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*Proceedings* of the Indiana Academy of Science

**Volume 122 Number 1 2013**



*Proceedings* of the INDIANA ACADEMY OF SCIENCE 2013 VOLUME 122, NUMBER 1

INDIANA ACADEMY of SCIENCE 1885

# *Proceedings* of the Indiana Academy of Science

**VOLUME 122 2013 NUMBER 1**

# *PROCEEDINGS* OF THE INDIANA ACADEMY OF SCIENCE

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*Cover*: *Galerucella* beetles are proving to be an effective biocontrol agent. They complete their life cycle (egg, larva, and adults stages) on the leaves of the invasive exotic plant purple loosestrife (*Lythrum salicaria*).

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Publication date: 7 April 2014

**This paper meets the requirement of ANSI/NISO Z39.48-1992 (Permanence of Paper).**

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2013. Proceedings of the Indiana Academy of Science 122(1):1–7

# IMPACTS OF SEWAGE WASTE WATER ON FEMINIZATION AND VITELLOGENIN EXPRESSION IN MALE FATHEAD MINNOWS

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**ABSTRACT.** Estrogenic compounds are commonly found in wastewater effluents. Exposure of male fish to these chemicals can lead to 'feminization', including decrease in secondary sex characteristics and production of female-specific proteins such as vitellogenin (VTG). We hypothesized that upon exposure to wastewater from the Muncie Water Pollution Control Facility, Indiana, adult male fathead minnows (*Pimephales promelas*) would respond with a decrease in secondary sex characteristics and increased expression of *vtg* if the effluents contained sufficient estrogens. Adult males were caged at two sites in the West Fork White River: the downstream group was placed directly below the outflow and the upstream group was placed 0.25 km upstream. A third group was housed indoors in aquaria and served as a control. After 21 d, body and organ measurements, secondary sex characteristics, and liver *vtg* gene expression were assessed. While no significant differences were observed in secondary sex characteristics between study groups, 'downstream' males had larger liver somatic index values and showed an up-regulation of liver *vtg* relative to the other two groups. Although our results agree with a previous study in this same area that found 'feminization' of native populations of bluntnose minnows (*P. notatus*), the estrogenic compounds that elicited this response remain unknown.

**Keywords:** Feminization, fish, estrogens, wastewater, White River, Indiana

## INTRODUCTION

An expanding amount of research suggests that effluents from domestic wastewater treatment plants (WWTP) can contain natural (estrone, E1; 17β-estradiol, E2; and estrone, E3) and synthetic (17α-ethinylestradiol, EE2) estrogens (see Limpiyakorn et al. 2011 for a review). The latter form of estrogen is the main ingredient of oral contraceptives and is considered to be the most potent environmental estrogen (Clouzot et al. 2008). Exposure of male fish to estrogens can result in a range of effects from complete sex reversal in the most severe cases to different degrees of 'feminization', including intersex (i.e. testes with oocytes) and decreased expression of secondary sex characteristics (Lange et al. 2012). In more extensive studies with fathead minnows (*Pimephales promelas*) exposed to municipal wastewater, greatly reduced reproductive capacities were observed (Rickwood et al. 2008, Thorpe et al. 2009). Further, female-biased sex ratios have been observed in feral populations of other teleost species such as white suckers (*Catostomus commersoni*) exposed to WWTP effluents (Vajda et al. 2008). The estrogenic ecological effects of these types of effluents are not well understood and are a cause for concern.

A commonly used biomarker of exposure to estrogens in fish is vitellogenin (VTG). Vitellogenin is a phospholipoprotein synthesized in the liver of egg-laying females after estrogen stimulus and is essential in the production of egg yolk proteins and thus embryo survival (Specker and Sullivan 1993). While VTG receptors are present in male fish, the gene is silent unless triggered in the presence of sufficient estrogen concentrations (Maitre et al. 1985; Copeland et al. 1986; LeGuellec et al. 1988). Thus VTG can serve as an efficient marker of estrogenic contamination in aquatic systems (Sumpter and Jobling 1995). Furthermore, induction of VTG in male fish has been

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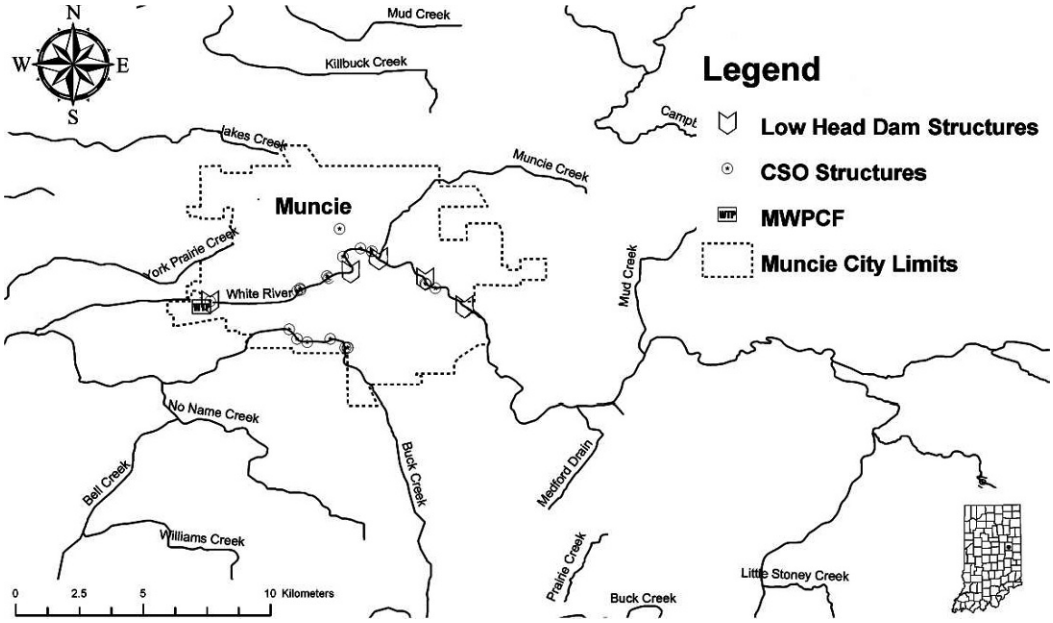


Figure 1.—Map of Muncie, Indiana (dotted lines), showing the location of the combined sewer overflow (CSO) and Muncie Water Pollution Control Facility (MWPCF) along the White River. Fish in this study were caged 0.25 km upstream and 5 m downstream of MWPCF. Adapted from Doll (2011).

associated with decreased sperm quality and decreased fertility, potentially resulting in poor reproductive success (Kidd et al. 2007).

The only study evaluating the potential effects of effluents from a WWTP in Indiana was published by Doll (2011). In this study, free-ranging adult bluntnose minnows (*Pimephales notatus*) were sampled from the West Fork White River (hereafter White River) at different distances up- and downstream from the Muncie Water Pollution Control Facility (MWPCF), Delaware County, Indiana. This facility is a conventional activated sludge treatment plant that discharges an average of 18 MGD (million gallons per day) into the White River. The MWPCF is located at river kilometer 501.5 where the drainage area is $\sim$ 635 km$^2$ (Hoggatt 1975). The MWPCF serves a population of 67,430 and includes one hospital and Ball State University. In addition, 13 combined sewer overflows are located on the White River within Muncie City limits. Secondary sex characteristics of male minnows collected downstream of the MWPCF had an average of 31.3% fewer tubercles and a 37.5% lower tubercle score compared to those sampled upstream of Muncie (Doll 2011). These results suggest that minnows were being exposed to estrogen-like compounds likely being released from the MWPCF and combined sewage overflows.

The main goal of our study was to further determine the potential estrogenicity of the MWPCF effluent. To do this we conducted an *in situ* exposure using fathead minnows, a commonly used species in ecotoxicology studies. We hypothesized that if estrogens are present at sufficient concentrations in this effluent, male fish caged downstream from the effluent discharge would respond with a decrease in expression of secondary sex characteristics and an induction of VTG.

## METHODS

**Experimental design.**—On June 1, 2011 adult fathead minnow males were exposed for 21 d to three different conditions: 1) caged (n = 4) located approximately 5 m below the MWPCF effluent; 2) caged (n = 3) 0.25 km upstream of the MWPCF (Fig. 1); or 3) kept indoors in the Bureau of Water Quality Laboratory ($\sim$ 38-L glass aquaria, n =4) containing reconstituted reverse osmosis water (APHA 2005) which was changed three times per week. Minnows in cages were allowed to eat naturally occurring food in the river whereas those in the tanks were fed 1 g of frozen brine shrimp every two

days to keep stress at a minimum. These three conditions are referred herein to as downstream, upstream, and control, respectively. This exposure length and species were chosen because they are commonly used in ecotoxicology for assessing impacts of endocrine disrupting compounds (US EPA 2002). Cages were galvanized round minnow traps 419 mm long, 222 mm wide at center, 178 mm wide at ends, and 6 mm wire bar mesh. Openings of each trap were closed off with 3 mm bar mesh fiberglass screen. Minnow cages and tanks contained three to five male adult males each. Dissolved oxygen (DO), temperature, and conductivity (river samples only) were measured with a portable YSI® meter (model 556MPS); turbidity (river samples only) was measured with an Oakton® turbidity meter (model T-100); ammonia, nitrite, and nitrate (indoor tanks only) was measured using Hach® water quality test strips (Hach, Loeveland, CO, USA) daily Monday through Friday. Meters were calibrated and water quality was measured daily. At the conclusion of the exposure, fish were euthanized with MS-222 (300 mg/L) and processed for data collection.

**Data collection.**—All fish were measured (total length, mm), weighed (g), and gonads and livers dissected and weighed (± 0.01 g) for determination of gonadosomatic index (GSI) and hepatosomatic index (HSI). These indices were calculated by dividing the weight of the organ by the weight of the fish multiplied by 100. A small section of each liver was placed in RNA*later* (Qiagen, Valencia, CA, USA) and stored at −80°C for analysis of *vtg* expression as described below. Secondary sex characters were measured from each male fish as already described (US EPA 2002; US EPA 2007; Doll 2011), which included tubercle counts and tubercle and fatpad scoring. Tubercle counts were the total number of tubercles present. The size of each tubercle was qualitatively ranked as 1 = present, 2 = enlarged, and 3 = pronounced. The tubercle score was the sum of all individual tubercle ranks per individual fish. The fatpad score is a qualitative ranking and was assigned a 1 = no fatpad visible, 2 = small fatpad evident, 3 = fatpad is clearly visible and is just above body surface, 4 = fatpad is prominent and is clearly above the body surface but not overhanging, and 5 = fatpad is very prominent and overhangs the body surface.

**Vitellogenin analysis.**—The expression of the gene *vtg*, which codes for the VTG protein, was measured as a marker of estrogen exposure. RNA was extracted (TriSure, Bioline, Taunton, MA, USA), quantified (Nanodrop 1000, Thermo Fisher Scientific, Waltham, MA, USA), DNase-treated (Fermentas Inc., Glen Burnie, MD, USA) and reverse transcribed to cDNA (Applied Biosystems, Foster City, CA, USA). The 260/280 ratio was used as an indicator of RNA quality, and only samples with a ratio of 1.8 or higher were used in gene analysis (33 samples of the 41 total). Elongation factor 1 (*ef1*) was used as the housekeeping gene. Primers for *vtg* and *ef1* were selected from primary literature (Biales et al. 2007; Mager et al. 2008) and purchased through Integrated DNA Technologies (Coralville, IA, USA). Polymerase chain reaction (PCR) products were sequenced on an ABI 3700 (Applied Biosystems, Foster City, CA, USA) at Purdue University's Genomics Core to validate specificity of gene amplification. Gene expression analysis was conducted by quantitative PCR (qPCR) on a Bio-Rad iQ5 (Bio-Rad Laboratories, Hercules, CA, USA) using a DyNAmo™ SYBR® green qPCR kit (Ratastie 2, 01620 Vantaa, Finland). Reactions were comprised of 6.0 µL 2X master mix, 360 nM primer, cDNA template synthesized from 45 ng of DNAse-treated total RNA, and molecular-grade water (12 µL total). Conditions used to amplify samples were: 94°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec for 40 amplification cycles. No template controls, negative reverse transcriptase controls, and a melt curve analysis were performed for each primer to determine whether nonspecific products were being amplified. Samples were rerun if standard deviation of the Ct values between duplicates was $> 0.5$. Expression of target genes was normalized relative to the expression of *elf1* ($\Delta Ct = Ct_{vtg\ gene} - Ct_{efl1}$). The relative expression of the target gene in experimental groups compared to the control group was quantified by the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001).

