of wildlife ecology and management (Gaulin & FitzGerald 1988; Slade et al. 1993; Whittaker et al. 1998; Anthony et al. 2005). To accomplish this, factors that influence capture rates, such as residual scent or season, must be identified.

Dirty traps are thought to be the preferred method for producing the largest yield in terms of small mammal captures (Boonstra & Krebs 1976; Heske 1987; Gurnell & Little 1992). It is thought that the residual scent of previously captured conspecifics entices others to investigate (Boonstra & Krebs 1976), though the exact reason for this (territorial defense, mate acquisition, curiosity) is unknown. However, the use of dirty traps has often been associated with sexually skewed capture rates (Whittaker et al. 1998; Wolf & Batzli 2002). Such biases typically involve higher male capture rates (Wolf & Batzli 2002), yet the cause of this bias remains undocumented. Territoriality of males offers one potential explanation, as males would enter the trap to defend their territory from the perceived threat of an unknown conspecific. However, female *Peromyscus* have been known to be equally, if not more, territorial than males (Metzger 1971; Korytko & Vessey 1991). Another explanation is that males are more active in their pursuit of reproductive females, and that the residual scent of females in dirty traps attracts males. While these mechanisms provide possible explanations for the prevalence of sexual biases in the literature, more empirical data are needed to understand sexually biased capture results in small mammals. Understanding and potential mitigation of this problem would be of great benefit to future studies requiring the acquisition of live, free-ranging conspecifics.

Disease transmission is another problem associated with the use of dirty traps since *Peromyscus* are known vectors for Hantavirus (Nichol et al. 1993; Mills et al. 1995). Nichol et al. (1993) documented a direct link between infection in humans and exposure to rodent excreta, particularly that of Deer Mice (*Peromyscus maniculatus*). Because dirty traps have excreta of previously captured mice, handling these traps may increase risk of human infection. Dirty traps also expose mice among and within populations to potential infections, as trappers move from one site to another. As

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such, the use of dirty traps previously occupied by infected individuals could facilitate the spread of diseases to new populations, increasing population mortality (Burthe et al. 2008), and the potential transmission to additional species (Kallio et al. 2006).

Our objective was to quantify those factors that influenced capture rates of *Peromyscus* spp. We wanted to determine if artificially scented traps (experimental traps) could be substituted for dirty traps, without a significant decrease in capture rates. We tested the following predictions: 1) experimental traps would not differ from dirty traps in capture rates, 2) experimental traps would cause less sexual bias than dirty traps, and 3) that the capture rates of *Peromyscus* spp. will vary seasonally.

METHODS

Trapping took place between 24 June 2010 and 31 October 2010 at study sites located in Muncie and Fort Wayne, IN and Miller City, IL. The Fort Wayne and Miller City study locations consisted of one site each. In Muncie there were three separate sites: Cooper Farm, Miller Wildlife Area, and Christy Woods. The study sites all consisted of stands of upland hardwood forest comprised primarily of red oak (*Quercus rubra* L.) and sugar maple (*Acer saccharum* Marshall), with relatively flat topography.

Although we could not definitively determine which species of *Peromyscus* (*P. leucopus* or *P. maniculatus*) were captured, based on the type of habitat where the trapping took place (upland hardwood forest), we believe that all individuals captured were *P. leucopus* (Whitaker & Mumford 2009), and will henceforth refer to them as such. Three adult *P. leucopus*, two males and one female, were captured and housed in individual cages lined with shredded paper towels for two weeks to collect scent. Mice were provided with pet mouse food (Extruded Global Rodent Diet, Harlan/Teklab Global) and water *ad lib*. Following scent collection, captive mice were released at their original site of capture. *Peromyscus* scent consisted of urine, feces, and other bodily odors that were absorbed into the paper towel pieces while the mice were caged. The shredded paper towels from male and female cages were placed in a sealed plastic bag, mixed, and laid in the sun for a few hours to volatilize scent chemicals to ensure an even mixture of all mice scent. Additionally, exposure to ultraviolet light reduces the vitality of infectious diseases such as Hantavirus (Prescott et al. 2005). The scented pieces were placed behind clean cotton in the back of the "experimental" Sherman traps ($22.9 \times 7.6 \times 8.9$ cm; H.B. Sherman Traps, Tallahassee, FL).

At each site, transects of 25 groups of three traps (one clean, one dirty, and one experimental) were established for each trap session, lasting three days (two nights; $n = 150$ trap nights). Trap groups were placed at least 1 m apart wherever *P. leucopus* and other small mammals were likely to be found, such as areas of high course woody debris or along fallen trees (Lee 2004; Whitaker & Mumford 2009). At each trap group, three non-folding Sherman traps were placed parallel to each other $\sim$ 2.5 cm apart, with the open ends facing the same direction. Dirty traps had previously captured *P. leucopus* and were never subjected to cleaning. Clean traps were either brand new or had been dismantled and thoroughly cleaned with Lysol® detergent (Reckett Benckiser Inc.). Experimental traps were clean traps with a large piece of *P. leucopus* scented paper towel. For each trap session, a new systematic trap-treatment order was implemented to ensure an unbiased placement of the three trap treatments throughout the course of the study. All six possible combinations were used in random order without replacement. If a clean or experimental trap captured an animal, that trap was removed and cleaned, and a trap of the appropriate treatment (clean or experimental) was set in its place. Traps were washed and scrubbed with a Lysol®-water mixture (following manufacture's directions), rinsed with water, and left to air dry. All traps were baited with sunflower seeds mixed in a small amount of peanut butter.

Traps were checked each morning from 0600–0900. Captured animals were identified by genus, sex, and recapture status. Captured animal's ventral surface was marked with a black permanent marker for short-term recapture identification and the animal's right ear was tagged (Model 1005-1; National Band and Tag, Newport, KY) for long-term recapture identification.

An Analysis of Variance (ANOVA) test was performed on the number of individuals captured per trap treatment per night to determine if differences existed among the three treatment types. Tukey's HSD tests were used post hoc

to determine which variables differed significantly. Separate ANOVAs and two-sample t-tests were also used to determine differences among and between male and female *Peromyscus* captured per trap treatment. Chi-squared goodness-of-fit tests were used on the total number of individuals captured throughout the study. ANOVAs were also used to test for differences in total (sexes combined), male, and female capture rates by month. However, because of uneven sampling effort among the months, we only analyzed capture rates and not total captures for our comparisons. All statistical tests were done using Minitab Statistical Software (Minitab Inc.) with $\alpha = 0.05$ to test for significance.

RESULTS

A total of 1650 trap nights (25 groups of 3 traps each night for 22 nights) resulted in 96 individual animals captured during the study. Two *P. leucopus* escaped from dirty traps before identification and were not included in any analyses. Clean traps captured 22 animals, experimental traps captured 28, and dirty traps captured 46. *Peromyscus leucopus* were the primary mammal captured ($n = 71$), with total captures of 20 clean, 22 experimental, and 29 dirty (Table 1). No recaptures occurred during this study. Of the total animals captured, 23 were non-mouse species, including 10 Eastern Chipmunks (*Tamias striatus*), nine Northern Short-tailed Shrews (*Blarina brevicauda*), and four Red Squirrels (*Tamiasciurus hudsonicus*).

There was no significant difference in capture rates (number captured per night) of *P. leucopus* among the different trap types ($F_{2,63} = 1.02$, $p = 0.366$; Table 1), nor was there a difference in the total number of *P. leucopus* captured over the duration of the study ($X^2 = 1.887$, *d.f.* = 2, $p = 0.389$; Table 1). There was also no significant difference in capture rates between males and females in clean ($t_{36} = 1.69$, $p = 0.100$) or experimental ($t_{38} = 1.56$, $p = 0.128$) traps; however, dirty traps caught more males than females ($t_{39} = 5.51$, $p < 0.001$; Table 1; Fig. 1). July had a higher total (both sexes) capture rate per night than September ($F_{4,17} = 2.96$, $R^2_{adj} = 0.272$, $p = 0.050$), with no significant differences among any other months. Separately, July had a higher capture rate of females than September and June ($F_{4,17} = 3.73$, $R^2_{adj} = 0.342$, $p = 0.023$; Fig. 2), whereas males showed no difference in

Table 1.—Results from Sherman live trap captures of *Peromyscus leucopus* ($n = 71$) based on trap treatment and gender. Averages were calculated from the number of mice captured per night with 25 trap stations per site ($n = 1650$ trap nights).

	Dirty			Clean			Experimental		
Gender	Male	Female	Total	Male	Female	Total	Male	Female	Total
n	25	4	29	14	6	20	14	8	22
Mean ± SE captured/night	1.14 ± 0.14	0.18 ± 0.11	1.32 ± 0.20	0.64 ± 0.18	0.27 ± 0.12	0.91 ± 0.25	0.64 ± 0.14	0.36 ± 0.11	1.00 ± 0.19

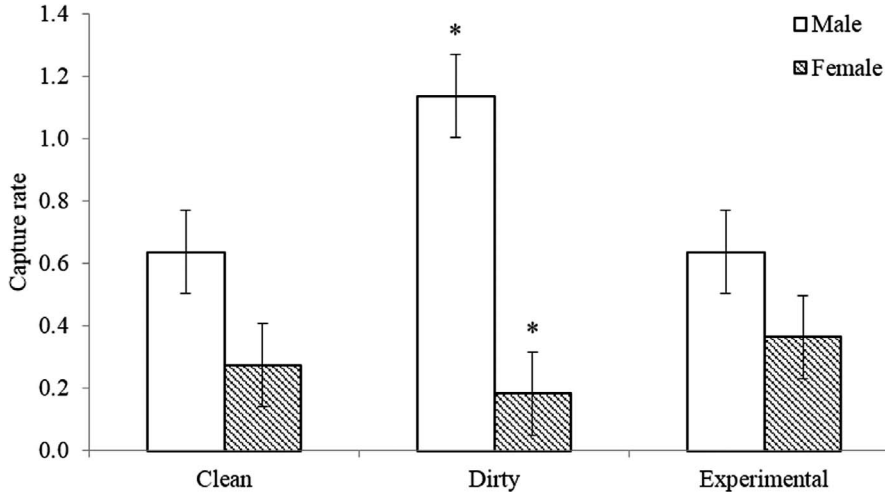

Figure 1.—Nightly ($n = 22$ nights; $n = 1650$ trap nights) capture rates for male ($n = 53$) and female ($n = 18$) *Peromyscus leucopus* among trap types (Mean $\pm$ SE). *Dirty traps had significantly higher male capture rates compared to females.

capture rates among months ($F_{4,17} = 2.00$, $p = 0.140$; Fig. 2).

DISCUSSION

Our results provide evidence that experimental traps represent a viable substitute for dirty traps in studies involving *P. leucopus*. These data (Table 1; Fig. 1) support our predictions that experimental traps would not differ significantly from dirty traps in the capture of *P. leucopus*, and that experimental traps would not have the sexual biases often associated with dirty traps (Fig. 1). It was clear that dirty traps had a much more prominent sexual bias, with a ratio of nearly 5:1 (males to females) compared to 2.3:1 in clean traps and 1.75:1 in experimental traps. These results provide supportive evidence that artificially scented traps represent a viable surrogate for dirty traps.

Our results support previous results that dirty traps cause sexual bias in capture results (Whittaker et al. 1998; Wolf & Batzli 2002). Such biases can result in erroneous population and demographic information (Burger et al. 2009) sufficient to raise questions about the legitimacy of previous studies reporting sex ratios. However, such biases were weakest in experimental traps, further supporting their use. What little difference was noted in sex ratios was likely the result of either random variation or seasonal variation in capture rates, possibly because of inactivity of breeding females. However, the underlying cause(s) of this sexual bias (territoriality, mate searching, etc.) remains unknown. Future studies should employ controlled experiments on both sexes, with various trap types, to determine the proximate cause(s) of sexually biased trap results.

The use of experimental traps also provides a mechanism to mitigate the transmission and spread of infectious diseases. Because *P. leucopus* are known vectors of infectious diseases, such as Hantavirus (Nichol et al. 1993; Mills et al. 1995), mitigating transmission between and among populations would be highly beneficial. Experimental traps are essentially clean traps; therefore, they are less likely to facilitate the spread of infectious diseases. Confirming that mice are free of infectious diseases prior to collection of the scented material also can reduce disease transmission. Additionally, exposing the scented material to ultraviolet light (i.e., sun or lamp), combined with previously mentioned methods, helps mitigate disease transmission to humans and wildlife.