**Data analysis.**—Water-quality parameters, *vtg* hepatic expression, and all morphological measurements with exception of tubercle and fatpad scores, were compared across sites using analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Tubercle and fatpad scores were compared across groups using chi-square. All tests were conducted using

Table 1.—Water quality taken from the White River in Muncie, Indiana, during the time of fathead minnow cage deployment (June 2 – June 22, 2011). Different small letters denote significant differences between the means (t-test, $p = < 0.05$). *Not measured.

| | Temperature(°C) | Conductivity(μS) | D.O.(mg/l) | Turbidity(NTU) |
|---|---|---|---|---|
| **Upstream (n = 13)** | | | | |
| Mean | 20.8[a] | 0.46[a] | 8.20[ac] | 63.5 |
| Median | 19.9 | 0.48 | 8.40 | 24.8 |
| Range | 5.60 | 0.20 | 2.50 | 283 |
| St. Dev. | 1.76 | 0.06 | 0.85 | 85.8 |
| **Downstream (n = 14)** | | | | |
| Mean | 18.9[b] | 0.81[b] | 9.20[b] | 34.0 |
| Median | 18.7 | 0.79 | 9.10 | 15.1 |
| Range | 2.70 | 0.63 | 1.80 | 149 |
| St. Dev. | 0.83 | 0.18 | 0.53 | 43.7 |
| **Control (n = 15)** | | | | |
| Mean | 22.0[c] | -[*] | 8.50[ac] | - |
| Median | 22.3 | - | 8.50 | - |
| Range | 3.41 | - | 1.52 | - |
| St. Dev. | 0.95 | - | 0.42 | - |

SAS 9.3, and significance was declared at $\alpha = 0.05$.

## RESULTS

**Water quality.**—There were significant differences in water temperature and DO across the three treatments; however, overall differences were relatively small (ranges of temperature 18–21°C and DO 6.6–8.9 mg/l) (Table 1). Conductivity was almost twice as high in the 'downstream' site compared to the 'upstream' site (0.81 ± 0.18 μS and 0.46 ± 0.06 μS, respectively). Nitrogenous compounds were measured in the control tanks only. Nitrate and nitrites were not detected, and total ammonia averaged 0.44 ± 0.32 mg/L during the experiment.

**Morphometric measurements.**—There were no mortalities during this experiment. At the time of dissection, four fish from the 'downstream' and two fish from the 'upstream' site were classified as females and were eliminated from further analyses. The 'upstream' fish were larger than the other two groups, but not significantly (Table 2). The only parameter that differed among treatments was LSI, with males from the 'downstream' site having the largest value (upstream 1.38 ± 0.8; downstream 2.04 ± 0.8; control 0.96 ± 0.3).

**Secondary sex characteristics.**—Secondary sex characteristics of male fathead minnows are summarized in Table 2. No significant differences were observed across treatments. However, the 'control' group had the highest tubercle count, fatpad score and weight of all the groups.

**Vitellogenin expression.**—While upstream males showed no significant difference in *vtg* expression compared to control fish, downstream males displayed an up-regulation in the expression of this gene (Figure 2).

## DISCUSSION

We documented a significant induction of *vtg* in male fathead minnows after a 21-d exposure to the MWPCF effluent. These males also had significantly higher LSI than the upstream and control group. However, secondary sex characteristics did not significantly differ among the three treatments. Although there were differences in water quality parameters between treatment groups, overall differences were relatively small (ranges of temperature 18–21°C and DO 6.6–8.9 mg/l; Table 1). These values fell well within acceptable limits for fish and would not have induced the differences in *vtg* expression and LSI observed. These results suggest presence and exposure of fish to estrogens or estrogen-mimic compounds. To our knowledge, the present study is only the second published evaluating the potential impacts of sewage effluents on fish in Indiana. The first study in Indiana also showed evidence of feminizing contaminants being released by

Table 2.—Means (± SD) of morphological parameters measured in male fathead minnows at the end of the study. Gonadosomatic index (GSI), liver somatic index (LSI), interocular distance (ID), widest head width (HW), tubercle count (TC), fatpad score (FPS), and fatpad weight (FPW). * Indicates significance p = 0.0001. [a] = only one fish measured.

| Location | Total weight (g) | Total length (mm) | GSI (%) | LSI (%) | ID (mm) | HW (mm) | TC | FPS | FPW (g) |
|---|---|---|---|---|---|---|---|---|---|
| Upstream | 3.99 | 71.6 | 0.86 | 1.38 | 6.63 | 8.5 | 12.1 | 1.25 | 0.04[a] |
| (n = 8) | (0.8) | (4.8) | (0.4) | (0.8) | (0.9) | (0.8) | (8.4) | (0.5) | |
| Downstream | 3.41 | 66.6 | 1.34 | 2.04* | 6.00 | 7.89 | 15.6 | 1.87 | 0.02 |
| (n = 15) | (0.6) | (2.6) | (1.0) | (0.8) | (0.7) | (0.7) | (3.7) | (0.8) | (0.02) |
| Control | 3.33 | 65.7 | 0.97 | 0.96 | 6.22 | 8.00 | 16.1 | 2.00 | 0.08 |
| (n = 18) | (1.1) | (6.7) | (0.4) | (0.3) | (0.8) | (1.0) | (5.9) | (1.0) | (0.08) |

the MWPCF, as wild bluntnose minnows exposed to effluent at the downstream site were observed to have reduced expression of secondary sex characteristics (Doll 2011).

Hundreds of studies around the country and the world have quantified natural and synthetic estrogens in effluents from sewage waste water treatment plants (Limpiyakorn et al. 2011). Although in most cases estrogens are found at very low concentrations (low ng/l), feminization and complete reproductive failure of male fathead minnows has been shown after chronic exposure to < 5 ng/l to EE2 (Parrott and Blunt 2005; Filby et al. 2007; Kidd et al. 2007). This reproductive failure in males can result from alterations in testicular development, but it can also be the result of more subtle effects such as VTG production. Indeed, VTG production by males can lead to decreased fertility and even kidney pathology (Zha et al. 2008). Alterations in behavior have also been reported after exposure of male fish to estrogens (Dammann et al. 2011). Fathead minnow males caged downstream from the MWPCF also had enlarged livers. Since VTG is produced by the liver, this hepatomegalia is consistent with these fish being exposed to estrogens (Gunnarsson et al. 2009). VTG induction and LSI have been shown to have a positive relationship in fathead minnows (Barber et al. 2007).

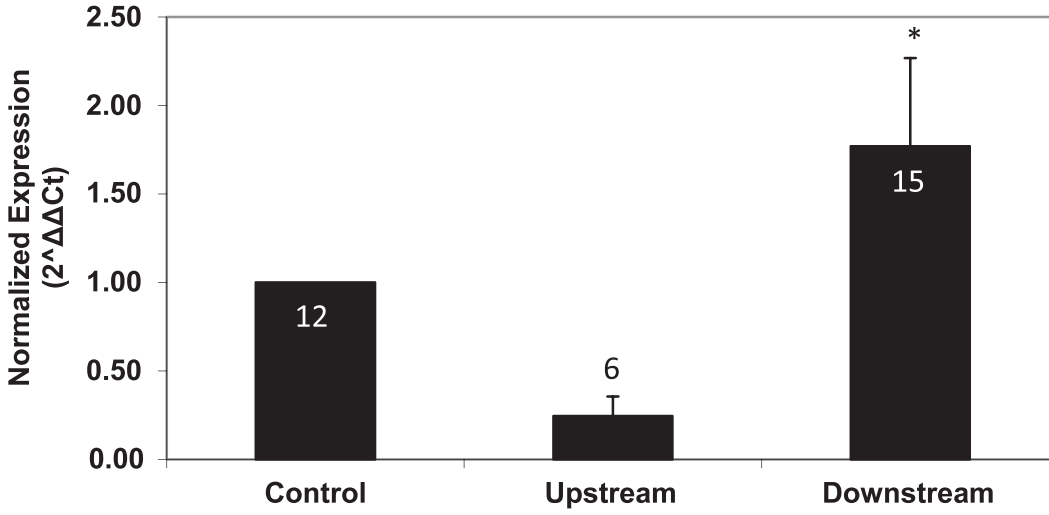


Figure 2.—Mean ± SD of relative *vtg* gene expression from livers of male fathead minnows caged downstream and upstream from the Muncie Water Pollution Control Facility (MWPCF) for 21 d in relation to the controls which were kept indoors in aquaria for the same length of time. Males downstream responded with an up- regulation (denoted with an asterisk; p = 0.05) in *vtg* expression compared back to the control males (assigned a value of 1). Numbers on bars indicate sample sizes.

Results of this study showed indications of feminization downstream from the MWPCF. The lack of feminization in male secondary sex characteristics indicates that the estrogenic contaminants are not potent enough to elicit feminizing effects after a relatively short exposure (i.e. 21 d). However, these contaminants are still of concern for chronic fish exposures, as seen with the decreased expression of secondary sex characteristics in feral fish found at this downstream site (Doll 2011).

In conclusion, effluents from the MWPCF are likely releasing estrogens into the White River. More studies are needed that measure the types(s) and concentrations of estrogens and potentially other types of contaminants from this effluent as well as the potential impacts on natural populations of fish and other aquatic organisms inhabiting this site.

## ACKNOWLEDGEMENTS

We thank the Muncie Sanitary District Bureau of Water Quality for funding this project. We also thank Kelly Sudhoff, Tylenia Oliphant, and Brandon Hollsinger for their help in the field. We are grateful to Rick Conrad and the two anonymous reviewers for helpful suggestions that greatly improved this manuscript.

Finally we thank Greg Bright, Commonwealth Biomonitoring, for providing the fathead minnows from their aquatic toxicity testing stock.

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*Manuscript received 15 December 2012, revised 18 May 2013.*

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# TEMPORAL AND SIZE-RELATED TRENDS IN FOOD HABITS OF INTRODUCED WESTERN MOSQUITOFISH AND NATIVE TOPMINNOWS

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**ABSTRACT.** Western mosquitofish (*Gambusia affinis*) are stocked in Indiana waters for the biological control of mosquitoes. However, this species has the potential to negatively impact native fishes. We examined the food habits and diet overlap of adult western mosquitofish, northern starhead topminnow (*Fundulus dispar*), northern studfish (*F. catenatus*), blackstripe topminnow (*F. notatus*), and banded killifish (*F. diaphanus*) in Indiana from April through October 2005 to evaluate trophic resource use by month and body length. Food habits for each species were similar, with the largest percentage of the diet composed of zooplankton (Cladocera) and non-culicid diptera (Chironomidae and Ceratopagonidae). There was no trend in the percentage of culicids (mosquito larvae) consumed by species, regardless of month and body length (range, 0–27%). Diet overlap index values between western mosquitofish and the topminnow species were high but there was no clear trend, regardless of month (range, 0.25–0.87) or body length category (range, 0.49–0.83). Because food habits for the fishes examined in this study were similar and there exists the high potential for negative behavioral impacts by western mosquitofish, we do not recommend stocking this species into Indiana waters that contain native topminnows.

**Keywords:** Western mosquitofish, Northern starhead topminnow, Northern studfish, Blackstripe topminnow, Banded killifish, Food habits, Diet overlap

The introduction of non-native fishes can lead to large-scale changes in aquatic communities (Fuller et al. 1999). However, long-term impacts to community structure resulting from these introductions are often unknown (Bonar et al. 2005). On a global scale, introductions of non-native fishes are one of the primary causes for ongoing declines of native fishes (Simberloff 2004; Vitule et al. 2008). These introductions may have far-reaching consequences, and can lead to the extirpation of native species (Rogowski & Stockwell 2006; Vitule et al. 2008).

The western mosquitofish (*Gambusia affinis*), and its congener the eastern mosquitofish (*G. holbrooki*), are the most widely distributed larvivorous fishes in the world (Courtenay and Meffe 1989). These species were broadly stocked outside their native distribution for mosquito control and often outcompete native fishes for habitat and trophic resources (Fuller et al. 1999; Rehage et al. 2005; Laha & Mattingly 2007; Matthews & Marsh-Matthews 2011). Although mosquitofish are efficient at mosquito control (Hoy and Reed 1971; Bence 1988), more recent studies suggest that they do not preferentially prey on mosquito larvae and that native fishes may more effectively control mosquitoes (Castleberry and Cech 1990; Blaustein 1992; Hurst et al. 2004). As a result, there is a need to evaluate the food habits of introduced mosquitofish relative to other native fishes.

Topminnows occupy a similar ecological niche as mosquitofish and are often negatively impacted by introductions of this species (Meffe 1985; Laha & Mattingly 2007). For example, when Sonoran topminnows (*Poeciliopsis occidentalis sonoriensis*) were exposed to western mosquitofish in laboratory experiments, the topminnows ceased to feed, retreated to areas with structure to escape aggression

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Table 1.—Fish species collected at Indiana sites from April - October 2005, and associated site numbers for Figure 1. Species are western mosquitofish (MSQ), blackstripe topminnow (BST), northern studfish (NSF), northern starhead topminnow (NST), and banded killifish (BAK).

| Site number | Site name | Latitude | Longitude | Fish species | System type | Land-use type |
|---|---|---|---|---|---|---|
| 1 | Loomis Lake | 41.5200° N | 87.0550° W | NST | Lake | Fallow Fields |
| 2 | Pine Lake | 41.6203° N | 86.7478° W | NST | Lake | Fallow Fields/ Residential |
| 3 | Upper Fish Lake | 41.5714° N | 86.5439° W | NST | Lake | Fallow Fields/ Residential |
| 4 | Silver Lake | 41.6303° N | 85.0644° W | BAK | Lake | Fallow Fields/ Residential |
| 5 | Golden Lake | 41.6037° N | 85.0650° W | BST | Lake | Residential |
| 6 | Lake Wawasee | 41.4006° N | 85.7022° W | BAK | Lake | Residential |
| 7 | Waubee Lake | 41.3875° N | 85.8292° W | BAK | Lake | Fallow Fields/ Residential |
| 8 | Moots Creek | 40.5378° N | 86.7804° W | BST | Stream | Agriculture |
| 9 | Martell Forest Pond | 40.4534° N | 87.0542° W | MSQ | Pond | Fallow Fields/Forest |
| 10 | Greenfield Bayou | 39.1930° N | 87.3237° W | MSQ | Wetland | Fallow Fields/ Agriculture |
| 11 | Connelly Ditch | 39.1524° N | 87.0908° W | MSQ | Stream | Agriculture |
| 11 | Connelly Ditch | 39.1524° N | 87.0908° W | BST | Stream | Agriculture |
| 12 | Sugar Creek | 39.3043° N | 85.5812° W | NSF | Stream | Fallow Fields/Forest |
| 13 | Flatrock River | 39.2149° N | 85.5118° W | NSF | River | Fallow Fields/Forest |
| 14 | Lewis Creek | 39.2355° N | 85.4938° W | NSF | Stream | Fallow Fields/Forest |

and predation, and experienced declines in growth, increases in mortality, and reductions in reproductive potential (Schoenherr 1981). Sutton et al. (2009, 2013) found that western mosquitofish initiated agonistic behaviors (i.e., chases and nips) on four native Indiana topminnow species (banded killifish [*Fundulus diaphanus*], northern studfish [*F. catenatus*], northern starhead topminnow [*F. dispar*], and blackstripe topminnow [*F. notatus*]) and caused changes in topminnow behavior in mixed-species microcosms. The first three topminnow species have restricted ranges in Indiana, while the latter species is ubiquitous throughout the state.

We evaluated the food habits of populations of introduced western mosquitofish and four species of native Indiana topminnows to determine similarity in prey consumption. We examined: (1) temporal and size-related patterns in food habits; (2) the percent consumption of mosquitoes in the diet; and (3) temporal and size-related patterns in diet overlap. This research will allow greater understanding of how western mosquitofish could potentially impact native topminnows in Indiana if they were to co-occur in the same aquatic systems.

## METHODS

Western mosquitofish and topminnows were collected from Indiana waters from April through October 2005 (Table 1; Figure 1). Fish sampling was conducted monthly at three sites per species (14 sites total; one site was sampled for two species each month). The wetland site (Greenfield Bayou) contained silt substrate and no aquatic vegetation, and only supported western mosquitofish. The pond site (Martell Forest Pond) contained silt substrate and coontail (*Ceratophyllum demersum*), and the dominant fish species was green sunfish (*Lepomis cyanellus*). Lake-edge sites contained gravel, sand, and silt substrates and supported water lilies (*Nymphaea* spp.), with the exception of Waubee Lake and Lake Wawasee which lacked aquatic vegetation. These sites contained diverse fish assemblages, with the most abundant species being bluegill (*L. macrochirus*), largemouth bass (*Micropterus salmoides*), and various minnow species. Stream and river-edge sites contained silt and gravel substrates and supported diverse fish assemblages, and were dominated by native minnows. Regardless of sampling site, fish were always collected in shallow water habitats,

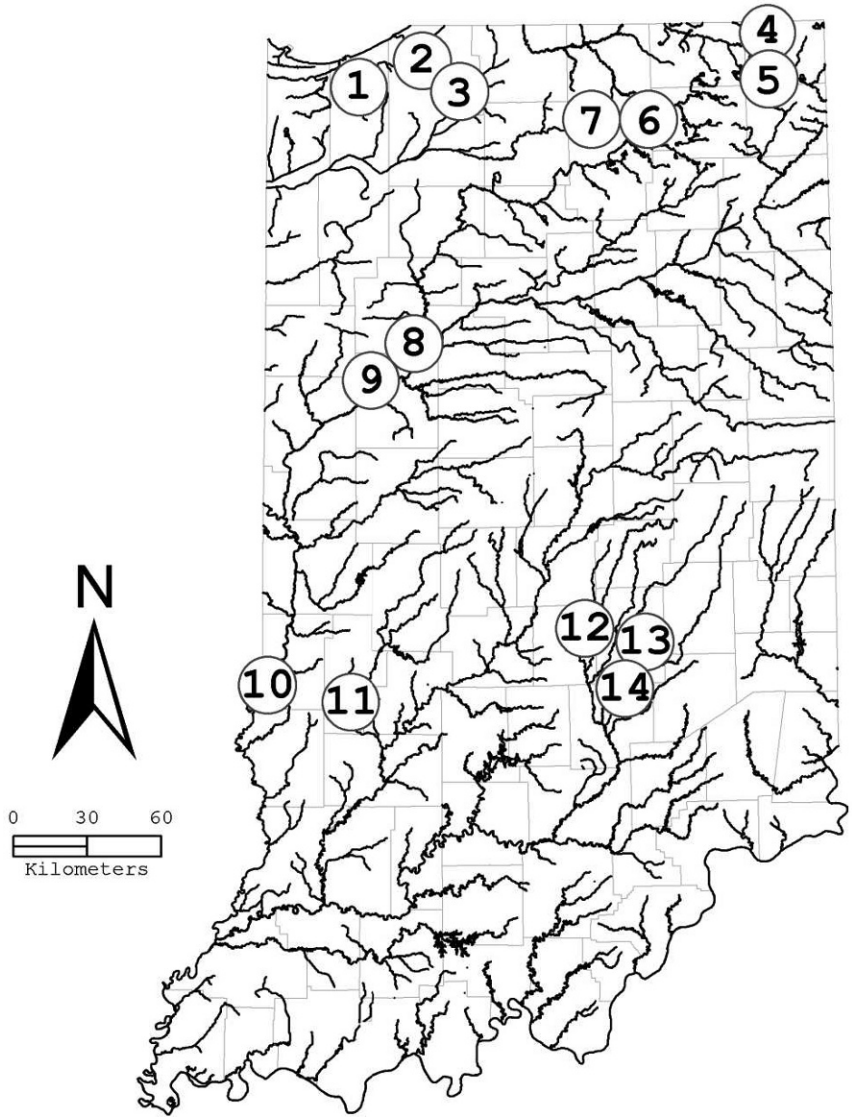


Figure 1.—Map of Indiana, with field sites sampled from April through October 2005 identified by the three-letter abbreviation. For the name of each site, see TABLE 1.

either backwater or side channels in stream and river-edge sites with no flow or shoreline areas of wetland, pond, and lake sites. Habitat types supporting these fishes were similar among sites.

A maximum of 20 adult fish of each species was collected each month from each sampling site. Sampling gears included a 3.18-mm mesh seine (length: 3.05 m; depth: 1.22 m) and a 3.18-mm mesh dip net (diameter: 40 cm). Fish were euthanized with an overdose of tricaine methanosulfonate and placed on ice to slow the digestion of stomach contents. In the laboratory, fish were measured for total length to the nearest 0.05 mm, weighed to the nearest 0.01 g, and fixed in 10% formalin pending laboratory analysis of stomach contents.

Stomach contents were extracted from each fish, blotted to remove excess formalin, and weighed to the nearest 0.0001 g. Consumed prey were examined under 2.5 X magnification with a stereomicroscope, and individual items were identified to family for diptera and order for all other prey types. The percentage of each

consumed prey type in relation to the entire stomach contents for each fish was recorded as the percent composition by weight following methods described in Garvey and Chipps (2012). Stomach contents were further analyzed to determine the proportion of mosquito (Culicidae) larvae consumed by fish species. To facilitate examination of trends in food habits, data from all three sampling sites for each fish species were combined for each sampling month because stomach contents did not differ for each species among sites (Chi Square, all $P > 0.08$. Because there was no difference in stomach contents between males and females for each species (Chi Square, all $P > 0.24$), both sexes were pooled for all analyses for each species.

The diets of western mosquitofish were compared each month and by 5-mm total-length category to the diets of each topminnow species using Schoener's diet overlap index (1970):

$$C_{xy} = 1 - 0.5\left(\sum |p_{xi} - p_{yi}|\right),$$

where $C_{xy}$ was the index value, $p_{xi}$ was the proportion of food type i consumed by species x, and $p_{yi}$ was the proportion of food type i consumed by species y. Calculated values ranged from 0.0 (no diet overlap) to 1.0 (complete diet overlap for consumed prey). Although this index cannot be used to make statistical comparisons of diet, index values greater than 0.60 indicate significant prey consumption overlap (Schoener 1970).

## RESULTS

A total of 1,816 fish was collected from April through October 2005. Western mosquitofish (n = 394) had a mean length of 32.25 mm and a mean weight of 0.54 g. Northern starhead topminnows (n = 346) were larger than mosquitofish, with a mean length of 40.16 mm and a mean weight of 0.88 g. Both blackstripe topminnows (n = 370) and banded killifish (n = 287) were similar in size to northern starhead topminnows. Blackstripe topminnows had a mean length of 42.63 mm and a mean weight of 0.98 g, while banded killifish had a mean length of 46.98 mm and a mean weight of 1.21 g. Northern studfish (n = 417) were the largest species, with a mean length of 49.71 mm and a mean weight of 2.01 g.

Non-culicid diptera (primarily chironomidae and ceratopagonidae) dominated the prey items consumed by topminnows and western mosquitofish each month (Figure 2). For northern starhead topminnows, non-culicid diptera comprised 34% of the diet, with the highest consumption in April (51%), May (47%), and October (61%). The highest percentage of culicid diptera consumed by this species occurred in May (23%) and September (18%). For northern studfish, non-culicid diptera comprised at least 40% of the diet each month, except during July and August when ephemeroptera, coleoptera, and other prey items (gastropods and nematodes) accounted for the majority of consumed prey items. The highest percentage of culicid diptera consumption for northern studfish occurred in April (20%) and August (10%). Non-culicid diptera comprised at least 38%, and coleoptera and hymenoptera comprised at least 25% of the stomach contents for blackstripe topminnows, regardless of month. Culicid diptera comprised their highest percentage of the diet in April (17%) for this species. Cladocera and non-culicid diptera composed the largest percentages of prey consumed by western mosquitofish (33 and 34%, respectively). The highest percentages of culicid diptera were consumed by this species in May (27%) and June (18%). Banded killifish consumed variable percentages of cladocera and non-culicid diptera, with the greatest consumption of non-culicid diptera in June. Culicid diptera were always a low percentage component of the diet for banded killifish.