Our results also document season (month) as a potential influence on the capture rates of small mammals (Fig. 2). Female *P. leucopus* were captured more frequently in July than either September or June. These trends are likely the result of cyclic breeding. Because *P. leucopus* from Indiana breed throughout the year with gestation periods of $\sim$ 21–25 days (Whitaker & Mumford 2009), females are likely less active

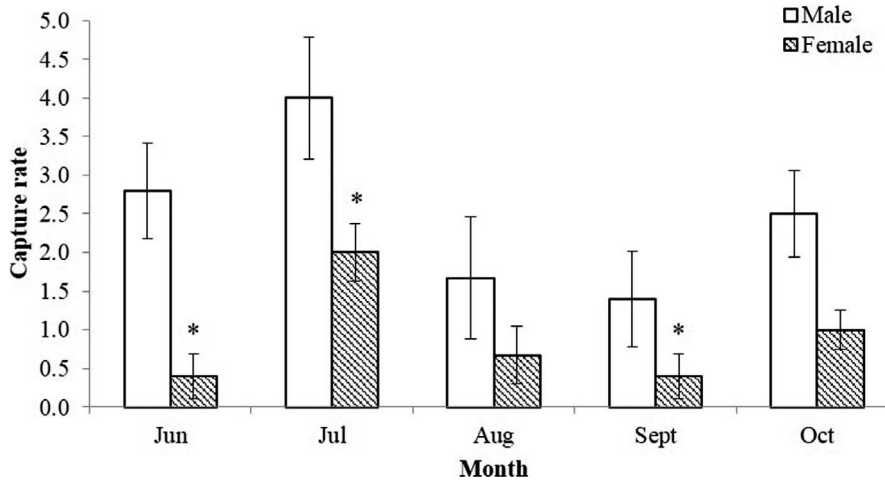

Figure 2.—Nightly (n = 22 nights; n = 1650 trap nights) capture rates for male (n = 53) and female (n = 18) *Peromyscus leucopus* for each month (Mean ± SE). *Female capture rates were significantly higher in July compared to June and September.

during peak periods of birthing. This could result in decreased capture rates some months. This likely explains the variation among monthly capture rates in female, but not male *P. leucopus*, as they are able to remain relatively active throughout the year. As such, special consideration should be given when interpreting capture data of *P. leucopus* with respect to season.

In summary, this study demonstrates that experimental traps can attract small mammals at rates equivalent to dirty traps. Reduced sexual bias, reduced risk of disease transmission, and similar capture rates clearly support the use of experimentally scented traps in field biology. Additionally, influence of season on the capture rate of *P. leucopus* should be considered when planning general surveys and interpreting data. Our results provide evidence for the efficacy of scented trap methodology and for the influence of season on small mammal trapping, and should encourage further investigations into questions relating to trapping protocols, such as whether potent or increased amounts of scented paper would increase capture yield.

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CONFIRMATION OF SUCCESSFUL CHESTNUT-SIDED WARBLER BREEDING IN SOUTH-CENTRAL INDIANA

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ABSTRACT. Reported here is the first documented successful chestnut-sided warbler (*Setophaga pensylvanica*) breeding attempt in south-central Indiana in 20 years. Although small breeding populations have historically utilized available habitat in the southern half of the state, Indiana birders have only recorded 22 chestnut-sided warbler sightings in this region during the breeding season (June—July) over the last 35 years. Constant-effort mist-netting was used to monitor six, 7-year-old clearcuts in the Morgan Monroe and Yellowwood State Forests (Morgan and Brown counties, IN) during the summer of 2015. Over the course of the breeding season, 16 chestnut-sided warblers: seven males, five females (four with a brood patch indicating breeding attempts), and four hatch-year birds (indicating successful breeding) were banded. In addition to one other report of confirmed breeding in northern Indiana (Miami County), this is the only confirmed chestnut-sided warbler breeding population in the state within the last decade. Breeding of chestnut-sided warblers in 2015 demonstrates the value of maintaining some early successional habitat in southern Indiana landscapes.

Keywords: banding, breeding, chestnut-sided warbler, early successional, Indiana, *Setophaga pensylvanica*

INTRODUCTION

Chestnut-sided warblers (*Setophaga pensylvanica*) breed in early successional deciduous forests in the northeastern United States (Byers et al. 2013). Thus, they respond positively to the creation of early successional habitat by modern silvicultural management and timber harvesting. Regenerating clearcuts (timber harvests $\geq$ 4 ha) serve as ideal pockets of habitat for the species, which uses the dense tangle of regrowth to hide open-cup nests from predators and to feed by gleaning small invertebrates from the underside of leaves (Greenberg 1983; Whitehead et al. 1995; Byers et al. 2013).

Although birds are sometimes reported during the summer months, all but the northernmost tier of Indiana counties fall outside of chestnut-sided warbler breeding range (Mumford & Keller 1984; Castrale et al. 1998). However, this does not necessarily mean chestnut-sided warblers have never bred within the state. In fact, the species was an uncommon but regular breeder in south-central Indiana during the late 1980s and early 1990s (Whitehead et al. 1995), and although they have not been detected within the region in the last 20 years, the historical breeding presence is still represented on the Birds of North America range map (Byers et al. 2013). Over the last 20 years, Indiana birders have reported an average of 14.8 birds/year during the breeding season (Ken Brock, pers. comm.). Of these reports, the majority are concentrated in the northern tier of counties, with only 22 reported June–July sightings in the southern half of the state in the past 35 years (Ken Brock, pers. comm.).

From 1985–1990 there were four confirmed chestnut-sided warbler breeding attempts in Indiana: three in northern counties (LaPorte, St. Joseph, LaGrange), and one in a south-central county (Brown) (Castrale et al. 1998). However, the most recent Breeding Bird Atlas (2005–2011) shows a decline in statewide breeding attempts, reporting only one confirmed successful breeding attempt in a northern county (Miami) and none in the south-central or southern regions (U.S. Geological Survey 2015). Historically, there have been reports of extra-limital breeding in south-central Indiana (Whitehead et al. 1995), but due to inadequate habitat renewal in the region (e.g., logging suppression and fire suppression), known breeding populations have gone undetected in this part of the state for the past 20 years (Forest Inventory 2015).

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Historically, logging has been strongly correlated with chestnut-sided warbler range expansion and breeding success. Prior to the Industrial Revolution and an increased national demand for timber, chestnut-sided warblers were a rare bird in the eastern United States (Askins 2002; Byers et al. 2013). Due to the low occurrence of naturally occurring early successional habitat creation, chestnut-sided warbler habitat, and thus population expansion, was limited. However, since the 1800s, chestnut-sided warbler populations have expanded in response to the increased provision of essential breeding habitat via forest logging (Askins 2002; Byers et al. 2013). Now a widespread spring (and fall) migrant across most of the eastern United States, chestnut-sided warblers can effectively find and colonize the ephemeral pockets of early successional habitat resulting from recently logged sites.

In Indiana, there was a noticeable peak in timber harvest (i.e., early successional habitat creation) in the late 1980s, corresponding with subsequent peaks in chestnut-sided warbler breeding activity within the state (Whitehead et al. 1995; Castrale et al. 1998; Forest Inventory 2015). However, a lull in logging activity in the 1990s and 2000s has limited the availability of adequate chestnut-sided warbler breeding habitat over the last twenty years. In this study, we documented successful chestnut-sided warbler breeding attempts in south-central Indiana clearcut sites that were harvested in 2008. This study highlights the potential conservation benefits of active management for early successional species in Indiana.

METHODS

During the summer of 2015, territoriality and productivity of breeding birds was monitored in six south-central Indiana clearcuts. These clearcuts, approximately 4 ha in size, were harvested in 2008–2009 as part of the ongoing Hardwood Ecosystem Experiment (HEE), a long-term, landscape-level study monitoring the social and ecological impacts of forest management within the Morgan-Monroe and Yellowwood state forests (MMYSF) in southern Indiana (Kalb & Mycroft 2013). The HEE was initiated in 2006 by the Indiana Department of Natural Resources, Division of Forestry, as a multidisciplinary 100 year collaborative study between scientists at Purdue University and other regional universities. Location maps of HEE clearcut sites (even-aged management treatments) are available in the Northern Forest Experiment Station General Technical Report (Kalb & Mycroft 2013). Harvested seven years ago, clearcuts now consist of a densely vegetated understory and a developing sapling overstory, ideal for chestnut-sided warblers (Byers et al. 2013). In addition, these sites represent some of the only appropriate chestnut-sided warbler habitat created in the region in the last two decades (Forest Inventory 2015).

Constant-effort mist-netting, following the Monitoring Avian Productivity and Survivorship (MAPS) protocol (DeSante et al. 2000), was used to monitor breeding activity in harvest-created gaps from May–August 2015. We banded all birds with a federal leg band, and recorded wing-chord length, tail length, culmen length, mass, presence of migratory fat, age, sex, and breeding status (presence of cloacal protuberance in males or brood patch in females). All birds were captured and handled in accordance with Federal Banding Permit #21781 and Purdue Animal Care and Use Committee guidelines (protocol # 110000078C002).

In clearcut sites, five 1.5 m wide net lanes were cleared, just large enough for 12 m long, 30 mm mesh, four-tier, black, tethered, nylon mist-nets. Nets were not positioned in a standardized grid, but rather in an attempt to maximize productivity and efficiency (DeSante et al. 2000). All nets were at least 50 m apart, as well as $\geqslant$25 m into the study site from the clearcut edge. This spacing allowed for adequate sampling of the clearcut habitat and efficient net checks to minimize injury and mortality. Nets were operated at each site for one day (6 net-hours) during each of nine consecutive 10-day sampling periods. Taking into account daily variation in net-hours caused by extenuating circumstances (e.g., weather conditions), nets were opened for a total of 1,562 net hours during the 2015 summer banding season.

In addition to banding data collected in summer 2015, 15 point counts were performed in and around the clearcuts between 20 May and 20 June, 2006–2015 (with the exception of 2013 when no point counts were done). During these point counts, observations on chestnut-sided warbler behavior and territorial defense were recorded. Point counts consisted of an unlimited-radius ten-minute count where all detected birds were recorded. Each point was surveyed twice during the 30-day period with a minimum of 7 days between repeated counts at any given

point. Items recorded included the time of first detection (for each individual), species, detection method (song, call, sight, etc.), sex (if possible), and approximate distance of bird from observer (10 m increments). Beginning in 2010 (two years post-harvest), it was also noted whether birds were in a harvest area, on the edge of a harvest area, or in the forest matrix. In 2015, digital recordings were taken at each point count.

RESULTS

During the 2015 banding season, 16 chestnut-sided warblers were caught at two of the six clearcuts, which we labeled the Northern and Southern sites. These two clearcuts (~ 0.5 km apart) were located in Yellowwood State Forest, Brown County, Indiana. Although these clearcuts were approximately the same size, age, and location, and the habitat differences between the two were indistinguishable, all but one individual was banded in the Southern site. Correspondingly, breeding activity was only observed in the Southern clearcut.

Observations.—Prior to the 2015 breeding season, 15 chestnut-sided warblers were detected in HEE point counts over a nine-year period. Of these 15 detections, nine chestnut-sided warblers (most likely migrants) were detected in May, during the first three days of the survey period (three, two, and four birds were detected in 2006, 2007, and 2014 respectively). However, in 2010, 2011, and 2014, six birds were detected during the month of June (two, three, and one bird(s) in 2010, 2011, and 2014 respectively). These June detections could be indicative of breeding attempts, although breeding success was unconfirmed.

Beginning in May 2015, potential breeding activity was observed in the Southern site. We observed males singing from exposed snags from 15 May–1 July (indicative of breeding territory establishment and defense). On 17 May, we observed a male chestnut-sided warbler defend its territory from a male prairie warbler (*Setophaga discolor*), and on 2 June during a point count we observed agonistic behavior between an unbanded male chestnut-sided warbler and a banded chestnut-sided warbler (sex unknown).

All seven banded males had an enlarged cloacal protuberance (indicative of active breeding status). Throughout the study, four females developed brood patches (indicative of breeding attempts). The first was banded on 3 June, the next two were banded on 11 June, and the final female with a brood patch (caught in the same net as a hatch-year bird) was banded on 1 July (see cover photo of this issue [124(1)]).