Diet composition of topminnows and western mosquitofish varied by length category, but largely consisted of non-culicid diptera (chironomidae and ceratopagonidae) and zooplankton (mostly cladocera; Figure 3). The percentage of zooplankton consumed by northern starhead topminnows declined for larger fish, whereas the percentage of non-culicid diptera remained high for individuals > 30 mm. More culicid diptera were consumed by this topminnow than any other species at lengths of 25 mm (17%) and 30 mm (21%). Nearly half of the prey items (49%) in northern studfish diets were comprised of non-culicid diptera, but the percentage of ephemeroptera and hemiptera consumed increased with fish length. This topminnow species consumed its highest percentage of culicid diptera at 20 mm in length (18%). Although most sizes of blackstripe topminnows contained similar percentages of prey items, non-culicid diptera comprised more

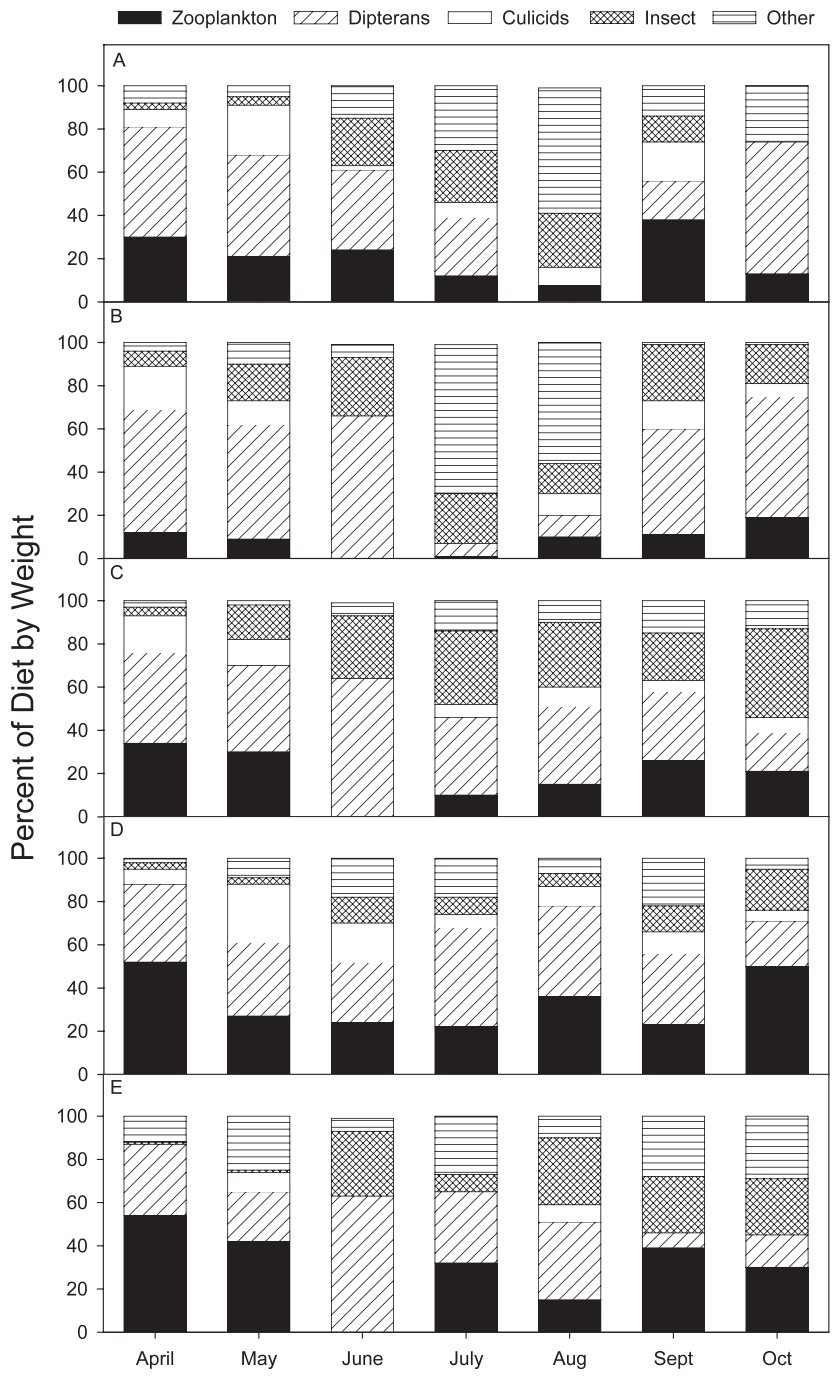


Figure 2.—Percent of diet by weight for (A) northern starhead topminnows, (B) northern studfish, (C) blackstripe topminnows, (D) western mosquitofish, and (E) banded killifish each month.

than half (55%) of the diet for 25-mm fish. Length classes for this species that consumed the most culicid diptera ranged from 35 to 45 mm (10% to 13%). Western mosquitofish of size 20 to 30 mm consumed similar percentages of zooplankton and non-culicid diptera (42% and 36%, respectively), but these prey items composed a lower dietary percentage for fish 35 to

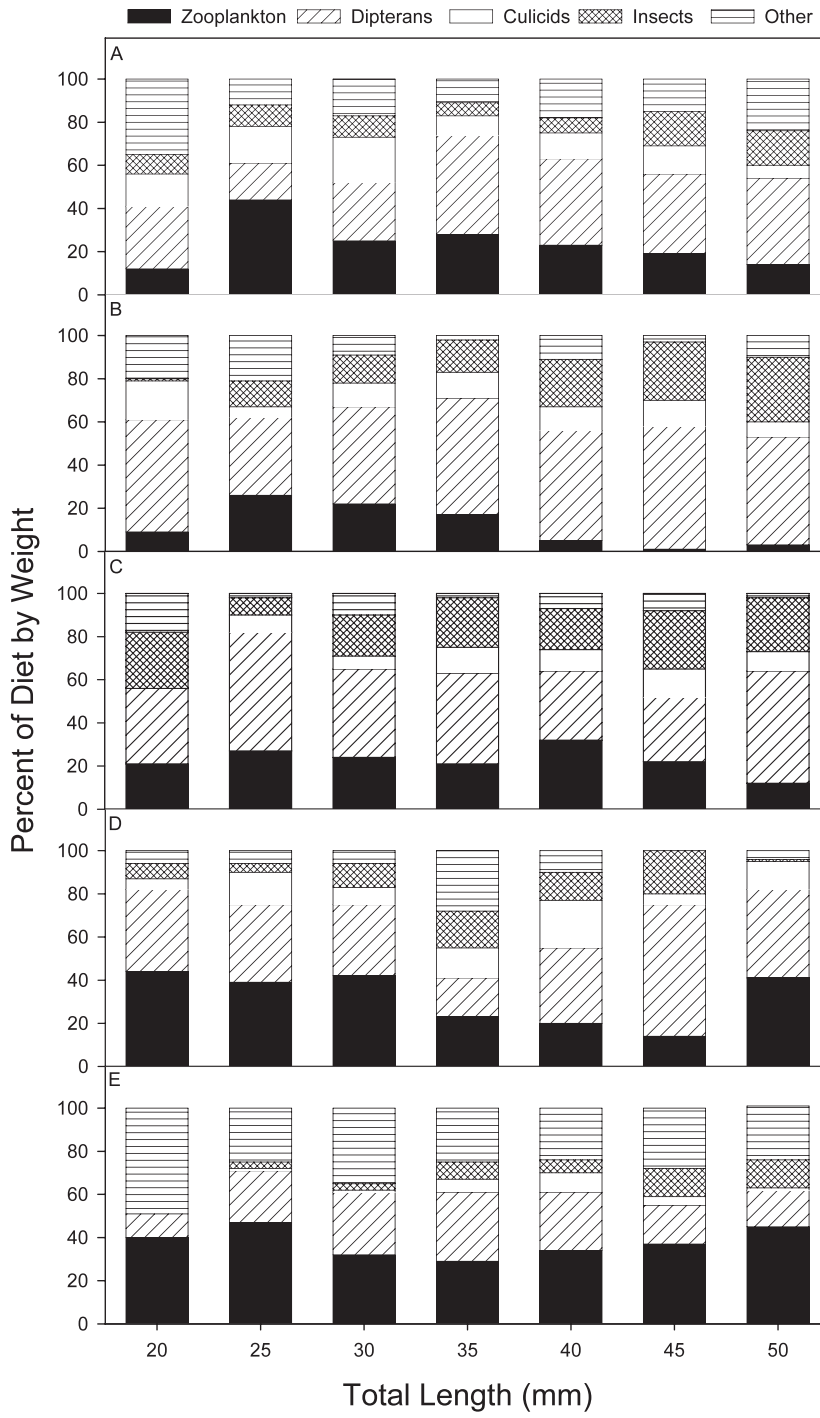


Figure 3.—Percent of diet by weight for (A) northern starhead topminnows, (B) northern studfish, (C) blackstripe topminnows, (D) western mosquitofish, and (E) banded killifish in each size category.

40 mm (mean = 22% and 27%, respectively). Non-culicid and culicid diptera were consumed at higher percentages by 35 (28% and 14%, respectively) and 40 mm (51% and 22%, respectively) fish. Banded killifish consumed mostly zooplankton (38%), except 20-mm fish where other prey items (amphipods) were the primary prey type (49%). Culicid diptera never comprised more than 9% of the diet for any length class of banded killifish.

There was no clear trend in diet overlap between western mosquitofish and topminnows from April through October 2005 (Table 2). Although no species pairings had significant diet overlap ($> 0.60$) for all months, western mosquitofish and blackstripe topminnows had significant overlap for all months except June (0.47). Diet overlap of western mosquitofish with banded killifish, northern starhead topminnows, and northern studfish was significant for five, four, and three months, respectively. The only month where diet overlap was significant between western mosquitofish and all topminnow species was May.

Diet overlap between western mosquitofish and topminnows did not follow a clear length-related trend (Table 3). For the 25- through 40-mm length categories, diet overlap was significant for all mosquitofish-topminnow pairings. Only the western mosquitofish-blackstripe topminnow pairing had significant diet overlap for all size categories. Diet overlap was not significant for the smallest and/or largest size categories between western mosquitofish and northern starhead topminnow (20 mm), northern studfish (20 and 50 mm), and banded killifish (20 and 45 mm).

## DISCUSSION

The food habits documented in this study for western mosquitofish and native Indiana topminnows are similar to the results of dietary studies reported for mosquitofish species in other evaluations. Although western mosquitofish consume culicids, they often consume other insects, small crustaceans, arachnids, and rotifera (Pflieger 1997). Eastern mosquitofish will switch from mosquitoes to other prey types with availability (Jenkins and Burkhead 1994). Northern starhead topminnows consumed insects, chironomidae, crustaceans, plant material, and algae, while blackstripe topminnows often ingested large percentages of insects, molluscs, cladocera, and copepods (Gunning and Lewis 1955; Schwartz and Hasler 1966). Northern studfish utilized more ephemeroptera, trichoptera, gastropods, and pelecypoda than other fish species, but also consumed diptera, nematodes, and coleoptera (McCaskill et al. 1972; Fisher 1981). Banded killifish consumed a variety of prey items, including small crustaceans, amphipods, cladocera, nematodes, chironomidae, and plant material (Becker 1983). All fishes examined in our study also consume terrestrial insects at the water surface (Becker 1983; Pflieger 1997), which was observed in our analyses.

Prey availability may be more important in prey selection of mosquitofish and topminnow than preferences for particular food items. For example, differences in the diet composition of mummichogs (*Fundulus heteroclitus*) reflected prey availability, regardless of habitat type occupied by this species (James-Pirri et al. 2001). In California rice fields, western mosquitofish reduced the abundance of mosquitoes, but preference for this prey type diminished in the presence of abundant zooplankton (Bence 1988). Native topminnows, such as the Plains killifish (*Fundulus zebrinus*), consumed mosquito larvae at rates equal to mosquitofish in outdoor mesocosms (Nelson and Keenan 1992). Prey availability data were not collected during this study, but it is well documented that fish are opportunistic feeders (Specziar 2004; Hinz et al. 2005; Quist et al. 2006). As a result, the lack of preference for mosquito larvae by western mosquitofish and similarity in food habits among the five species examined in this study may be more a function of availability rather than predilection for particular prey types.

Similarities in feeding strategies, behavior, and habitat use for western mosquitofish and topminnows may increase their potential for high niche overlap. All species examined in this study have an upturned mouth and flattened head to facilitate prey consumption at the water surface (Pflieger 1997). All five species occur at shallow depths ($< 15$ cm) near aquatic vegetation, with the exception of banded killifish and northern studfish which occur over gravel and sand substrates devoid of plant material. These similarities could facilitate interspecific competition when they co-occur. For example, western mosquitofish and blackstripe topminnows were often collected during this study in the same seine haul from Connelly Ditch, indicating co-occurrence in the same system.

Table 2.—Diet overlap values for April - October 2005 between western mosquitofish (MSQ) and blackstripe topminnow (BST), northern studfish (NSF), northern starhead topminnow (NST), and banded killifish (BAK).

| Month | MOSQ-BST | MOSQ-NSF | MOSQ-NST | MOSQ-BAK |
|---|---|---|---|---|
| April | 0.81 | 0.59 | 0.77 | 0.87 |
| May | 0.77 | 0.62 | 0.83 | 0.68 |
| June | 0.47 | 0.47 | 0.74 | 0.47 |
| July | 0.70 | 0.25 | 0.56 | 0.64 |
| August | 0.70 | 0.41 | 0.26 | 0.69 |
| September | 0.72 | 0.66 | 0.63 | 0.54 |
| October | 0.67 | 0.64 | 0.35 | 0.66 |

Both western and eastern mosquitofish have been shown to negatively impact native fish species where resource overlap is high. For example, western mosquitofish had a significant negative effect on the abundance and biomass of the threatened White Sands pupfish (*Cyprinodon tularosa*; Rogowski and Stockwell 2006). Sympatric populations of western mosquitofish and blackstripe topminnows in Indiana were observed to consume similar prey items, indicating the potential for high diet overlap between these two species (Clem and Whitaker 1995). In our study, the similarity in food habits (i.e., high diet overlap) indicates that these fishes have the potential for significant competition in prey consumption. The high similarity in food habits, coupled with the aggressive behaviors that western moquitofish exhibit towards other fishes (Schoenherr, 1981; Laha and Mattingly 2007; Sutton et al. 2009, 2013), suggest that introductions of this species could lead to deleterious results for native Indiana topminnows.