On 21 June, successful breeding of chestnut-sided warblers was confirmed in the Southern clearcut by netting and banding a fledgling. The tail length, degree of feather molt, presence of yellow lining around gape, and limited flight capacity all indicate that the bird was recently fledged from a nearby nest. The fledgling was released back at the net where it was caught, because it was most likely still dependent on parental care. All together, four hatch-year chestnut-sided warblers were banded during the 2015 breeding season (months of June and July). Hatch-year birds were banded on 21 June, 1 July, 24 July, and 31 July.

DISCUSSION

Prior to this study, the most recent breeding confirmation of chestnut-sided warblers in south-central Indiana was documented by Whitehead et al. (1995). This south-central region (including Brown, Jackson, and Lawrence counties, Indiana) has historically been known as an extra-limital breeding pocket outside of the main chestnut-sided warbler breeding range (Whitehead et al. 1995; Byers et al. 2013). This study confirms continued breeding in this region following a 20 year period in which chestnut-sided warbler breeding presence went unnoticed.

In this study, chestnut-sided warbler breeding success was documented in one of two clearcut sites in the Yellowwood State Forest. Both clearcuts were harvested at the same time, and no discernible difference in vegetation was observed between Northern and Southern sites that would explain the discrepancy in breeding preference. In contrast, Whitehead et al. (1995) observed chestnut-sided warblers in nearly every clearcut in the Yellowwood sites (Donald R. Whitehead, pers. comm.). One potential reason for the perceived differences in site use between our study and Whitehead et al. (1995) could be the size of the harvest openings. In our study, clearcuts were approximately 4 ha (Kalb & Mycroft 2013). Upon further review, it appears that the Yellowwood clearcuts described in Whitehead et al. (1995) might be better described as a series of small patch cuts (0.3–1.5 ha) in close proximity to one another (Michael Spalding, pers.

comm.). However, while increasing forest opening size within a forest-dominated matrix is positively correlated with breeding bird species richness (Taylor & Taylor 1979; Costello et al. 2000), chestnut-sided warblers are known to colonize both small selection cuts as well as larger clearcut stands (Costello et al. 2000; Tozer et al. 2010). Thus, the difference in clearcut size between the present study and Whitehead et al. (1995) may not explain the discrepancy in site use.

In the present study there was a 7-year time lag between habitat creation and confirmation of chestnut-sided warbler breeding. This is three years longer than the lag previously reported for chestnut-sided warblers in south-central Indiana (Whitehead et al. 1995), and five years longer than the minimum colonization period reported in DeGraff & Yamasiki (2003). Although the summer of 2015 was the first season mist-netting was used to confirm chestnut-sided warbler breeding in clearcuts, point count data were collected at these sites during the previous nine summers (including two years of pre-harvest data collection). Two and three chestnut-sided warblers were detected during point counts in June 2010 and 2011, respectively. Thus, it is a distinct possibility that breeding chestnut-sided warblers, present in low numbers, could have colonized HEE clearcuts two and three years post-harvest. However, based on the available data, 2015 is the first summer in which chestnut-sided warbler breeding was confirmed in HEE clearcuts.

Early successional habitat management within a forest-dominated matrix can benefit a wide variety of birds (Pagan et al. 2000; DeGraff & Yamasaki 2003; Porneluzi et al. 2014). Many avian species require early successional habitat for several components of their life history (e.g., yellow-breasted chat [*Icteria virens*], prairie warbler, and northern bobwhite [*Colinus virginianus*]). In addition, several birds that require mature forest habitat for breeding (e.g., scarlet tanager [*Piranga olivacea*], ovenbird [*Seiurus aurocapilla*], wood thrush [*Hylocichla mustelina*], and worm-eating warbler [*Helmitheros vermivorum*], depend on the availability of nearby early successional habitat during the post-fledging period (Pagen et al. 2000; Vitz & Rodewald 2006; Streby et al. 2011). In Indiana, ruffed grouse (*Bonasa umbellus*; another species that occupies early successional habitat) populations have been in decline for the past 25 years (Backs & Castrale 2014). In fact, the 2015 ruffed grouse hunting season was suspended in Indiana due to statewide declines.

Given the ephemeral nature of early successional habitat, continuous regeneration is needed within the landscape to maintain adequate availability of suitable breeding habitat (DeGraff & Yamasaki 2003). The present study documented breeding seven years post-harvest, but in light of existing data on habitat viability, management plans have been developed that recommend new patch generation every 10—15 years (DeGraff & Yamasaki 2003). Although active forest management (i.e., timber harvesting) is sometimes opposed by the general public, clearcutting can be one of the most effective methods of early successional habitat creation (Askins 2002). Based on this study and others (Askins 2002; DeGraff & Yamasaki 2003), we suggest implementation of regular timber harvest rotation in Indiana to maximize benefits to early successional species while maintaining mature forest structure and species composition.

In summary, chestnut-sided warbler breeding success in HEE clearcuts illustrates one potential ecological benefit of active forest management in the state of Indiana. The benefits of early successional habitat management, however, extend beyond providing breeding habitat for chestnut-sided warblers. Forest openings (e.g., clearcuts) create breeding habitat for many early successional specialists as well as mature forest species (Askins 2002; Pagen et al. 2000; Byers et al. 2013). A balance of both early successional and mature forest habitat conservation is essential for maximizing species richness and biodiversity within the state.

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LENGTH-WEIGHT RELATIONSHIPS ASSOCIATED WITH GENDER AND SEXUAL STAGE IN THE NORTHERN CLEARWATER CRAYFISH, *ORCONECTES PROPINQUUS* GIRARD, 1852 (DECAPODA, CAMBARIDAE)

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ABSTRACT. The northern clearwater crayfish, *Orconectes propinquus* Girard 1852, is a species native to eastern Canada and the northern United States. Growth patterns and relationships of body morphometrics were evaluated to understand its basic niche requirements. Growth and size relationships for gender, sexual phase for adults and juveniles, and chelae length and width relationships to interpret information on sexual dimorphism were determined. The log transformed carapace length-weight relationship for male form I ($y = 3.3201x - 4.0205$, $r^2 = 0.9645$, $F = 1302.9$, $p = <0.001$), and juveniles ($y = 3.2792x - 4.0171$, $r^2 = 0.8721$, $F = 709.2$, $p = <0.001$) showed positive allometric growth rates, whereas male form II ($y = 2.8831x - 3.4474$, $r^2 = 0.8268$, $F = 248.2$, $p = <0.001$) and female ($y = 2.9184x - 3.5313$, $r^2 = 0.9395$, $F = 1879.3$, $p = <0.001$) showed negative allometric rates of change with increasing length. Relationships between log transformed carapace length and carapace width ($y = 1.0425x - 0.3841$, $r^2 = 0.8964$, $F = 1945.8$, $p = <0.001$), carapace depth ($y = 0.9468x - 0.2816$, $r^2 = 0.9018$, $F = 2065.2$, $p = <0.001$), abdomen width ($y = 1.0413x - 0.4443$, $r^2 = 0.864$, $F = 1429.2$, $p = <0.001$), chelae width ($y = 1.2927x - 0.9998$, $r^2 = 0.6255$, $F = 375.9$, $p = <0.001$), and chelae length ($y = 1.3083x - 0.6281$, $r^2 = 0.7439$, $F = 653.6$, $p = <0.001$) for the total collected adult population grew at a negative allometric rate. According to the body condition index b, growth rates showed increasing weight with length but were within the expected allometric growth.

Keywords: morphometrics, allometry, growth, length-weight relationships, body condition Index

INTRODUCTION

Basic life history information is missing for the majority of the nearly 650 described species of crayfish (Moore et al. 2013), and there is limited understanding of growth and length-weight relationship information (Stein 1976; Romaire et al. 1977; Rhodes & Holdich 1984; Garvey & Stein 1993; Mazlum et al. 2007; Wang et al. 2011; Simon & Stewart 2014). Understanding patterns in growth can provide important community management information based on size, weight, and body condition between native and invasive species introductions.

The native distribution of northern clearwater crayfish, *Orconectes propinquus* Girard 1852 includes the northern USA and Canada (Page 1985; Hobbs 1989). It is native to Illinois, Indiana, Ohio, Pennsylvania, New York, southern Quebec and Ontario, with populations extending west into Iowa and Minnesota (Page 1985). *Orconectes propinquus* is commonly found in streams across the state of Indiana and can be found in nearly every region of the state (Simon 2001).

The competitive advantage of *O. propinquus* is speculated to be based on its large size compared to other sympatric native species (Loughman & Simon 2011; Simon & Stewart 2014). This size advantage may be the result of size and weight differences caused by unequal growth of body parts (Lockwood et al. 2013). Change in growth of select structures is sexually dependent and rates may be characterized as either allometric or isometric based on the regression slope constant b (Mazlum et al. 2007). The b constant is considered a measure of body condition based on the cube law (Froese 2006). Growth is isometric when $b = 3$, and allometric when $b < 3$ or $b > 3$. This suggests that positive allometric growth occurs when organism weight increases more than length ($b > 3$) and negative allometric growth occurs when length increases more than weight ($b < 3$). Allometry may change

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during growth and sexual stage. These growth rate changes can result in differential expression based on gender or sexual maturation. This divergent expression may not provide a competitive advantage for females and juveniles.

The current study evaluates the relationships between growth, gender, and body morphology that would enhance competitive advantage among the northern clearwater crayfish. Length and weight, carapace, chelae, and abdomen relationships among male form I and II, female, and juvenile individuals of *O. propinquus* were investigated. This information will contribute to baseline information needs for evaluating introduced species life history attributes.

METHODS

All specimens (n = 333) used for measurement of *Orconectes propinquus* morphometry were collected from mostly ambient natural streams (n = 51), man-made ditches (n = 2), a mitigated wetland site (n = 1), and a few unknown locations across the state of Indiana (n = 6). Surveys were conducted in Indiana during May 2005 until May 2013. Sampling was restricted to daylight hours and all available habitats within a reach were sampled within a linear distance of 15 times the wetted stream width or 150 m minimum distance in lakes and wetlands along the shoreline margin using backpack electrofishing equipment. Data over this species native range was pooled in order to examine the relationships between growth, gender, sexual stage, and size.

A total of 123 females, 104 males (n = 50 form I and n = 54 form II), and 106 juveniles were measured using digital calipers to the nearest 0.1 mm. Individuals were segregated by gender and sexual stage groups. Two people measured the crayfish samples (n = 76 and n = 257) and measures were compared so that less than 5% variation in measurement error was achieved. All measured individuals had a full complement of chelae and walking legs. Regenerated chelae were not measured. Each individual crayfish was externally classified according to sex and reproductive state. All individuals were measured for morphometric variables and for wet weight (W_{WT}). (Technically, since our measurements are in grams, not newtons, this should be mass, not weight. However, since the literature going back almost 200 years has always referred to this as weight, we are using weight instead of mass.) Weight (W_{WT}) was measured by placing the individual on paper towel to remove excess water, and then weighed with an accuracy of 0.001 g. Seven morphometric characteristics were measured for each specimen (see Simon & Stewart 2014) – carapace length (CL), postorbital carapace length (POCL) [from the anterior margin of postorbital spine to the posterior margin of the carapace], carapace width (CW), carapace depth (CD), chelae length (ChL), chelae width (ChW), and abdomen width (ABW). Based on similar studies of *Procambarus* (Mazlum et al. 2007; Wang et al. 2011) and *Orconectes* (Simon & Stewart 2014) crayfish species these morphological characters are related to sexual dimorphism and are influenced by environmental conditions and food resources.

Juvenile and adult specimens were distinguished by the presence or absence of reproductive organs (Hobbs 1989). Any possible relationship between smaller (CL $<$ 25 mm) and larger (CL $>$ 25 mm) specimens were determined by comparing the ratios between the means of above measurements and mean carapace length (CL/ABW, CL/POCL, CL/CW, CL/CD, CL/ChL, CL/ChW) of all individuals in each group.

The carapace length was considered as the independent variable for all relationships performed as it appears to be minimally affected by growth variations and sexual maturation among Decapoda (Lovett & Felder 1989). Regression analyses to determine the relationship between all measurements versus CL was investigated for each sex separately using the multiplicative model: $Y = aX^b$, where Y and X are the morphological dimensions and a and b the regression constants (StatSoft 2010). The relationships obtained were log transformed to the form $\log_{10}Y = \log_{10} a + b \log_{10} X$. The log transformation is preferred in order to better satisfy the assumptions of regression analysis (Sokal & Rohlf 1981). This allows the derivation of a single value from the analysis for the scaling relationship between the two-morphometric parameters. The allometry pattern for each parameter was established by testing the slope (b) of the regression equations against isometry (H_o: $b = 1$) applying the Student's t-test. Analysis of Covariance (ANCOVA) was used to compare the slopes b and carapace length between sexes, sizes, and sampling period (Zar 1984). The Kruskall–Wallis test (Zar 1984) was used

to identify possible differences in time, area, and size, at the 95.0% confidence level. The Mann–Whitney test compared independent samples, at the 95.0% confidence level (Sokal & Rohlf 1981), while a simple regression analysis was used to examine the relationship between *O. propinquus* morphological characters with the sex as a covariate.

RESULTS

The samples in the study area showed differences in mean carapace length between male and female, specifically the male carapace was larger than those of females (Mann–Whitney test, $p > 0.001$). The carapace length of sampled females ranged from 11.78 to 35.67 mm (average 23.96 $\pm$ 5.65 mm) and that of male form I from 12.16 to 34.16 mm (average 24.63 $\pm$ 5.11 mm), while male form II ranged from12.22 to 33.49 mm (average 23.95 mm $\pm$ 5.14 mm).

Mean carapace length (CL $\pm$ SD) and mean weight (W_{WT} $\pm$ SD) for the entire population of male and female were 24.12 $\pm$ 5.40 mm (range = 11.78–35.67 mm) and 3.93 $\pm$ 2.53 g (range = 0.34–12.09 g), respectively (Table 1). Mean carapace length (CL $\pm$ SD), mean weight (W_{WT} $\pm$ SD), and their range were calculated for male and female as: CL_{male} = 24.28 $\pm$ 5.11 mm (range = 12.16–34.16 mm), $W_{WT\ male}$ = 4.24 $\pm$ 2.54 g (range = 0.34–11.06 g); $W_{WT\ female}$ 3.67 $\pm$ 2.50 g (range = 0.43–12.09 g), respectively. The normalized ($\log_{10}$) length-weight relationship for male form I was explained by the linear equation, where y = 3.3201x - 4.0205, $r^2 = 0.965$, $F = 1302.9$, $p \leq 0.001$, male form II was explained by the linear equation, y = 2.8831x - 3.4474, $r^2 = 0.863$, $F = 248.2$, $p \leq 0.001$, female length and weight (y = 2.9184x - 3.5313, $r^2 = 0.940$, $F = 1879.3$, $p \leq 0.001$), and juveniles (y = 3.2792x - 4.0171, $r^2 = 0.872$, $F = 709.2$, $p \leq 0.001$) (Fig. 1). Only male form I and juveniles showed positive allometric rates of weight change with increasing length (Table 1).

Mean carapace width (CW $\pm$ SD), mean carapace depth (CD $\pm$ SD), and their range for male and female were CW_{males} = 11.69 $\pm$ 2.78 mm (range = 5.74–18.17 mm), CD_{male} = 10.81 $\pm$ 2.35 mm (range = 5.06–15.47 mm), $CW_{females}$ = 11.25 $\pm$ 2.91 mm (range = 5.69–18.42 mm), and CD_{female} = 10.52 $\pm$ 2.48 mm (range = 5.93–17.19 mm) (Table 1). The relationships between carapace length with carapace width (y = 1.0425x - 0.3841, r^2 = 0.896,

Table 1.—Descriptive statistics, estimated parameters ($\log_{10}$), and growth type of length-weight relationships for 333 individual *Orconectes propinquus*. SE = standard error of *b*; CL = 95% confidence limits of *b*; n = number of crayfish; +A = positive allometric growth; -A = negative allometric growth.

		Carapace length (mm)			Weight (g)			Parameters of the relationships					
Sex and sexual form	n	Mean (SD)	Min	Max	Mean (SD)	Min	Max	*a*	*b*	SE (*b*)	CL (*b*)	r^2	Type of growth
Male (I)	50	24.63 (5.11)	12.16	34.16	4.60 (2.76)	0.38	11.06	−4.021	3.320	0.092	3.135-3.505	0.964	+ A
Male (II)	54	23.95 (5.14)	12.22	33.49	3.91 (2.30)	0.34	9.91	−3.447	2.883	0.813	2.516-3.250	0.863	− A
Female	123	23.96 (5.65)	11.78	35.67	3.67 (2.50)	0.43	12.09	−3.531	2.918	0.067	2.785-3.052	0.940	− A
Juvenile	106	10.03 (1.94)	6.51	15.45	0.21 (0.15)	0.02	0.92	−4.017	3.279	0.123	3.035-3.523	0.872	+ A
Total	333		6.51	35.67		0.02	12.09						

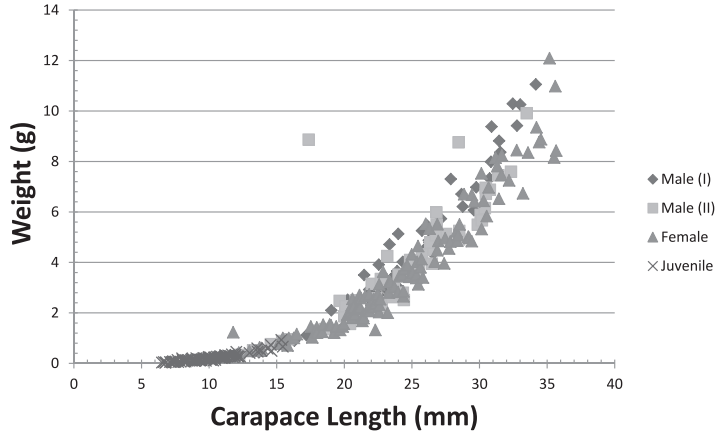

Figure 1.—Length-weight relationships for *Orconectes propinquus* Girard 1852 sexual phases. Diamonds = form I males; boxes = form II males; triangles = females; and x = juveniles.

$F = 1945.8$, $p \leq 0.001$), carapace length with carapace depth (y = 0.9468x - 0.2816, $r^2 = 0.902$, $F = 2065.2$, $p \leq 0.001$), carapace length with abdomen width (y = 1.0413x - 0.4443, $r^2 = 0.864$, $F = 1429.2$, $p \leq 0.001$), carapace length with chelae length (y = 1.3083x - 0.6281, $r^2 = 0.744$, $F = 653.6$, $p \leq 0.001$), and carapace length with chelae width (y = 1.2927x - 0.9998, $r^2 = 0.626$, $F = 375.9$, $p \leq 0.001$) for the total adult specimens grew at a negative allometric rate. Mean carapace width (CW $\pm$ SD), mean carapace depth (CD $\pm$ SD), and their range were calculated for form I male, form II male, female, and juveniles respectively as: CW_{MI} = 11.87 $\pm$ 2.81 mm (range = 5.74–18.17 mm), CD_{MI} = 10.92 $\pm$ 2.34 mm (range = 5.55–15.47 mm), CW_{MII} = 11.52 $\pm$ 2.76 mm (range = 5.86–16.96 mm), CD_{MII} = 10.72 $\pm$ 2.39 mm (range = 5.06–15.33 mm), CW_{female} = 11.25 $\pm$ 2.91 mm (range = 5.69–18.42 mm), CD_{female} = 10.52 $\pm$ 2.48 mm (range = 5.93–17.19 mm), CW_{juv} = 4.25 $\pm$ 0.98 mm (range = 2.32–7.38 mm), and CD_{juv} = 4.29 $\pm$ 0.93 mm (range = 1.84–6.47 mm).

Sexual stage differences were observed between adult and juveniles. The carapace width (CW) growth rate increased at a negative allometric rate with weight for male form I, male form II, females, juveniles, and the entire population. ANCOVA tests showed that length-weight regression slopes and intercepts were not significantly different among sexes or sexual stage ($p > 0.05$). In addition, our results shows that form II male were 1.04 times heavier than form I male and 1.18 times heavier than females. Form I males were 1.95 mm larger than females, while form II males were 1.67 mm larger than females. Mean total length and weight did not differ between males and females ($p > 0.060$); the only significant differences were detected among sexual stages ($p < 0.0001$).

Relationships among chelae length and width measurements for the population were evaluated for gender and sexual stage. Mean chelae length (ChL $\pm$ SD), mean chelae width (ChW $\pm$ SD), and their range were calculated for form I males, form II males, and females, respectively, as ChL_{MI} = 18.55 $\pm$ 6.08 mm (6.56–32.58 mm), ChW_{MI} = 7.69 $\pm$ 2.48 mm (3.05–12.63 mm), ChL_{MII} = 17.00 $\pm$ 5.76 mm (7.19–27.57 mm), ChW_{MII} = 6.82 $\pm$ 2.99 mm (2.72–19.17 mm), and ChL_{female} = 13.73 $\pm$ 4.33 mm (5.53–25.70 mm), ChW_{female} = 5.63 $\pm$ 1.93 mm (2.03–11.40 mm) (Table 2).

No significant difference was observed in mean ChL between form I and form II males ($p > 0.05$), but a significant difference was detected in mean ChL between males and females ($P < 0.05$). Form I and form II male had longer ChL than females. A similar trend was observed in mean ChW for form I and form II males, but a significant difference was observed between males and females ($p < 0.05$). Chela lengths and width increased with CL for both genders and sexual stages (Table 2). In addition, chelae length-weight relationships were positively correlated with gender and sexual states (Table 2). Although the slope and intercepts of regressions for ChL and ChW were similar for form I and form II males, the slope and intercepts of regression of females

Table 2.—Mean and standard deviation (SD) for chelae length (ChL) and chelae width (ChW) characteristics and parameters of the relationship between log ChL and log ChW of each sex and sexual form. SE = standard errors of *b*; CL = 95% confidence limits of *b*; N = number of crayfish; r^2 = coefficient of determination.

Sex and sexual form	n	Chelae length (mm)			Chelae width (mm)			Parameters of the relationships				
		Mean (SD)	Min	Max	Mean (SD)	Min	Max	*a*	*b*	SE (*b*)	CL (*b*)	r^2
Male (I)	50	18.55 (6.08)	6.56	32.58	7.69 (2.48)	3.05	12.63	−0.234	0.880	0.036	0.751–1.010	0.795
Male (II)	54	17.00 (5.76)	7.19	27.57	6.82 (2.99)	2.72	19.17	−0.340	0.945	0.085	0.774–1.117	0.702
Female	123	13.73 (4.33)	5.53	25.70	5.63 (1.93)	2.03	11.40	−0.904	1.191	0.073	1.046–1.336	0.685
Juvenile	106	5.41 (1.43)	2.14	10.09	2.12 (0.65)	0.78	4.13	−0.421	0.011	0.066	0.879–1.142	0.691

were significantly different (ANCOVA $p < 0.05$) from form I and form II males (Table 2). The relative growth rate of the abdomen in males did not present statistically significant results; however, females were significantly different from males (ANCOVA $p < 0.001$).

DISCUSSION

Studies focused on length-weight relationships reveal that sexual dimorphism is common in freshwater crayfish species (Lindquist & Lahti 1983; Holdich 2001; Mazlum et al. 2007; Wang et al. 2011; Simon & Stewart 2014). Difference in sexual dimorphism is a function of the rapid disproportionate growth of chelae in male compared to female genders. Differences in body size among sex and sexual stage was consistent with those reported in other studies (Stein 1976; Romaire et al. 1977; Rhodes & Holdich 1984; Garvey & Stein 1993; Mazlum et al. 2007; Wang et al. 2011; Simon & Stewart 2014). Juvenile crayfish grew at a positive allometric rate and rapidly attained adult sizes as in other members of the genus *Orconectes* (Simon & Stewart 2014).

The relative growth between the sexes differs only slightly as indicated by morphometric relationships, which is similar to *Orconectes virilis* (Simon & Stewart 2014). A positive allometry of all body relationships observed in male form I and juvenile, reflects the decreasing growth rate of these morphological characters in relation to CL, which is attributed to a sex-related variation (Wetzel 2002). Variation in abdomen width is commonly found in freshwater crayfish, but is always related to sex, sexual maturity, and size (Wetzel 2002). Widening of female abdomen width (ABW) reflects a sexually active female that is correlated with either swollen or white glair, dependent offspring, or remnants of egg stalks attached to pleopods. Wetzel (2002) found that only form I females mated with form I males and reinforced the view that wide abdomens are a reflection of the act of mating and rearing offspring. Reasons for female variation may include presence of ovigerous stages of ova development, instar development during the prolonged period of recruitment, and larval growth (Wetzel 2002). No reproductively active females were present in this study; none exhibited the widened ABW.