Table 3.—Diet overlap values by length category between western mosquitofish (MSQ) and blackstripe topminnow (BST), northern studfish (NSF), northern starhead topminnow (NST), and banded killifish (BAK). Length categories were 20 (20.01–25 mm), 25 (25.01–30.0 mm), 30 (30.01–35.0 mm), 35 (35.01–35.0 mm), 40 (40.01–45.0 mm), 45 (45.01–50.0 mm), and 50 (50.01–55.0 mm) mm.

| Size Category | MOSQ-BST | MOSQ-NSF | MOSQ-NST | MOSQ-BAK |
|---|---|---|---|---|
| 20 | 0.63 | 0.56 | 0.53 | 0.54 |
| 25 | 0.77 | 0.75 | 0.75 | 0.71 |
| 30 | 0.79 | 0.79 | 0.72 | 0.67 |
| 35 | 0.70 | 0.64 | 0.64 | 0.64 |
| 40 | 0.81 | 0.70 | 0.82 | 0.72 |
| 45 | 0.69 | 0.83 | 0.72 | 0.49 |
| 50 | 0.64 | 0.55 | 0.62 | 0.60 |

The potential impacts of western mosquitofish on native Indiana topminnows proposed in this study need to be interpreted with caution because rarely was the former species found in the same systems as topminnows. Western mosquitofish were only found at one site (Connelly Ditch) during this study that also contained a native topminnow species (blackstripe topminnow). Diet overlap between these two species at this site was high (month: range, 0.53–0.86; length: range, 0.60 to 0.85; Zeiber 2007), suggesting that the potential for trophic competition is also high. Although western mosquitofish are native to southern and southwestern Indiana, they have been stocked throughout much of the state to reduce West Nile Virus *Flavivirus* transmission by mosquitoes (G. Polston, Marion County Health Department, personal communication). As a result, the potential for trophic competition is high and could result in niche shifts due to changes in behavior and/or prey/habitat utilization, which may have deleterious effects on native Indiana topminnows as has been shown in other studies (Schaeffer et al. 1994; Arthington and Marshall 1999; Fuller et al. 1999; Rehage et al. 2005; Laha and Mattingly 2007; Matthews & Marsh-Matthews 2011). Additional research is required to determine if these potential impacts will lead to realized outcomes if western mosquitofish are introduced or escape into aquatic systems in Indiana which currently support topminnow species.

Western mosquitofish and the Indiana topminnows examined in this study appear to be trophic equivalents and have nearly identical habitat requirements. These similarities, coupled with the unknown potential impacts of resource competition and aggressive and deleterious behaviors often exhibited by mosquitofish toward topminnows, make it likely that western mosquitofish would negatively impact topminnow species in Indiana waters if they cooccurred in the same aquatic system. We do not recommend stocking mosquitofish into permanent or even ephemeral water bodies where the possibility of escapement by mosquitofish into nearby waterways exists. If there is a need for mosquito control in Indiana waters, we suggest that blackstripe topminnow be considered as an alternative to western mosquitofish because

they are the most trophically similar topminnow species in Indiana. Sutton et al. (2012) observed that blackstripe topminnows exhibited almost no aggressive behaviors toward northern starhead topminnows, northern studfish, and banded killifish and did not change the behavior of these fishes in laboratory evaluations. However, this recommendation also requires additional research to ensure that blackstripe topminnows would not negatively impact other fishes if introduced into aquatic systems in which they are not native.

## ACKNOWLEDGEMENTS

We would like to thank M. Boone, L. Edenfield, M. León, H. Patrick, A. McAlexander, S. Shaw, and D. Rajchel for their assistance in the field and laboratory. The experimental procedures used in this research were approved by the Purdue University Animal Care and Use Committee as protocol 01-058. Permits for the collection of fish were provided by the Indiana Department of Natural Resources (#s 05-3089 and 05-3266). This project was funded through the Indiana Nongame Fund and State Wildlife Grants T-7-R-1 through the Indiana Department of Natural Resources. Additional funding was provided by the Purdue University Department of Forestry and Natural Resources and the American Fisheries Society Hutton Junior Fishery Biology Program.

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*Manuscript received 17 October 2012, revised 17 July 2013.*

2013. Proceedings of the Indiana Academy of Science 122(1):18–19

# A NEW JUNIOR SYNONYM FOR THE HOLARCTIC SPECIES *AMETROPUS FRAGILIS* ALBARDA, 1878 (INSECTA: EPHEMEROPTERA: AMETROPODIDAE)

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**ABSTRACT.** Based on two previously published and independent studies, the Nearctic species *Ametropus neavei* McDunnough, 1928 (Insecta: Ephemeroptera: Ametropodidae) should be considered a junior synonym of the Holarctic species, *A. fragilis* Albarda, 1878 [=*A. neavei*, **new synonym**].

**Keywords:** Mayflies, systematics, taxonomy, aquatic insects

The mayfly genus *Ametropus* Albarda, 1878 (Insecta: Ephemeroptera: Ametropodidae) has a Holarctic distribution. The distinctive larvae may be found in large rivers and smaller mountain streams with a shifting-sand substrate, where they may be found on submerged logs or leafpacks. Larvae are agile swimmers and sometimes behave as semi-burrowers, when they hide in the substratum with only eyes and antennae exposed (Bauernfeind & Soldán 2012).

McCafferty (2001) considered the Nearctic species *Ametropus albrighti* Traver, 1935 to be a junior synonym of the also Nearctic *A. neavei* McDunnough, 1928, based on the two species being similar and stable in structure, but variable with respect to coloration and maculation of the body, especially the abdomen. *Ametropus neavei* (sensu McCafferty 2001) has been reported from Alaska, east to North Dakota and south to New Mexico; a disjunct population has been reported from the Upper Peninsula of Michigan (McCafferty et al. 2012, Rinella et al. 2012).

Jacob (2006) subsequently considered *A. albrighti* to be a junior synonym of the Palearctic species *A. fragilis* Albarda, 1878, thereby recognizing a Holarctic distribution for the latter species. However, he did not treat *A. neavei* in the same detail as the other two species he recognized in the genus, nor did he list the publication by McCafferty (2001) among his cited literature.

Based on the combination of these two independent studies, *A. neavei* should be considered a junior synonym of the Holarctic species *A. fragilis* Albarda, 1878 [=*A. neavei* McDunnough, 1928, **new synonym**; =*A. eatoni* Brodsky, 1930 (syn. by Landa 1969); =*A. albrighti* Traver, 1935 (syn. by Jacob 2006; syn. under *A. neavei* by McCafferty 2001)].

Only two extant species of *Ametropus* are known, and both are found in North America. *Ametropus ammophilus* Allen & Edmunds, 1976 occurs only in western North America (McCafferty et al. 2012), and it is distinguished from *A. fragilis* by its much larger size, cleft penes of the male genitalia, a more complex abdominal color pattern (McCafferty 2001) and its more restricted distribution. The two species are known to overlap in their geographic distributions only in a small area of Alberta (McCafferty 2001, McCafferty et al. 2012). In North America, *A. fragilis* is the much more widespread of the two species (Rinella et al. 2012), but it may be endangered in all or part of its Nearctic range (McCafferty et al. 2012).

## MATERIALS EXAMINED

***Ametropus fragilis.*** One male adult, reared from larva, with associated exuviae (one subimago, three larval sets), USA, Wyoming, Sweetwater County, Black's Fork River at I-80, west of Green River City, 6-VII-1968, R&D Koss, deposited in the Purdue University Entomological Research Collection, West Lafayette, Indiana, USA. Note: This material is typical of the *albrighti* variant of *A. fragilis*.

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*Manuscript received 24 June 2013, revised 12 September 2013.*

2013. Proceedings of the Indiana Academy of Science 122(1):20–26

# DISPERSAL AND DISTRIBUTION OF BIOLOGICAL CONTROL AGENTS FOR *LYTHRUM SALICARIA* IN INDIANA

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**ABSTRACT.** The invasive wetland perennial, *Lythrum salicaria* has spread throughout Indiana wetlands since 1900. Four insect species were approved for release as biological control agents. These species included: *Hylobius transversovittatus*, *Nanophyes marmoratus*, *Galerucella calmariensis*, and *G. pusilla*. The distribution of these beetles has been monitored by the Indiana Department of Natural Resources since 1994. This project aimed to develop an updatable GIS database, expand the existing data on locations of these control agents, and estimate their distribution throughout the state. *Nanophyes marmoratus* and *Hylobius transversovittatus* have spread slowly between wetlands, which limited the analysis of this work to the spread of *Galerucella*. Geospatial analyses of *Galerucella* spp. indicates that they have become widely distributed in the northern region of the state. By calculating distances and date of initial observation between sites, it was estimated that *Galerucella* spp. spread at a rate of least 491 meters per year with a maximum rate of 1,822 m/yr. This simplified calculation of dispersal rates and GIS mapping allows for visualization of areas for potential future releases in order to maximize the control of *L. salicaria*. Additionally, it suggests that *Galerucella* spp. have become widely established in northern Indiana.

**Keywords:** *Lythrum salicaria*, *Galerucella*, *Nanophyes marmoratus*, *Hylobius transversovittatus*, biological control, invasive species, geospatial distribution

*Lythrum salicaria*, or purple loosestrife, is an invasive wetland perennial in North America (Thompson et al. 1987). It can form clumps of 30–50 stems arising from a single taproot and a terminal spike of tightly clustered flowers over 1 meter in length (Mal et al. 1992). This vigorous branching and flowering allows a single plant to produce upwards of 2.5 million seeds each year (Malecki et al. 1993).

North American introductions of *L. salicaria*, which began as early as 1814 in New England (Mal et al. 1992), were likely from inadvertent transport in shipping ballast and imported wool and intentional introduction by immigrants, who used the plant as a medicinal herb (Thompson et al. 1987). Anthropogenic activities, such as the development and use of canals, contributed to the further spread of *L. salicaria* into the Midwest (Thompson et al. 1987). Other uses of the plant, both as an ornamental and as a nectar plant for beekeeping, resulted in intentional introductions. In Indiana the earliest record of *L. salicaria* is from 1900, although Stuckey (1980) and Deam (1940) note very little spread through 1940. Today, *L. salicaria* is distributed throughout Indiana but is most common in the northern counties.

*Lythrum salicaria* forms dense stands (Malecki et al. 1993) that can become dominant in wetland seed banks (Welling and Becker 1990), reduce wildlife habitat quality (Whitt et al.1999, Rawinski 1982, Lor 2000), interfere with pollination of native species (Brown et al. 2002, Da Silva and Sargent 2011, Templer et al. 1996), and alter wetland function (Emery and Perry 1996, Bärlocher and Biddiscombe 1996). It may also outcompete native plants in a variety of wetlands (Gaudet and Keddy 1995, Weihe and Neely 1997, Gabor et al. 1996, Mal et al. 1997).

Control efforts against *L. salicaria* began in the 1950's, and included attempts at flooding, cutting, and burning (Skinner et al. 1994). Initial efforts were largely unsuccessful for all

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but the smallest patches of *L. salicaria* (Blossey et al. 2001, Skinner et al. 1994). Glyphosate, 2,4-D, or triclopyr have been used in chemical control but because of extensive seed banks (Welling and Becker 1990), spraying must be repeated often (Blossey et al. 2001, Skinner et al. 1994). The non-selective nature of chemical control also reduced populations of sedges, grasses, cattails, and other native wetland plants (Skinner et al. 1994, Gabor et al. 1996). The lack of effective methods of control and continued dispersal of *L. salicaria* resulted in the formation of a program to establish a biological control program for *L. salicaria* in North America. An overview of this process is provided by Malecki et al. (1993), while Blossey et al. (2001), and Hight and Drea (1991) examine the process in more detail. Four insect species eventually were approved for release. These species included: *Hylobius transversovittatus* Goeze (a root-mining weevil), *Nanophyes marmoratus* Goeze (a flower feeding weevil), *Galerucella calmariensis* L., and *G. pusilla* Duft (two leaf-beetles) (Blossey et al. 2001).

Following the approval of *Galerucella* spp., *N. marmoratus*, and *H. transversovittatus* for release, all three were released at sites in Indiana by the Indiana Department of Natural Resources (IDNR), Division of Nature Preserves. *Galerucella* spp., the earliest to be approved, were first released in 1994. Release of *N. marmoratus* and *H. transversovittatus* did not begin until eight years later, in 2002. A protocol for raising captive populations of *Galerucella* was quickly established. As releases of *Galerucella* spp. continued, and as populations accumulated, individuals were collected from existing populations and released to sites where they were previously absent. Thus, of the three genera of insects, *Galerucella* spp. were most widely released in Indiana because they were approved first and were easily raised.

The aim of this project was to develop an easily updateable geodatabase for tracking the distribution of purple loosestrife biological control agents across the state. The development of this database is important for improving our understanding of the specific dispersal range of *Galerucella* spp. as well as the general dispersal patterns of biological control agents. It was also a goal of this project to understand the current spread and patterns of dispersal, especially of *Galerucella* spp., by field checking numerous sites throughout northern Indiana in 2011 supplemented by 17 years of continued observations made by IDNR personnel. Furthermore, knowledge of the current range of *Galerucella* spp. is important for the planning of future releases of the insects in order to maximize the effectiveness of the biological control program for *L. salicaria*.

## METHODS

The primary files in the geodatabase are point feature classes of locations where beetle populations have been located. To maintain these records, any new release site is added to the files and time each spring is spent checking locations where the beetles are likely to have spread. This surveying involves driving to areas around known release or dispersal sites and identifying areas infested by *L. salicaria*. Once *L. salicaria* is found, the area is checked for presence of beetles and their abundance is ranked.

During the summer of 2011, particular effort was made to survey a number of sites within spatial gaps in the current data. In order to check a large number of sites over a short period of time most of the sites examined were boat ramps. In addition to ease of checking for *L. salicaria* and biological control agents, boat ramps are disturbed habitats, often are in full sunlight (favored by *L. salicaria*), and boats and trailers provide a means of spreading loosestrife seed. As a result of this additional work, 103 sites were examined, with nearly all of them being boat ramps.

Geospatial analysis, processing, and map construction was performed using ArcGIS Desktop 10 (ESRI 2011). In addition, the Geospatial Monitoring Environment (Beyer 2012) was used to calculate distances between release and non-release sites. A point feature class file containing the sites where insects were located was created for each beetle species. These files also contained the tabular data for each location, including whether insect were released, the county, insect abundance, first observation date, and latest observation date. A series of maps were created to show the dispersal of *L. salicaria* biological control insects in Indiana.

Finally, the geospatial data were analyzed to determine the likely dispersal rate of *Galerucella* spp. Non-release sites were joined to each release site manually, using data exported to spreadsheets. Using the *convert.tabletolines*

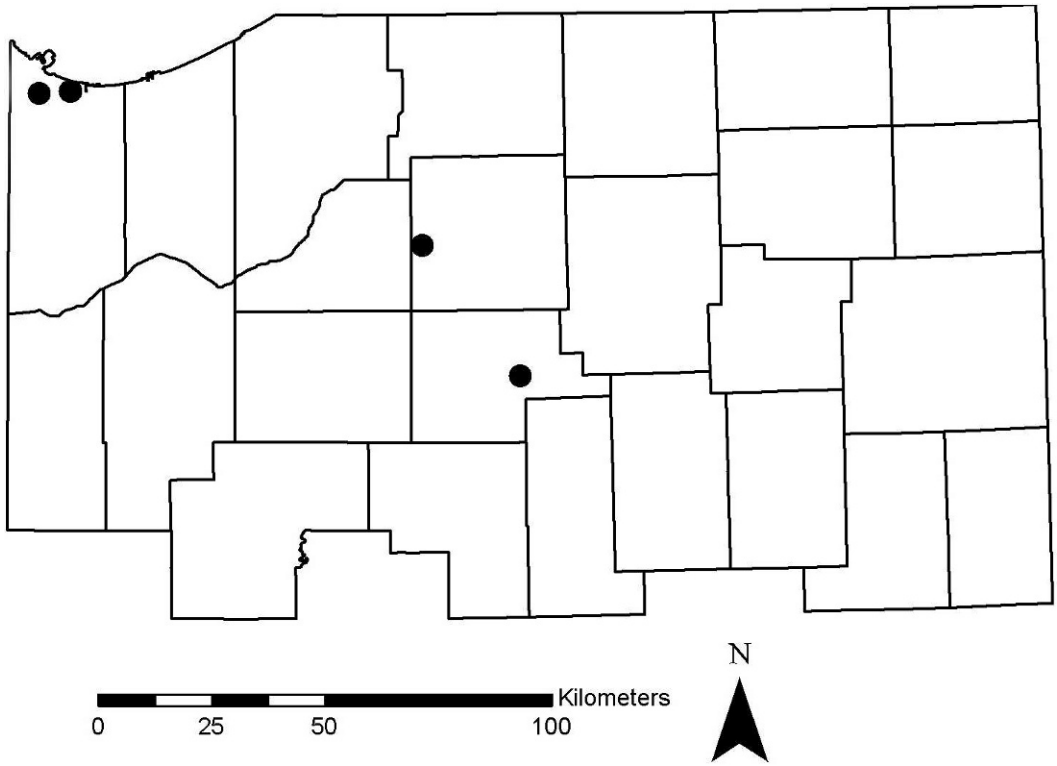


Figure 1.—*Hylobius transversovittatus* sites in Indiana. All sites are release sites.

command in the Geospatial Modeling Environment (Beyer 2012), the distance between each set of points was determined and added to the table. For each pair of sites the number of years between the release of *Galerucella* spp. at the release site and the first observation at the non-release site was determined. The distance between sites was divided by the years between these dates to calculate the average distance traveled per year. The assumption was made that the smallest distance travel per year was the most likely source of the beetles at the new site, and other pairs of points were removed. One record remained for each non-release site. The maximum distance per year (1,822 m/yr) and the mean distance per year (491 m/yr) from this list were then used to draw buffers around known *Galerucella* spp. locations in ArcMap. The calculated dispersal distance per year was multiplied by the number of years since the first observation (or release date) at each site. The buffer was then drawn at this distance. The buffer distance around a newer site is smaller, while that of an old site is much larger.

## RESULTS AND DISCUSSION

This project sought to examine the distribution of all three biological control insects throughout Indiana. However, locating *H. transversovittatus* proved to be very difficult. They have not been located at new sites (Figure 1) and only occasionally at past release sites; this is likely due to their secretive life cycle (Ferrarese and Garono 2010). The adult beetles are nocturnal (Blossey et al. 1994), so surveying is usually conducted for beetles in the larval stage. Since this stage is completed below ground, surveying is labor intensive and requires uprooting the plants for close examination (Malecki et al. 1993). Therefore, it is difficult to draw conclusions as to their distribution within Indiana.

*Nanophyes marmoratus* have been found at some non-release sites, but all of these were located near a release site, suggesting that their dispersal has been limited (Figure 2). In addition, their dispersal may be limited by the success of *Galerucella* spp. Large populations of *Galerucella* spp. may reduce the flowering of

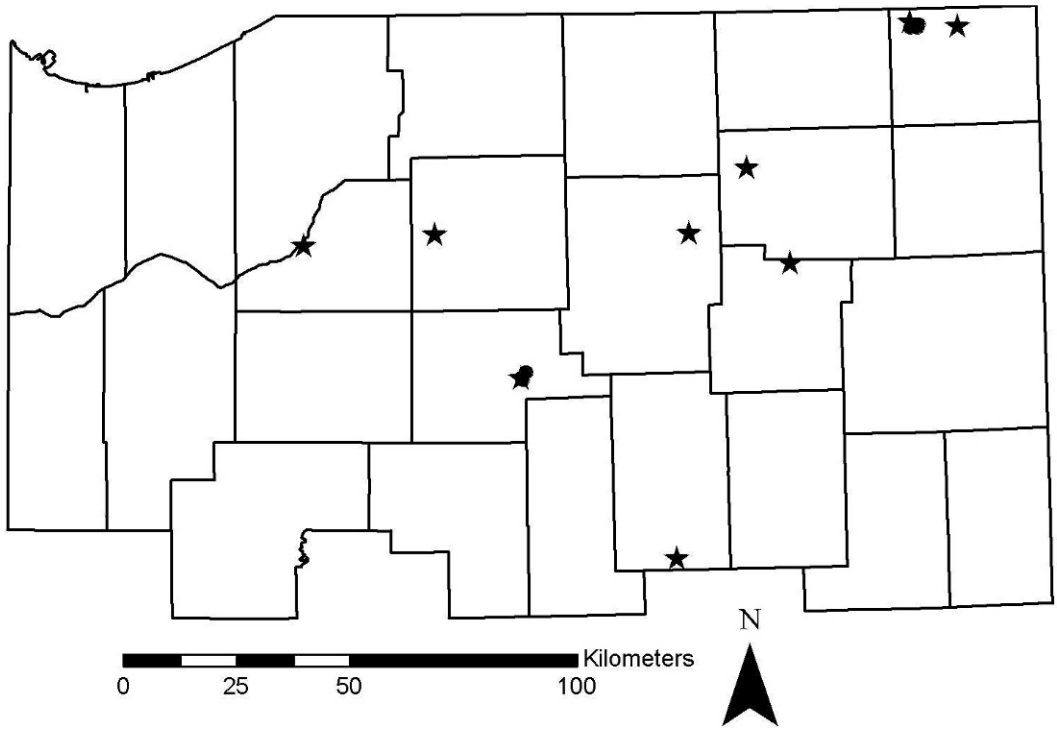


Figure 2.—*Nanophyes marmoratus* release sites (★) and non-release (●) in Indiana.

*L. salicaria* (Blossey and Skinner 2000), flowers necessary to the life cycle of *N. marmoratus*. Additionally, *N. marmoratus* has been release at far fewer sites in Indiana than *Galerucella* spp., further reducing the potential for locating new sites for *N. marmoratus*.

In contrast to *Hylobius* and *Nanophyes, Galerucella* spp. have dispersed quite well and were relativity easy to locate, resulting in 156 confirmed sites (Figures 3). In particular, *Galerucella* spp. have spread across northern Indiana but, in the remainder of the state where *L. salicaria* is infrequent, has only 5 reports (one in Brown, Marion, and Scott Counties; two in Morgan County). The mean annual dispersal distance of *Galerucella* spp. was calculated at 491 m/yr and the maximum was 1,822 m/yr. The buffered areas in Figure 4 approximate the potential area to which *Galerucella* spp. may have dispersed. These areas cover a total of 5,294 $km^2$ for the dispersal of 491 m/yr and 33,348 $km^2$ based on 1822 m/yr. Using the National Wetland Inventory as a basis for *L. salicaria* habitat, the potential *Galerucella* spp. habitat is reduced greatly to 747 $km^2$ (491 m/yr) and 2,799 $km^2$ (1822m/yr).

When compared to previous studies, these dispersal estimates fall within the wide range of values observed. Albright et al. (2004) found *Galerucella* spp. 9 km from the nearest release site after 4 years. If the dispersal is estimated over four years, the distance of 2,250 m/yr is slightly larger than the 1822 m/yr found in this study. However, Dech and Nosko (2002) suggest a very limited dispersal rate of both *Galerucella* species. After four years *Galerucella* spp. were found a maximum of approximately 50 meters from the release location.

Based upon additional observations made during the surveying for *Galerucella* spp. it appears that the beetles are well distributed to potential habitat within the 491 m/yr buffer area. Dispersal throughout the larger buffer area is more sporadic, but a large number of sites do occur (Figure 4). While the assumption was made that *Galerucella* spp. dispersal occurs in a uniform fashion over time ($x$ m/yr) this is almost certainly not the case. This assumption allows for an approximation of the potential

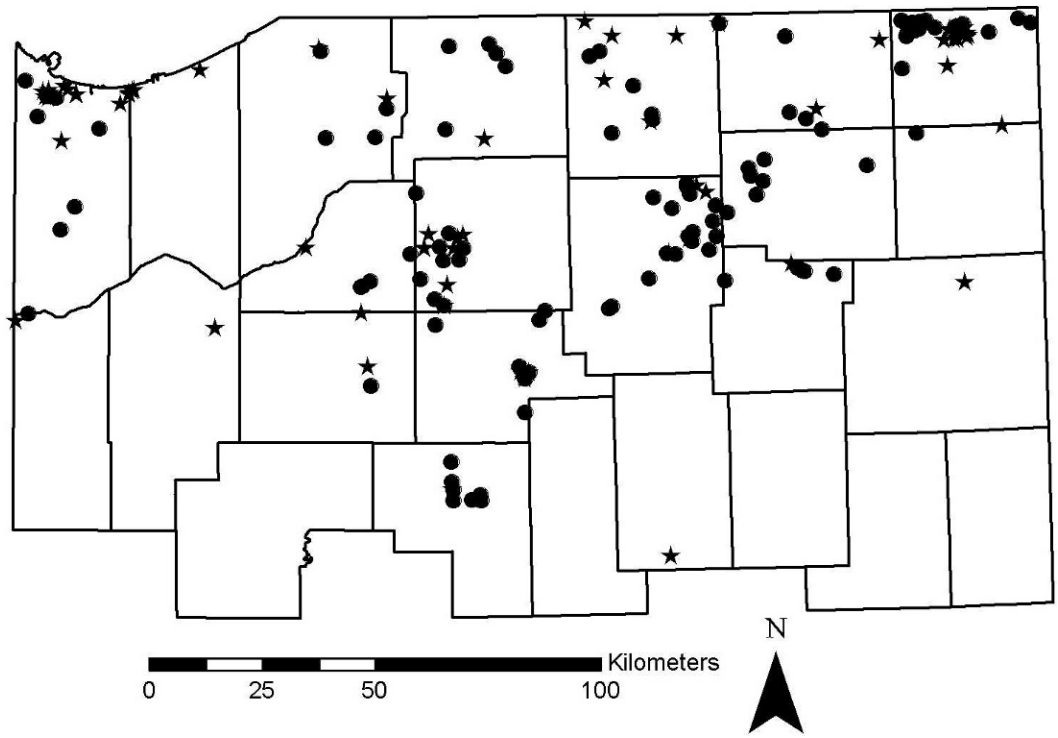


Figure 3.—*Galerucella* spp. release sites (★) and non-release sites (●) in northern Indiana.