In this study, the length-weight relationships showed that males were heavier than females

Table 3.—Comparison of crayfish species body condition index factors (*b*) reflecting body condition for length-weight relationships.

Species and sexual form	Body condition factor: Length-weight	Study
Orconectes propinquus		Current study
Male	3.320	
Male form I	2.883	
Male form II	2.918	
Female	3.279	
Orconectes virilis		Simon & Stewart 2014
Male	3.85	
Male form I	3.66	
Male form II	3.95	
Female	3.73	
Procambarus acutus		Mazlum et al. 2007
Male	3.30	
Male form I	3.61	
Male form II	3.26	
Female	3.50	
Procambarus clarkii		Wang et al. 2011
Male	3.63	
Male form I	3.60	
Male form II	3.60	
Female	3.35	

of the same length. The largest male (34.16 mm CL) was shorter, but heavier (11.05 g) than the longest female (35.67 mm, weighing 8.429 g). No statistical difference in mean weight was observed; however, this is attributed to the accelerated development of the chelae in sexually mature form I males, whereas chelae of females grow slower throughout life. The relatively longer chelae of form I and form II males are due to sexually dimorphic change.

Body condition rates are used to evaluate competition and ecological relationships among a wide variety of species including crayfish (Lindquist & Lahti 1983). Body Condition Index factors comparisons among crayfish species show that *O. propinquus* exhibit the lowest body condition rates (Table 3). Condition factors (*b*) can be predictive to evaluate competition based on length-weight growth rates. Based on condition factors of *O. virilis* (Simon & Stewart 2014), *Procambarus acutus acutus* (Romaire et al. 1977), and *P. clarkii* (Wang et al. 2011), *O. propinquus* would not be dominant in any introduced scenario between these four species (Table 3).

In summary, the length-weight relationship and condition factors observed in *O. propinquus* show the lowest body mass related to length among the crayfish species studied. We observed little morphometric or growth related differences between sex or sexual phase, including various male forms and female sexual phases, with the exception of female abdomen width. With increasing emphasis on the attainment of basic life history information for crayfish it will be necessary to consider differences among populations and possible intraspecific body shape differences associated with different habitats and water qualities.

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SUPPLEMENTAL MATERIALS

Indiana: Brown County, Jackson Twp., Gravel Creek, Gravel Creek Rd, Story, 39.10718 N, −86.22673 W, 2 July 2008; Brown County, Middlefork Salk Creek, Poplar Rd, Story, 39.12683 N, −86.13673 W, 2 July 2008, Field No. MFS06US, INBS 3831; Brown County, Middlefork Salk Creek, Poplar Rd, Story, 39.12683 N, −86.13673 W, 2 July 2008, Field No. MFSC06DS, INBS 3832; Brown County, Gravel Creek, Gravel Creek Rd, Story, 39.10718 N, −86.22673 W, 2 July 2008, Field No. MFSC14US, INBS 3833; Brown County, Gravel Creek, Elkinsville Rd, Story, 39.09169 N, −86.28108 W, 2 July 2008, Field No.. MFSC15US, INBS 3835; Brown County, Hamilton Creek, Mt. Neb. Rd, 39.08792 N, −86.17157 W, 2 July 2008, Field No. MFSC21US, INBS 3839; Brown County, Hamilton Creek, Christianburg Rd, Christianburg, 39.08693 N, −86.15695 W, 2 July 2008, Field No. MFSC23US, INBS 3841; Brown County, Hamilton Creek, Christianburg Rd, Christianburg, 39.08693 N, −86.15695 W, 2 July 2008, Field No. MFSC23DS, INBS 3842; Brown County, unnamed creek, Blue Creek Rd, Elkinsville, 39.05613 N, −86.27207 W, 2 July 2008, Field No. MFSC25US, INBS 3843; Brown County, Happy Hollow Lake Creek, Bellsville Pike, New Bellsville, 39.14452 N, −86.10815 W, 2 Jul 2008, Field No. MSC48US, INBS 3849; Brown County, Washington Twp., unnamed trib. of Salt Creek, D/S bridge at Crooked Creek Rd., 0.1 mi from SR 46, 2 mi. E Belmont, (Simon), 39.154756 N, −86.305369 W, 6 July 2010, Field No. TPS 92-75 Cob; Brown County, Washington Twp., Middle Fork Salt Creek, SR 46 Bridge, Nashville, (TP Simon), 39.20079 N, −86.248267 W, 1 June 2005, Field No. TPS05-50; Brown County, Hamble Twp., unnamed trib. of Salt Creek, D/S bridge at intersection of N. Peoga Ridge Rd. and Gatesville Rd .2 mi N, TRS: 9N 4E 25/36/30 SE ¼, (Simon), 39.261817 N, −86.143267 W, 6 July 2011, Field No.TPS-92-6814JUL2010; Clay County, Jackson Twp., 300 E Shady Lane, N W, 2 June 2006; Clay County, Jackson Twp., TPS 92-91 COB, N W, 3 June 2006, Clay County, Jackson Twp., TPS 92-68, N W, 4 June 2006; Clay County, Jackson Twp., TPS 92-103, N W, 5 June 2006; Clay County, Jackson Twp., TPS 92-94, N W, 6 June 2006; Clay County, Jackson Twp., unnamed trib., Boon, Hoosierville, (J Burskey, M. Herbert, TPS), 39.47431 N, −87.09138 W, 8 June 2006, Field No. JLB06062, INBS 2292; Clay County, Dick Johnson Twp., unnamed trib., 950 N Bee Ridge, (J Burskey, M, Herbert, T), 39.52639 N, −87.16421 W, 2 June 2006, Field No. JLB06065, INBS 2299; Clay County, Dick Johnson Twp., unnamed trib., 200 W Perth, (J Burskey, M. Herbert, TPS), 39.58467 N, −87.14317 W, 2 Jun 2006, Field No. JLB06066, INBS 2301; Clay County, Van Buren Twp., Croys Creek, 1300N Shady Lane, (J Burskey, M Herbert, TPS), 39.57536

N, −87.03456 W, 2 June 2006, Field No. JLB06068, INBS 2308; Clay County, Brazil Twp., Otterco, SR 59, Cardonia, (J Burskey, M Herbert, TPS), 39.12525 N, −87.12524 W, 2 June 2006, Field No. JLB06070, INBS 2315; Clay County, Dick Johnson Twp., N BR Otter Creek, Rockruch Church, Perth, (J Burskey, M Herbert, TPS), 39.59304 N, −87.17594 W, 2 June 2006, Field No. JLB06071, INBS 2316; Clay County, Cass Twp., unnamed creek, 700 E Poland, (J Burskey, M, Herbert, T), 39.4429 N, −87.9706 W, 3 June 2006, Field No. JLB06074, INBS 2323; Clay County, Washington Twp., McIntyre Creek, 200 N LAP corner, (J Burskey, M Herbert, T), 39.41501 N, −87.02196 W, 8 June 2006, Field No. JLB06076, INBS 2328; Clay County, Perry Twp., unnamed trib., 300 S Hickory Island, (J Burskey, M Herbert, TPS), 39.34651 N, −87.237943 W, 8 June 2006, Field No. JLB06081, INBS 2339; Clay County, Harrison Twp., unnamed creek, 1100 S Barrick Corner, (J Burskey, M Herbert, TPS), 39.22697 N, −87.09078 W, 9 June 2006, Field No. JLB06084, INBS 2345; Clay County, Harrison Twp., unnamed creek, 900 S Barrick Corner, (J Burskey, M Herbert, TPS), 39.25607 N, −87.0766 W, 3 June 2006, Field No. JLB06083, INBS 2349; Clay County, Harrison Twp., Connelly Ditch, Clay City, (J Burskey, M Herbert, TPS), 39.30834 N, −87.12373 W, 9 June 2006, Field No. JLB06090, INBS 2358; Gibson County, Montgomery Twp., Coffee Bayou, CR 50 S Bridge 3 mi E Skelton, (TP Simon), 38.35591 N, −87.715187 W, 23 May 2005, Field No.TPS05-79; Greene County, Stockton Twp., Buck Creek, 1100W Hoosier, (J Burskey, M Herbert, TP Simon), 39.06857 N, −87.14802 W, 26 May 2006, Field No. JLB06141, INBS 2467; Greene County, Highland Twp., unnamed creek, 650 N Calvertville, (J Burskey, M Herbert, TP Simon), 39.12044 N, −86.901183 W, 26 May 2006, Field No. JLB061358, INBS 2456; Greene County, Stockton Twp., Black Creek, White Rose, (J Burskey, M Herbert, TP Simon), 39.01565 N, −87.21257 W, 3 May 2006, Field No. JLB06146, INBS 2484; Knox County, Widner Twp., Maria Creek, Kerns Rd, Freelandville, (J Burskey, M Herbert, TP Simon), 39.84276 N, −87.36537 W, 11 May 2005, Field No. JLB06032, INBS 2229; Knox County, Widner Twp., Unnamed, BBRD Freelandville, (J Burskey, M Herbert, TP Simon), 38.87047 N, −87.33417 W, 5 June 2006, Field No. JLB06033, INBS 2232; Knox County, Busseron Twp., Marsh Creek, 875 N Oaktown, (J Burskey, M, Herbert, TP Simon), 38.85416 N, −87.40137 W, 7 June 2006, Field No. JLB06038, INBS 2245; Knox County, Ralmyra Twp., Kessingger ditch, SR 50/150, Fritchton, (J Burskey, M Herbert, TP Simon), 38.67584 N, −87.37235 W, 6 Jun 2006, Field No. JLB06040, INBS 2250; Lawrence County, Pleasant Run Twp., Guthrie Creek, D/S SR 50 Bridge, 0.2 from Back Creek Rd. 2.5 mi N Leesville, (TP Simon), 38.874025 N, −86.306256 W, 18 July 2010, Field No. TPS05-70; Monroe County, Monroe Creek, Monroe Creek Rd, 39.1146 N, −86.46969 W, 2 July 2008, Field No. LM63US, INBS 3850; Monroe County, Monroe Creek Tributary, Monroe Creek Rd, 39.1145 N, −86.46818 W, Field No. LM63US, INBS 3860; Monroe County, Monroe Creek Tributary, Huges Rd, Handy, 39.11539 N, −86.47111 W, 2 July 2008, Field No. LM37DS, INBS 3866; Monroe County, Monroe Creek Tributary, Huges Rd, Handy, 39.11612 N, −86.47137 W, 2 July 2008, Field No. LM38DS, INBS 3869; Monroe County, Indian Creek Twp., unnamed trib. of Clear Creek, U/S W. Tom Phillips Rd., between S. Burch Rd. and S. Breeden Rd., 1 mi. S. Stanford, (TP Simon), 39.076031 N, −86.668358 W, 18 July 2010, Field No. TPS92-97; Monroe County, Perry Twp., Clear Creek, d/s Country Club Road bridge 6 mi S Bloomington, (TP Simon), 39.13557 N, −86.5335 W, 24 May 2012, Field No. TPS12-011; Monroe County, Bloomington Twp., Jordan River wetland, u/s Jordan River garage 5 mi E Bloomington, (TP Simon), 39.16779 N, −86.51395 W, 10 May 2012, Field No. TPS12-005; Monroe County, Bloomington Twp., West Br Jackson Creek, u/s Rhorer Road, 6 mi SE Bloomington (TP Simon), 39.13581 N, −86.50811 W, 22 May 2013, Field No. TPS12-010; Owen County, Washington Twp., Rattlesnake Creek, 75 S Southport, (J Burskey, M Herbert, TP Simon), 39.27888 N, −86.80404 W, 12 June 2006, Field No. JLB06151, INBS 2495; Owen County, Franklin Twp., unnamed creek, Hoot Rd, Arney, (J Burskey, M Herbert, TP Simon), 39.24731 N, −86.88777 W, 12 June 2006, Field No. JLB06152, INBS 2496; Owen County, Franklin Twp., unnamed creek, Hoot Rd, Arney, (J Burskey, M Herbert, TP Simon), 39.24731 N, −86.88777 W, 12 June 2006, Field No. JLB06153, INBS 2499; Owen County, Morgan Twp., unnamed creek, 500 N Beamer, (J Burskey, M Herbert, TP Simon), 39.36384 N, −86.91877 W, 12 June 2006, Field No. JLB06155, INBS 2506; Owen County, Harrison Twp., unnamed creek, Quincy Rd, Quincy, (J Burskey, M Herbert, TP Simon), 39.45584 N, −86.70132 W, 13 Jun 2006, Field No. JLB06161, INBS 2523; Owen County, Wayne Twp., unnamed creek, Moore Rd, Carp, (J Burskey, M Herbert, TP Simon), 39.39566 N, −86.71476 W, 13 Jun 2006, Field No. JLB06166, INBS 2536; Ripley County, Washington Twp., South Hogan Creek, CR625E 5 mi. E of Versailles, (TP Simon), 39.076031 N, −86.668358 W, 17 May 2005, Field No. TPS05-60 Burrowers Clear; Sullivan County, Jefferson Twp., Maria Creek, 1050 S Carlisle, (J Burskey, M Herbert, TP Simon), 38.92437 N, −87.33298 W, 22 May 2006, Field No. Field No. JLB06099, INBS 2373; Sullivan County, Curry Twp., Turman Creek, 100 N Farharsburg, (J Burskey, M Herbert, TP Simon), 39.23004 N, −87.4095 W, 23 May 2006, Field No. JLB06107, INBS 2392; Sullivan County, Fairbanks Twp., W Fk Turman Creek, 850 N Scott City, (J Burskey,

M Herbert, TP Simon), 39.2082 N, −87.47838 W, 24 May 2006, Field No. JLB06120, INBS 2422; Vigo County, Fayette Twp., Coal Creek, Libertyville Pl, Libertyville, (J Burskey, M Herbert, TP Simon), 39.58861 N, −87.51873 W, 8 May 2006, Field No. JLB6001, INBS 2140; Vigo County, Fayette Twp., Coal Creek, Shepardsville Rd, Shepardsville, (J Burskey, M Herbert, TP Simon), 39.6064 N, −87.41516 W, 8 May 2006, Field No. JLB6002, INBS 2141; Vigo County, Lost Creek Twp., Snake Creek, Mainstreet, Seelyville, (J Burskey, M Herbert, TP Simon), 39.50836 N, −87.26758 W, 9 May 2006, Field No. JLB06005, INBS 2149; Vigo County, Lost Creek Twp., Miami Garden Rd, Ehrmandale, (J Burskey, M Herbert, TP Simon), 39.54218 N, −87.19978 W, 9 May 2006, Field No.. JLB06007, INBS 2155; Vigo County, Prairie Creek Twp., Prairie Creek, French Drive, (J Burskey, M Herbert, TP Simon), 39.28839 N, −87.50438 W, 10 May 2006, Field No. JLB06014, INBS 2157; Vigo County, Sugar Creek Twp., E Little Sugar Creek, Concannon Rd, Terre Haute, (J Burskey, M Herbert, TP Simon), 39.50947 N, −87.47329 W, 8 May 2006, Field No. JLB06015, INBS 2179; Vigo County, Riley Twp., Little Honey Creek, Old Riley Rd, Riley, (J Burskey, M Herbert, TP Simon), 39.41353 N, −87.34412 W, 10 May 2006, Field No. JLB06021, INBS 2196; Vigo County, Honey Creek Twp., unnamed creek, Eaton Rd, Youngstown, (J Burskey, M Herbert, TP Simon), 39.36852 N, −87.36021 W, 10 May 2006, Field No. JLB06022, INBS 2200.

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ADDITIONAL ULTRASTRUCTURAL OBSERVATIONS OF THE GILL EPITHELIUM OF THE WATER FLEA *DAPHNIA MAGNA* WITH REFERENCE TO IONIC AND MACROMOLECULAR TRANSPORT

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ABSTRACT. The ultrastructural features of the gill epithelium of adult *Daphnia magna* are consistent with their dual function of ion transport from the surrounding medium to the hemolymph and transport of macromolecules from the hemolymph towards the cuticle. The thin cuticle of the gill epithelium displays short, thin epicuticular tubercles and pits. Silver grains penetrate the cuticle in $AgNO_3$ treated specimens. The single layer of flat epithelial cells is of one type only, the dark cells. The epithelial cells display extensive infoldings of the apical and basal plasma membranes associated with mitochondria and delicate, folded lateral membranes enclose narrow intercellular spaces. They have large irregular nuclei with prominent nucleoli. Their cytoplasm is rich in mitochondria, rough and smooth endoplasmic reticulum, microtubules, and vesicles, while Golgi complexes are sparse. The basal cytoplasm displays dense tubular elements, coated vesicles, multivesicular bodies, and lysosomes. These ultrastructural features are characteristic of ion transporting epithelia and of cells engaged in protein and lipid synthesis.

Keywords: *Daphnia magna*, gill epithelium, membrane infoldings, mitochondria, rough endoplasmic reticulum, dense tubules, multivesicular bodies, coated vesicles, cuticle, lysosomes

INTRODUCTION

Animals living in fresh water are faced with the problem of maintaining osmotic pressure and ionic composition of body fluids higher than that of the surrounding medium. To solve this problem aquatic animals have developed mechanisms to absorb ions into their blood (Krogh 1939; Rankin & Davenport 1981). Aquatic larvae of dipterous genera, such as *Aedes* and *Chironomus*, have developed anal papillae that absorb sodium and chloride ions into the hemolymph (Stobbart 1960; Wright 1975). In many crustaceans the gills play a role not only in respiration but also act as osmoregulatory organs (Prosser 1973). In hyperosmotic crustaceans sodium influxes from dilute media have been reported (Sutcliffe 1968; Harris 1970). The activity of Na^+/K^+ ATPase enzyme, which is strongly implicated in sodium transport, has been measured in the pleopod gills of isopod *Idotea wosnesenskii*, acclimated to different media, suggesting their role in inward ionic transport from dilute media (Holliday 1988). Kirschner in his review article (2004), Freire et al. (2008), Bianchini & Wood (2008), and Tsai & Lin (2014) all have implicated gills and other structures in the ion regulatory mechanisms of crustaceans.

Ultrastructural studies of ion transporting organs include larval anal organ/papillae of semiaquatic *Drosophila melanogaster* and aquatic *Chironomus tentans* (Jarial 1987, 1995); chloride cells of *Callibaetis* sp. (Ephemeroptera) (Komnick & Able 1971); labium of *Cenocorixa bifida* (Jarial 2003); "gills" (= endopodites) of terrestrial isopods like *Oniscus* (Kűmmel 1981); and the gills of crayfish, *Pacifastacus leniusculus* (Morse et al. 1970), of euryhaline Chinese crab, *Eriocheir sinensis* (Barra et al. 1983), and of shrimp, *Penaeus japonicus* (Bouaricha et al. 1994). In these studies epithelial cells of these organs were characterized by highly folded apical and basolateral plasma membranes associated with many mitochondria. The extensive plasma membrane infoldings associated with mitochondria provide increased surface area and energy for active ion transport. Similar ultrastructural features are exhibited by transporting epithelia throughout the animal kingdom (Berridge & Oschman 1972).

The ultrastructure of the gill epithelium of *Daphnia magna* acclimated to different salinities was investigated by Kikuchi (1983). The purpose of the present study was to further elucidate the

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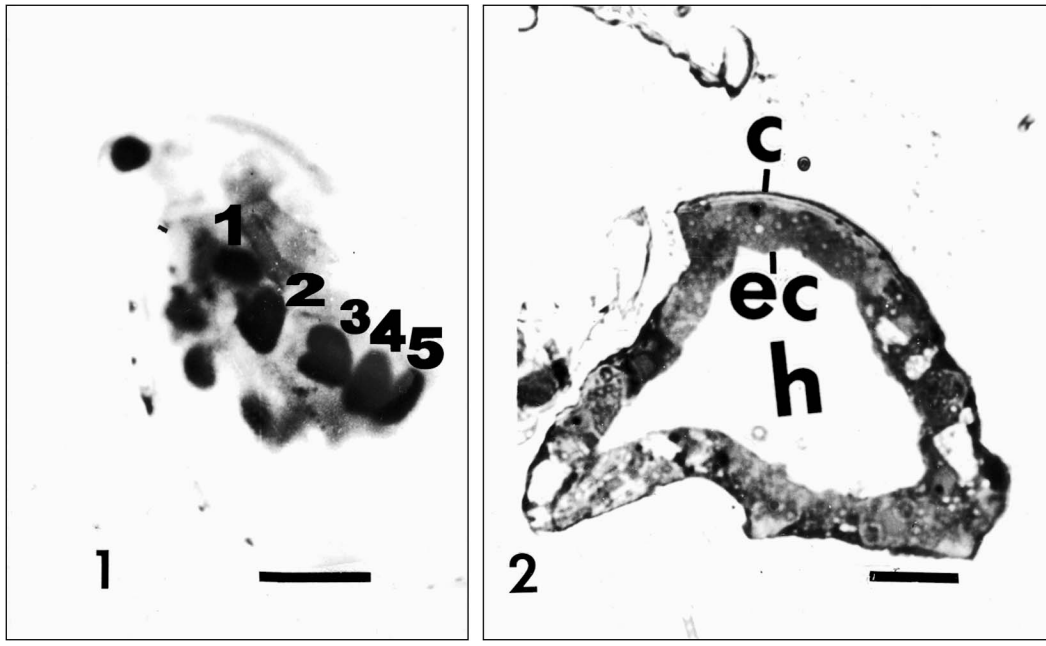


Figures 1 & 2.—Gills of *Daphnia magna*. 1. Lateral view of a specimen treated with $AgNO_3$ solution showing five darkly stained gills (1-5). Scale bar = 0.3 mm. 2. Light micrograph of a cross section (2 μm) of a gill stained with azure II. c = cuticle; ec = epithelial cells; h = hemocoel. Scale bar = 20 μm.

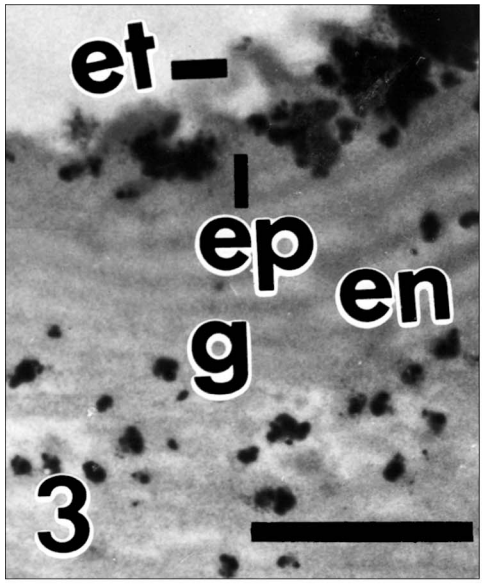
subcellular features of the gill epithelium of *Daphnia magna* and to relate them with their function in ionic uptake from the external medium and transport of macromolecules from the hemolymph to the cuticle.

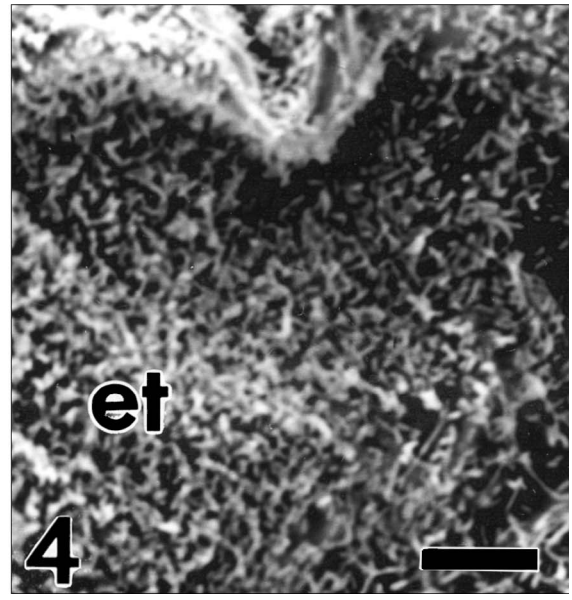
METHODS

Adult *Daphnia magna* used in this study were obtained from Carolina Biological Supply Company, Burlington, NC. Six specimens were used immediately upon arrival. For transmission electron microscopy (TEM) the animals were fixed by immersion at room temperature in 2.5% glutaraldehyde and 2% paraformaldehyde (1:1) in 0.1M cacodylate buffer at pH 7.4 (Millonig 1976), washed in two changes of buffer, post-fixed in 1% osmium tetroxide in the same buffer, and washed in two changes of buffer. Permeability of silver ions was demonstrated by the silver nitrate ($AgNO_3$) method of Ewer & Hattingh (1952). A few animals were quickly rinsed in distilled water and immersed in 1% $AgNO_3$ solution for 5 minutes followed by a quick rinse in distilled water to remove any adherent $AgNO_3$. The animals were then exposed to Kodak D-19 developer to reduce silver to metallic black silver, rinsed in distilled water, photographed, and immersed in the aforementioned fixative mixture. The gills were removed with sharp scissors, dehydrated in an ethanol series, transferred to propylene oxide, embedded in LX112 (Ladd Industries) (Luft 1961), and polymerized at 60°C overnight. Ultrathin sections were cut on a Porter-Blum MT-2 ultra-microtome, stained with uranyl acetate and lead citrate, and examined with a Hitachi 600 TEM. For scanning electron microscopy (SEM), similarly fixed material was chemically dried in hexamethyl-disilazane (Polysciences), coated with gold/palladium, and examined with a Cambridge S-90 SEM. Thick (2 μm) sections were cut, stained with azure II, and examined with an American optical series 20 light microscope (LM).