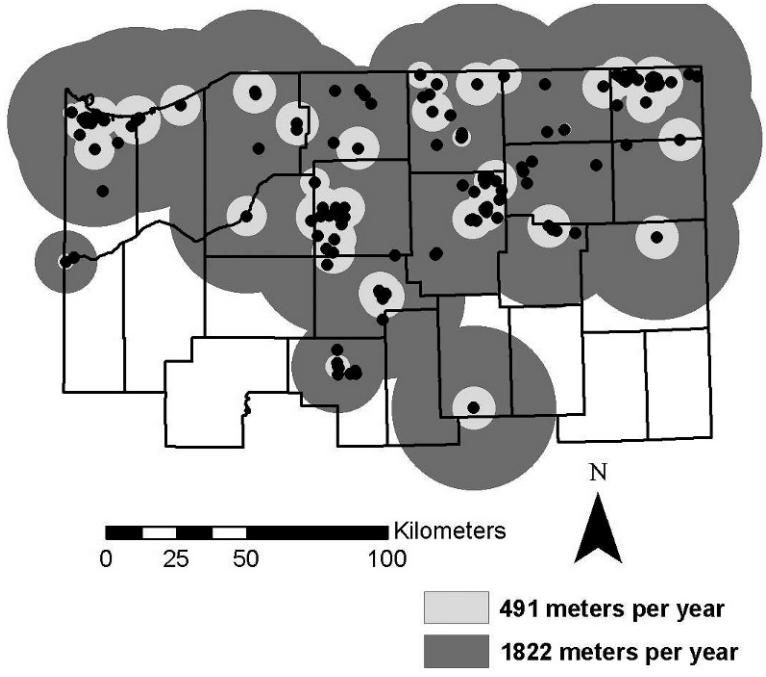


Figure 4.—*Galerucella* spp. release sites and non-release sites in Indiana with buffers showing potential area currently occupied by *Galerucella* spp. The small buffer area is based on 491 m/yr and the larger on 1822 m/yr.

area inhabited by *Galerucella* spp., but their dispersal patterns are more complex. Bartelt et al. (2008), Hambäck (2010), and Grevstad and Herzig (1997) have shown the importance of pheromones released by *Galerucella* spp. and the resulting aggregation behavior. Additionally, when large *Galerucella* spp. populations accumulate they can defoliate nearly all *L. salicaria* in the area (Landis 2003). Following this defoliation, beetles have been observed to disperse in large numbers to new sites, further complicating the dynamics and patterns of dispersal.

While the areas defined in Figure 4 are potential areas of occurrence, they provide an approximation for future biocontrol action. Using these maps, the areas least likely to be currently occupied by *Galerucella* spp. can be identified and additional releases can be performed in these areas. Additionally, the results of this study improve our understanding of the dispersal range of *Galerucella* spp. over time. Future work will be able to continue adding new locations to the geodatabase and as data are added the estimations of dispersal range will improve. Additionally, because the methodologies are easily applied to any case where the release locations have been documented, the methods used here may be applied in other locations where *Galerucella* spp. have been released in order to understand any regional differences that may occur.

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*Manuscript received 6 January 2013, revised 26 July 2013.*

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# CONODONT BIOSTRATIGRAPHY OF A SHALE LENS OVERLYING THE BUCKTOWN COAL MEMBER OF THE DUGGER FORMATION (PENNSYLVANIAN, DESMOINESIAN), PIKE COUNTY, INDIANA

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**ABSTRACT.** We collected conodonts from black and gray shale lenses containing limestone nodules lying immediately above the Bucktown Coal Member of the Dugger Formation (Pennsylvanian, Desmoinesian) in a Solar Sources Pride Creek pit 2 miles south of Petersburg in Pike County, Indiana. The shale lens varied from approximately 0.6 to 0.92 m in thickness and was approximately 190 m wide. Our objectives included describing the fauna, interpreting the paleoenvironment, and continuing the establishment of regional correlations in the Midcontinent. We processed ten samples, nine productive, from four intervals along the pit face and identified 507 elements to species level. Conodont faunas are dominated by *Idiognathodus* and *Adetognathus*. *Neognathodus*, *Hindeodus*, and *Idioprioniodus* are rare.

*Adetognathus* is uncommonly abundant, indicating that the depositional environment of the shales was very restricted, low energy, shallow-water, possibly a lagoon or a small embayment. The extremely high juvenile to adult ratio of *Idiognathodus* suggests that the paleoenvironment was harsh and resulted in a high juvenile mortality rate. The rare *Idioprioniodus* elements further suggest low-energy, reducing conditions but the fragmentation of the *Idioprioniodus* and *Hindeodus* elements may indicate introduction into the depositional site by irregular storm events. The rarity of *Neognathodus* shows its lack of tolerance for highly euryhaline conditions.

The restricted environmental conditions resulted in a lack of generic and specific diversity, particularly the rarity of *Neognathodus*, the primary biostratigraphic indicator for the Desmoinesian Series. Thus, there are insufficient data for regional correlations.

We interpret the geographically restricted basal black shales overlying the coal to be of flotant marsh origin, representing a localized flooding event. Distributary channels associated with a deltaic system subsequently delivered fresh water that resulted in deposition of brackish water gray shales. The evidence supports a model of localized control on sedimentation rather than sedimentation controlled by glacially influenced eustatic sea level change.

**Keywords:** Conodonts, Bucktown Coal Member, Dugger Formation, Pennsylvanian

## INTRODUCTION

The objectives of our investigation of the conodonts of the Desmoinesian Bucktown Coal Member of the Dugger Formation in the eastern part of the Illinois Basin in Indiana included describing the conodonts, furthering the development of a stable Pennsylvanian conodont taxonomy, interpreting paleoenvironments, and continuing the establishment of regional correlations in the Midwest.

We collected a total of ten 1-kg samples, nine productive, from four closely spaced locations in the Solar Sources Pride Creek Pit about 3 km south of Petersburg in Pike County, Indiana, in the vicinity of coordinates 38° 27′ 01.59″ N, 87° 16′ 19.98″ W (fig. 1). These ditch samples were collected over intervals of lithologic homogeneity not exceeding 0.5 m in thickness (fig. 2). The locations sampled were approximately 60 m apart along the face of the pit with Location A to the west and Location D to the east (fig. 3). The samples are from marine shales, both gray

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Figure 1.—Map of Indiana showing collecting locality for the Bucktown Coal Member of the Dugger Formation. Solar Sources Pit is in the vicinity of 38° 27′ 01.59″ N, 87° 16′ 19.98″ W.

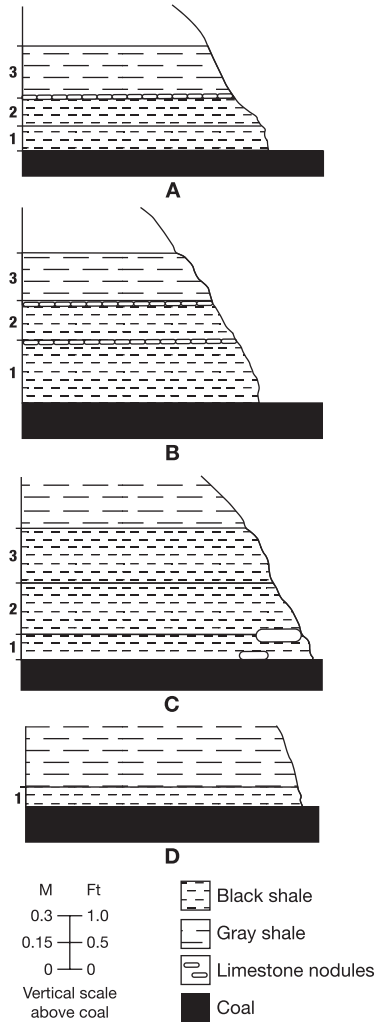


Figure 2.—Columnar sections showing general lithologies and sample numbers for intervals collected for the Bucktown Coal Member of the Dugger Formation.

and black, containing limestone nodules, with the black shale lying directly on the Bucktown Coal. The marine shale itself was just about 2 m thick and was not continuous as it extended for only about 180 m. Our focus in this study is on the conodonts that we extracted from this geographically restricted lens of shale overlying the Bucktown Coal.

Recovery in conodont bearing-samples ranged from one element from the black shale in Location D and from the gray shale at the top of Location B to 160 elements from the gray shale at the top of Location A. In total, 507 identifiable specimens representing five genera and four named species were recovered (Table 1). About 86 percent are Pa elements. This reflects the usual overrepresentation of Pa elements reported for most Pennsylvanian studies. With the exception of Location B, conodonts were generally more abundant in the upper gray shales than in the lower black shales and generally increased upward in the section (fig. 3). We interpret the irregular element distribution to show variation in environmental conditions in time and also laterally within a very limited geographic area.

## STRATIGRAPHY AND SEDIMENTATION

In Indiana, the Dugger Formation of the Carbondale Group was first recognized by Wier (1950) for exposures of sandstone, shale, coal, and limestone 3 km northeast of the town of Dugger in Sullivan County, Indiana. He placed the lower boundary of the formation at the unconformity above the Alum Cave Limestone Member (Wier, 1950, 1952) but the boundary was later lowered to include both the Alum Cave and black shale underlying it in the Dugger Formation (Wier and Gray, 1961). The Tri-State Committee on Correlation of the Pennsylvanian System in the Illinois Basin (2001) defined the Dugger to include the strata from the top of the Springfield Coal Member of the Petersburg Formation to the top of the

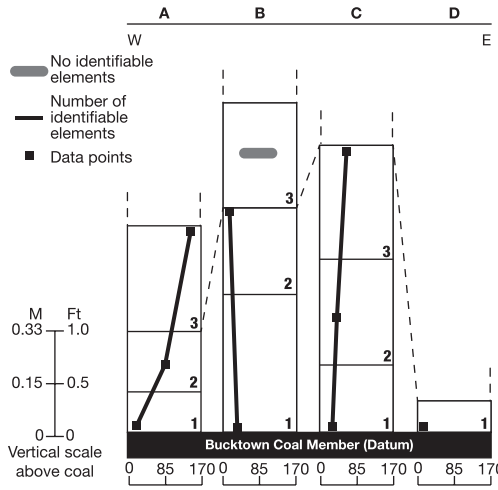


Figure 3.—Lithologic units, sampling locations, and sampling intervals. Scale of identifiable elements is at the base of each column. Approximately 60 m separate each sampling location.

Danville Coal Member. The strata include, in ascending order, the following named units: the Alum Cave Limestone, Antioch Limestone, Bucktown Coal, Herrin Coal, Providence Limestone, Hymera Coal, Anvil Rock/Bridge Junction Sandstone, Universal Limestone, and Danville Coal, and intervening unnamed beds of shale, sandstone, and coal (fig. 4).

The Bucktown Coal Member was first proposed by Wier in an unpublished manuscript and was first published by Burger and Wier (1970) who designated a type section near Bucktown in Sullivan County, Indiana. It was proposed for a coal that ranges from 0.3 to 1.2 m and averages 0.4 m in thickness but in places contains a shale parting as much as 3.4 m thick. The coal lies stratigraphically 3 to 20 m above the Springfield Coal Member of the Petersburg Formation and is commonly overlain by a black shale. The Bucktown has been correlated with the Briar Hill, formerly 5A, Coal Member of the Carbondale Formation in Illinois (fig. 4) (Hopkins and Simon, 1975). It has also been correlated with the Briar Hill, or Western Kentucky Number 10, Coal of the Carbondale Formation in Kentucky (Burger and Wier, 1970; Greb and others, 1992). Greb and others (2003), however, did not accurately represent the Tri-State Commission (2001) correlations in their study (see their fig. 2). They attribute Briar Hill to the Tri-State nomenclature, but the Tri-State Commission nowhere addresses Briar Hill or Briar Hill correlations.