RESULTS

Adult *Daphnia magna* have five pairs of small sac-like gills that protrude externally from their thoracic appendages. They become darkly stained when exposed to dilute silver nitrate solution (Fig. 1). Each gill measures approximately 110 μm in its long axis. The gill epithelium is composed of a single layer of large, flattened





Figures 3 & 4.—Features of the gill cuticle. 3. Electron micrograph of the gill cuticle from a $AgNO_3$ treated specimen composed of two layers, an inner laminated endocuticle (en) and outer epicuticle (ep) bearing short, narrow epicuticular tubercles (et) facing externally. Silver grains (g) penetrate both layers of the cuticle. Scale bar = 1 μm. 4. Scanning electron micrograph of the external surface of a gill displaying small epicuticular tubercles (et). Scale bar = 10 μm.

epithelial cells containing dense cytoplasm. Lateral membranes of these cells are not distinguishable by light microscopy. The epithelial cells basally are in contact with the hemocoel while the cells apices are covered by a thin cuticle measuring about 1.5 μm in thickness (Fig. 2). In electron micrographs, the cuticle is composed of two layers, an inner lamellate endocuticle and an outer epicuticle (Fig. 3). In transmission and scanning micrographs, the epicuticle displays short, narrow epicuticular tubercles and pits (Figs. 3, 4, 5 inset, 9). In specimens exposed to silver nitrate solution, the silver grains are seen penetrating both layers of the cuticle to reach underlying epithelium (Fig. 3). The apical plasma membrane of the epithelial cells located under the thin cuticle is organized into numerous narrow, closely spaced infoldings, their tips closely associated with many large mitochondria (Fig 5). The cytoplasm contains numerous mitochondria, smooth and rough endoplasmic reticulum, vesicles, microtubules, ribosomes, and dense tubules (Figs. 5, 7, 9). The epithelial cells contain centrally placed large, irregularly shaped nuclei with prominent nucleoli (Fig. 6). The basal plasma membrane (facing the hemocoel) is supported by a conspicuous basal lamina that is elaborately infolded to form invaginations closely associated with mitochondria. These basal membrane infoldings form a labyrinth of intracellular channels that anastomose freely in the basal cytoplasm (Figs. 8, 9). The basal cytoplasm contains free ribosomes, abundant rough endoplasmic reticulum in the form of whorls and cisternae, multi-vesicular bodies, dense tubules, coated and smooth vesicles, and lysosomes, while the Golgi complexes appear sparse (Figs. 7, 9). The delicate lateral membranes are folded forming interdigitations among adjacent epithelial cells. They enclose narrow intercellular spaces and are joined apparently by septate desmosomes that link the lateral membranes (Figs. 5, 7, 8). Figure 9 summarizes the ultrastructural organization of the gill epithelium of *D. magna* as revealed by this study.

DISCUSSION

The silver staining technique has been used to locate ion transporting organs in a number of aquatic insects and crustaceans (Krogh 1939; Ewer & Hattingh 1952; Copeland 1967; Jarial et al. 1969; Morse et al. 1970; Barra et al.

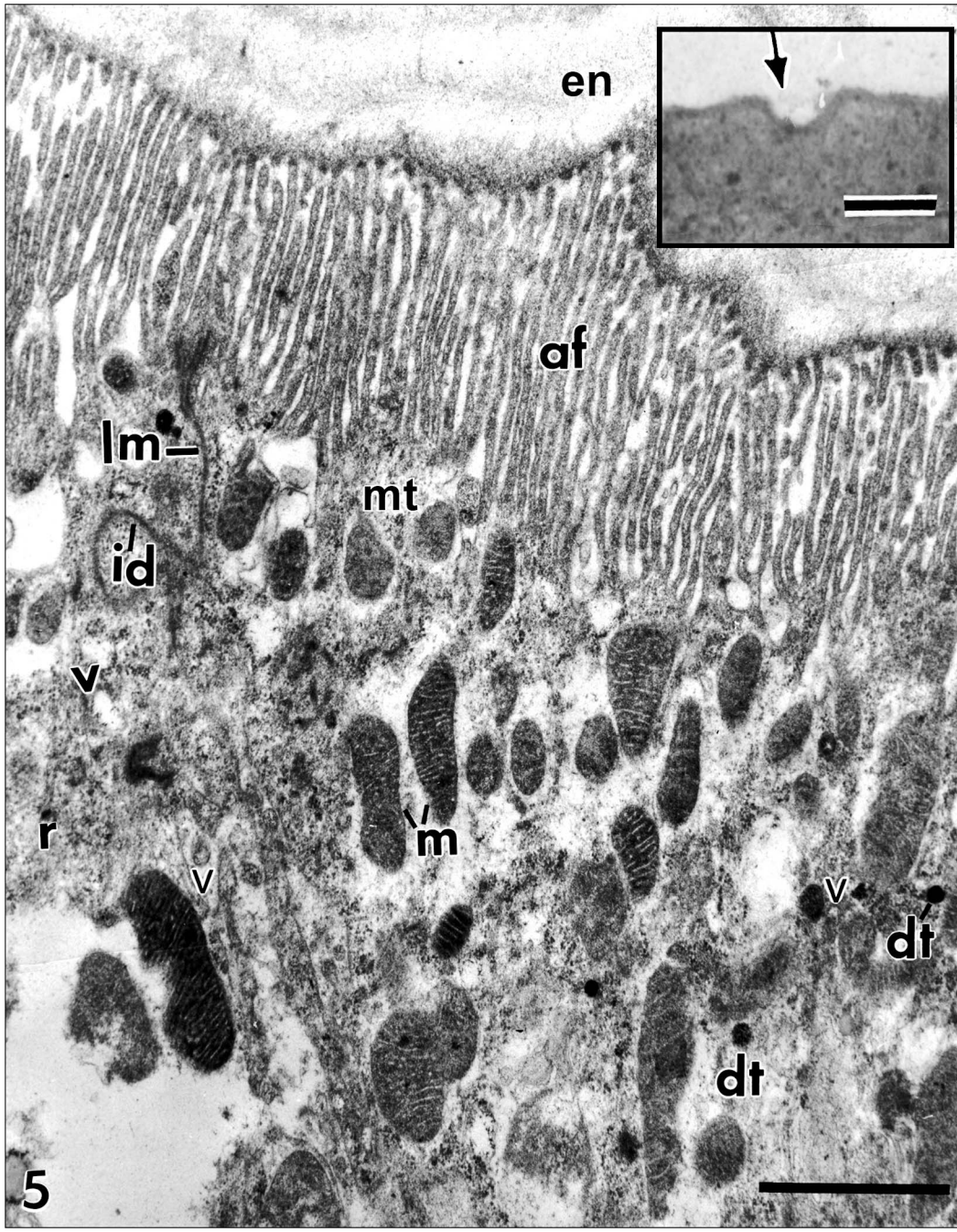
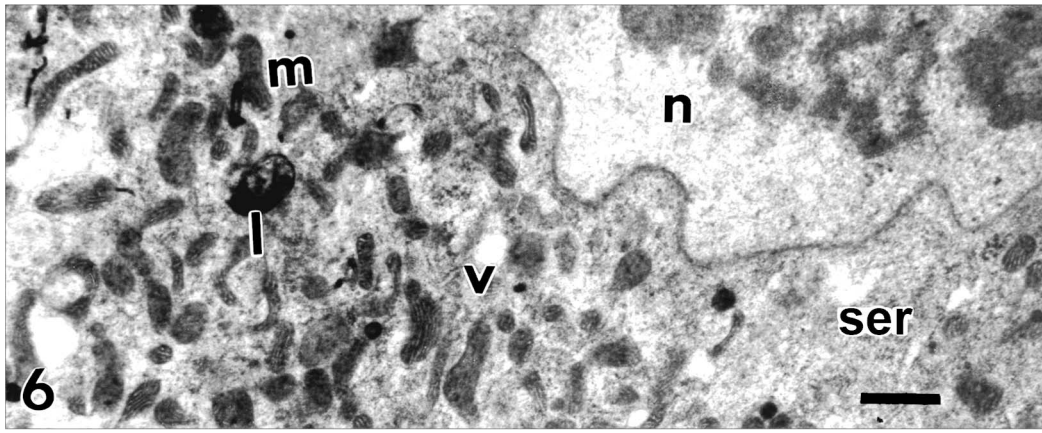
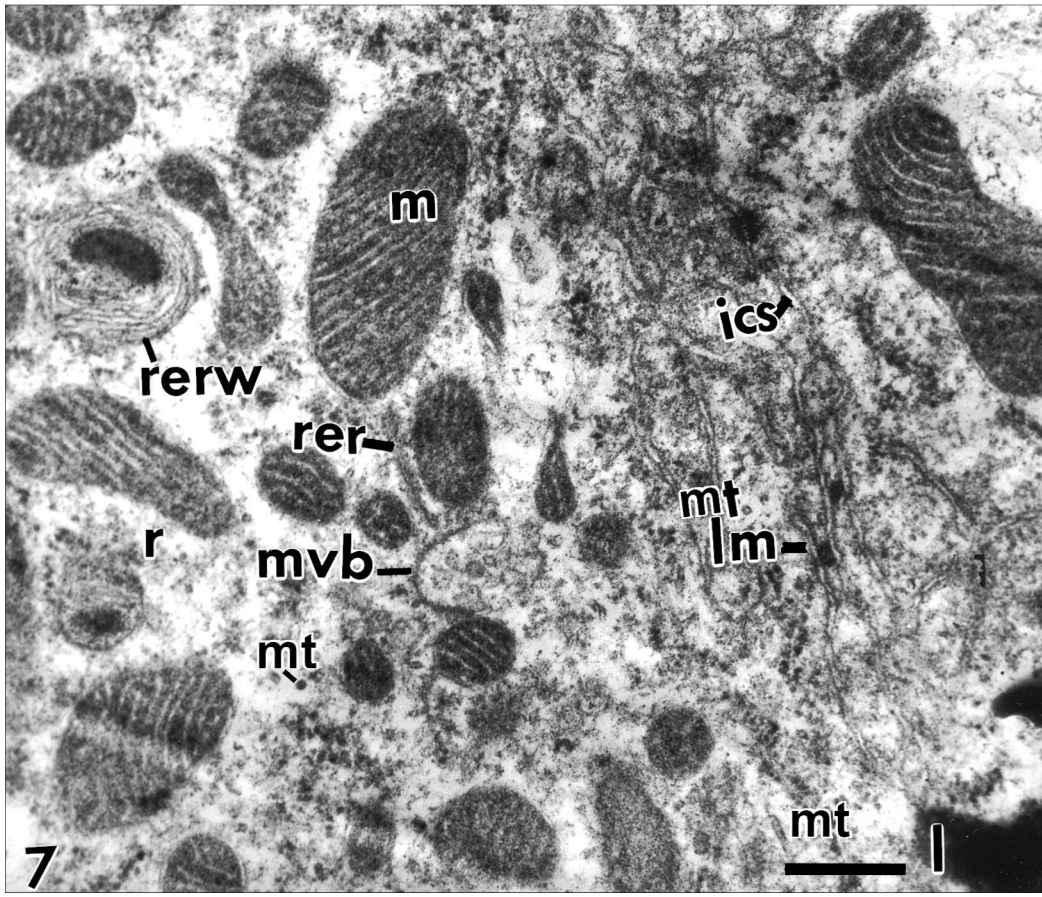


Figure 5.—Electron micrograph of apical region of a gill epithelial cell showing numerous apical membrane infoldings (af) and large mitochondria (m). en = endocuticle; dt = dense tubules; id = interdigitation between adjacent cells; lm = lateral membranes; r = ribosomes; mt = microtubules; v = vesicle. Scale bar = 0.5 μm. Inset: A portion of epicuticle showing a pit (arrow). Scale bar = 0.2 μm.





Figures 6 & 7.—Cytoplasmic detail of the central region of gill epithelial cells. 6. Nuclear region of an epithelial cell showing a large, irregular nucleus (n), numerous mitochondria (m), smooth endoplasmic reticulum (ser), lysosomes (l), and vesicles (v) in the cytoplasm. Scale bar = 1 μm. 7. Higher magnification electron micrograph shows large mitochondria (m), rough endoplasmic reticulum whorls (rerw), and cisterns (rer) in the cytoplasm. ics = intercellular space; l = lysosome; lm = lateral membrane; mt = microtubules; mvb = multivesicular body; r = ribosomes. Scale bar = 0.25 μm.

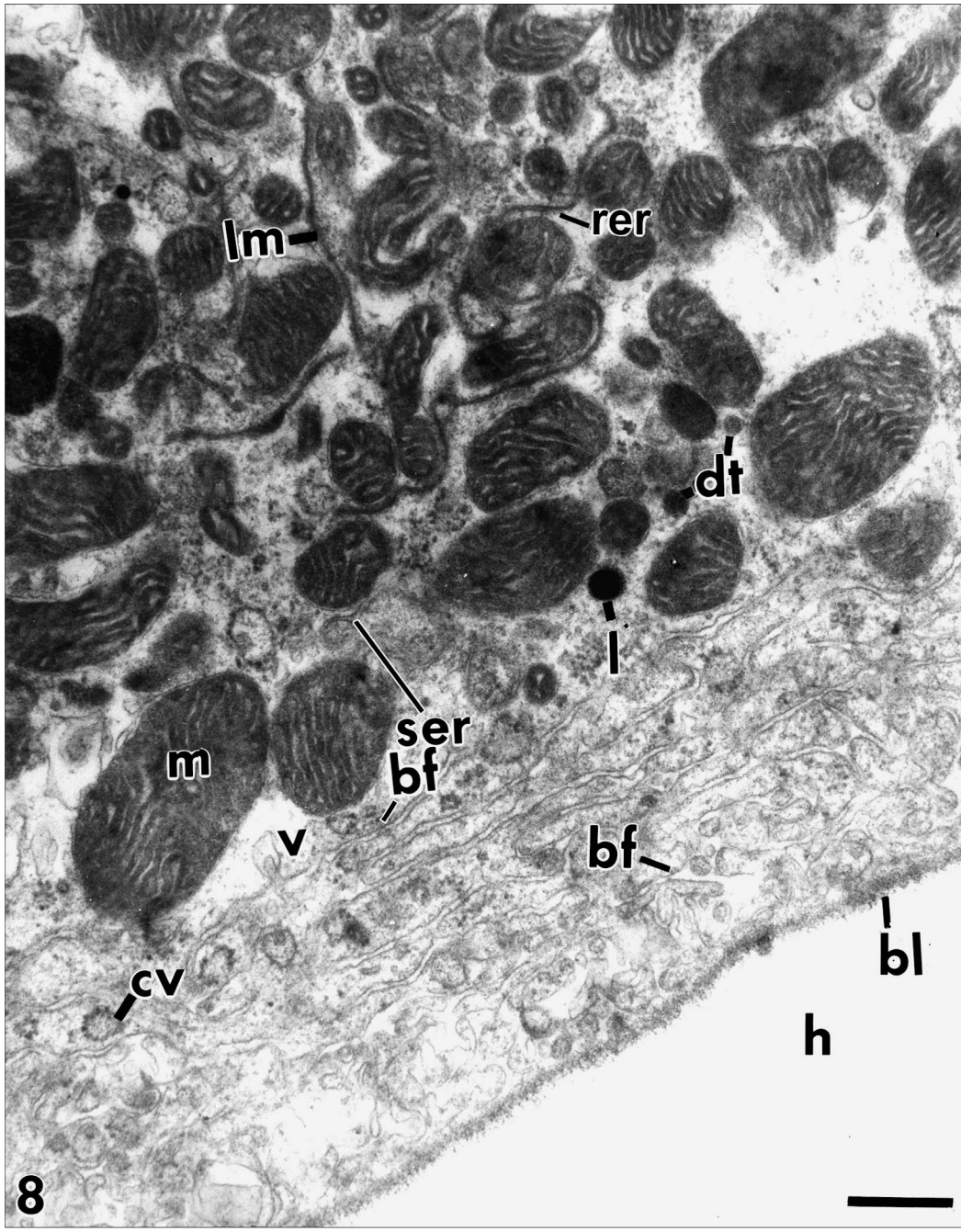


Figure 8.—Electron micrograph of the basal region of a gill epithelial cell displaying basal membrane infoldings (bf) resting on a prominent basal lamina (bl). cv = coated vesicle; dt = dense tubule; h = hemocoel; l = lysosome; lm = lateral membrane; m = mitochondrion; ser = smooth endoplasmic reticulum; v = smooth vesicle. Scale bar = 0.25 μm.

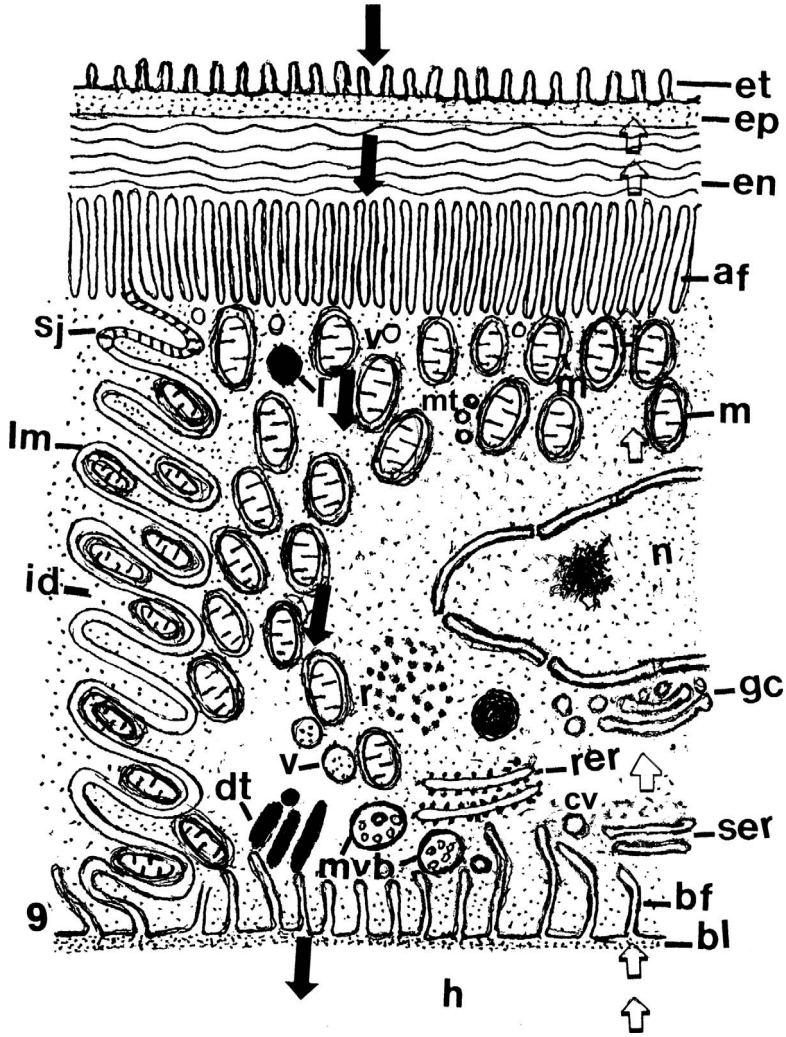


Figure 9.—Diagrammatic representation of the ultrastructural features of a gill epithelial cell of *Daphnia magna*. Solid arrows represent transport of ions from external medium into the epithelial cells and open arrows represent transport of macromolecules from the hemocoel into the epithelial cells and the cuticle. af = apical membrane infoldings; bf = basal membrane infoldings; bl = basal lamina; cv = coated vesicles; dt = dense tubules; en = endocuticle; ep = epicuticle; et = epicuticular tubercles; gc = golgi complex; h = hemocoel; id interdigitations; l = lysosome; lm = lateral membrane; m = mitochondria; mt = microtubules; mvb = multivesicular bodies; n = nucleus; r = ribosomes; rer = rough endoplasmic reticulum; ser = smooth endoplasmic reticulum; sj = septate junction; v = vesicles.

1983; Kikuchi 1983; Holliday 1998). X-ray microanalysis has demonstrated that in tissues stained with $AgNO_3$, chloride ions released from the tissues were captured as AgCl precipitates on the tissue surfaces (Barra et al. 1983). Silver staining of the gills and ultrastructural localization of silver grains in the epicuticle, endocuticle, and apical region of the epithelial cells suggest that the gill epithelium of *D. magna* is permeable to chloride ions and possibly to other ions.

The ultrastructural features of the gill epithelium of *D. magna* are essentially similar to those of the dark cells of *Daphnia* described by Kikuchi (1983), but the light cells were not observed. Differences noted by us include the absence of large cytoplasmic tubules or any connection of such structures with the basal or lateral

membranes. This may be due to the different fixative used in our study. Also, in the current study the intercellular spaces bounded by lateral cell membranes appear too narrow to play any significant role in fluid transport. Lastly, the basal membrane infoldings of the epithelial cells are relatively short.

Ultrastructural features of the gill epithelium of *D. magna* include extensive infoldings of the apical and basal plasma membranes, as well as folded lateral membranes that are closely associated with many large mitochondria. The later provide a large surface area and energy for fluid transport. Such ultrastructural features are commonly exhibited by epithelia that specialize in ion and water transport from dilute media (Berridge & Oschman 1972). In contrast, the salt-secreting gill epithelial cells of brine shrimp, *Artemia salina* (Copeland 1967), the epithelial cells of the pereopodal discoid osmoregulatory organ of esturine amphoid, *Melita setiflagella* (Kikuchi & Matsumasa 1995), and the epithelial cells of the anal papillae of the saltwater mosquito larva, *Aedes campestris* (Meredith & Phillips 1973), are characterized by shallow apical membrane infoldings that are not closely associated with mitochondria. These are further characterized by deep extensive basal membrane infoldings associated with many mitochondria that apparently play a role in removing excess salts from the hemolymph.

The ultrastructural organization of the gill epithelium of *D. magna* bears close resemblance to that of anal papillae of aquatic larval forms of insects (Copeland 1964; Jarial 1995) and the gills of crustacea (Barra et al. 1983; Bouaricha et al. 1994) that engage in the active transport of ions from dilute media (Stobbart 1960; Sutcliffe 1968).

The unique ultrastructural features of the gill epithelial cells of *D. magna* are the presence of dense tubular elements, coated vesicles, multivesicular bodies, and lysosomes in the basal cytoplasm. These features are characteristic of cells that take up colloidal materials and synthesize proteins (Miller 1960; Maunsbach 1966; Locke 1974). The network of basal membrane infoldings allows the uptake of macromolecules from the hemolymph into the epithelial cells. Once in the epithelial cells, the macromolecules are degraded and used in the synthesis of proteins in the well-developed rough endoplasmic reticulum (Palade 1975) and lipid synthesis in the smooth endoplasmic reticulum (Cormack 1987) to help fuel cellular metabolism and contribute to the protein and lipid constituents of the cuticle covering the gill epithelium (Hadley 1994; Neville 1998). The microtubules function in maintaining the shape of the gill epithelial cells and apparently play a role in moving organelles like vesicles in the cytoplasm (Mescher 2010).

In conclusion, the ultrastructure of the gill epithelium of *D. magna* suggests that this structure is engaged in the active transport of ions from the medium into the hemolymph to maintain osmotic constancy of the body fluids, as well as trans-epithelial transport of hemolymph macromolecules for synthesis of protein and lipid components of the cuticle covering these organs. This study provides the basis for future functional studies that may help elucidate further how the gill epithelium works.

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