Table 1.—Distribution of conodonts in the primary 1-kg samples of the Bucktown Coal Member of the Dugger Formation in Indiana by sample and location (see Figures 1 and 2 for locations and sample intervals).

| | Location | A | | | B | | C | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sample | 1 | 2 | 3 | 1 | 2 | 1 | 2 | 3 | D | Totals |
| *Idiognathodus delicatus* adult | Pa | 1 | 2 | 7 | - | 1 | - | 1 | 2 | - | **14** |
| *I. delicatus* juvenile | Pa | 9 | 48 | 76 | 13 | 43 | 12 | 7 | 35 | 1 | **244** |
| *Neognathodus* sp. | Pa | 1 | - | - | - | - | - | - | - | - | **1** |
| I/N ramiforms | Pb | - | 2 | 10 | 2 | 8 | 3 | 2 | 10 | - | **37** |
| | M | - | - | 2 | - | - | - | 1 | - | - | **3** |
| | Sb | - | - | 1 | - | - | 1 | 1 | - | - | **3** |
| | Sc | - | - | 1 | - | 1 | - | 5 | 1 | - | **8** |
| *Hindeodus minutus* | Pa | - | - | - | - | - | - | 2 | - | - | **2** |
| | M | - | 1 | - | - | - | - | 1 | - | - | **2** |
| | Sb | - | - | - | - | - | - | 1 | - | - | **1** |
| *Idioprioniodus conjunctus* | Pa | - | - | - | - | - | 1 | - | - | - | **1** |
| | M | - | - | - | - | - | - | - | 1 | - | **1** |
| | Sa | - | - | 1 | - | - | - | - | - | - | **1** |
| | Sc | - | - | 1 | - | - | - | - | - | - | **1** |
| *Adetognathus lautus* | Pa | 1 | 29 | 60 | 7 | 47 | 12 | 11 | 10 | - | **177** |
| | Pb | - | - | - | - | - | - | 1 | - | - | **1** |
| | M | 1 | 1 | - | - | - | 1 | - | 1 | - | **4** |
| | Sc | - | - | 1 | - | - | - | 4 | 1 | - | **6** |
| **Totals** | | **13** | **83** | **160** | **22** | **100** | **30** | **37** | **61** | **1** | **507** |

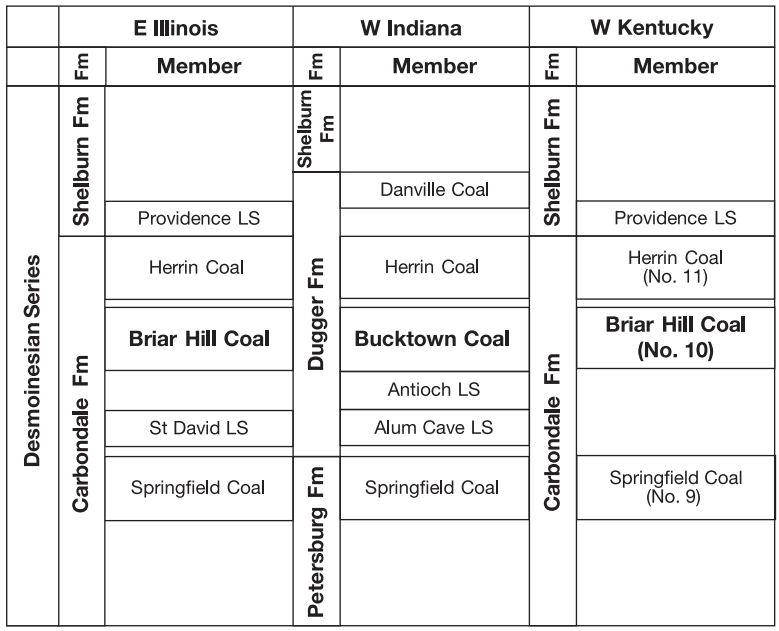


Figure 4.—Chart showing correlations of named stratigraphic units for part of the Desmoinesian Series in Indiana. No thicknesses are implied by the position of the names. Modified from Tri-State Committee on Correlation of the Pennsylvanian System in the Illinois Basin, 2001.

## THE CONODONT FAUNA

As is commonly the case with Pennsylvanian conodont studies, Pa elements of *Idiognathodus* dominate the collection. We assign these morphotypes to *Idiognathodus delicatus* Gunnell. *Adetognathus lautus* (Gunnell) is found in 90 percent of the samples and it ranges from 9 percent to 52 percent of the total Pa elements. Elements of *Hindeodus minutus* (Ellison) and *Idioprioniodus conjunctus* (Gunnell) are rare and we recovered only one broken element of *Neognathodus* sp. cf. *medexultimus* Merrill. The collection is reposited in the Indiana Geological Survey/Indiana University repository as numbers 18,225 through 18,267.

Many workers recognize that early ontogenetic specimens of *Idiognathodus* have morphologic features of another genus, *Streptognathodus* (for example, Van den Boogaard and Bless, 1985: Sweet, 1988). *Streptognathodus*-like morphologies develop paedomorphically from *Idiognathodus* in that *Streptognathodus* retains a trough into the adult stage, whereas in *Idiognathodus*, the trough fills (Brown and Rexroad, 2009). In our collection we have an excellent example of the ontogenetic growth stages of *Idiognathodus* (Plate 1). In most juvenile specimens of *Idiognathodus*, the carina extended to the posterior tip of the platform. During ontogeny the oral surface of the platform surrounding the carina gradually filled in progressively from the posterior tip towards the anterior part of the platform. This process resulted in the filling of the trough with the transverse ridges that define adult *Idiognathodus*. Additionally, ancillary nodes began to develop and grow with ontogeny. We believe that infilling of the trough and ancillary lobe development was environmentally driven and carries no taxonomic significance. Thus, we do not agree with those workers who split species on the basis of numbers of transverse ridges, morphology of the transverse ridges, or number or morphology of the ancillary nodes (for example, Lambert and others, 2003). Instead we accept variation in platform morphologic features as attributable to both ontogeny and the effects of environmental conditions on growth.

We think that the complex relationships that exist between *Idiognathodus* and *Streptognathodus* must be investigated from the inception of these morphologies, from Morrowan and Atokan time, and that all troughed and partially troughed forms prior to Missourian

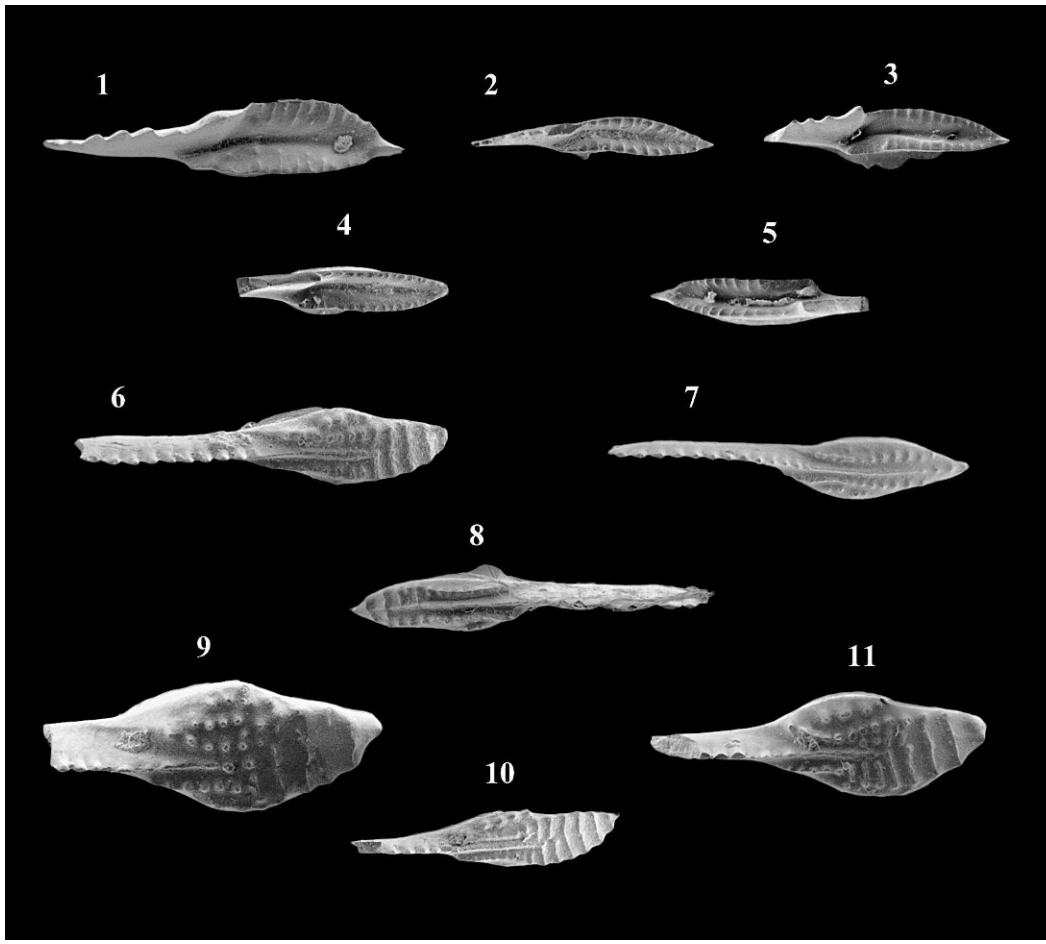


Plate 1.—Scanning electron microscope photographs. Magnifications are X40; locality and sample numbers are in parentheses following the repository number.

should be excluded from *Streptognathodus*. Therefore, in the Desmoinesian, we recognize only one genus, *Idiognathodus*.

We base Desmoinesian zonations on populations of *Neognathodus* species (Merrill 1972, 1975, 1999; Brown and others, 1991; Rexroad and others, 2001; Brown and Rexroad, 2009). Unfortunately, in this study we recovered only one broken Pa element on *Neognathodus*. The carina appears to exhibit a greater amount of fusion of the nodes than is common in this species and we attribute this to the influence of harsh environmental conditions. Based on only one specimen, we cannot meet our goal of contributing to regional correlation other than to say that the specimen exhibits characteristics of Desmoinesian neognathodids.

## PALEOECOLOGY

Even though we recognize that the number of elements in our collection is relatively small, we propose an interpretation of the environmental setting that resulted from the deposition of the black and gray shales that overlie the Bucktown Coal Member. The section represents generally shallow-water, low-energy, moderately to highly stressed, euryhaline marine conditions. However, there is great variability in conodont distributions, both vertically and laterally.

Conodonts are more abundant in the upper gray shale of Location A than they are in the lower black shales of all locations (fig. 3). But the opposite is true of Location B, in which the upper gray shale is almost barren. Thus the gray shales do not represent a uniform

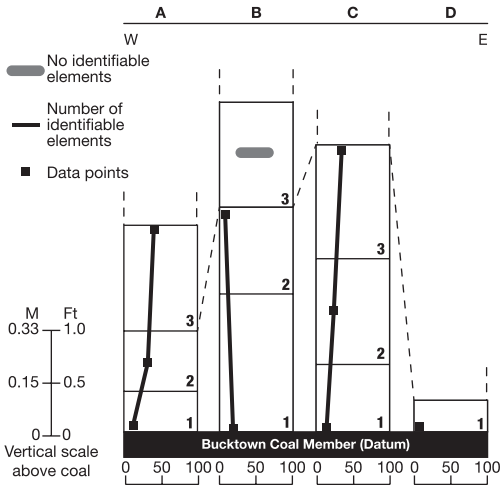


Figure 5.—Percentage of *Adetognathus* Pa's as a part of the total Pa's by sampling interval. Approximately 60 m separate each sampling location.

depositional environment, but instead environmental conditions vary in a relatively short distance such as between Location A and B. This lateral variation resulted in an environment inhospitable to conodonts in Location B. Further, the conodonts in Locations A, B, and C increase in abundance upward in the black shale. We interpret this distribution to show marine conditions above the coal changing from the relatively anoxic and restricted conditions that produced the lower black shales to very quiet low-energy conditions represented by the gray shales.

*Adetognathus* tolerates stressed euryhaline environmental conditions (Merrill and von Bitter, 1976) and is uncommonly abundant in this locality. We recovered *Adetognathus* from all but one sample. Its abundance indicates a very restricted, low-energy, shallow-water environment, possibly lagoonal or perhaps representing a small embayment; we interpret its presence to indicate moderately stressed to harsh conditions. *Adetognathus* Pa elements comprise from 9 percent to 52 percent of Pa elements in nine samples and this element is the dominant genus in two samples (fig. 5). If one accepts assignment to the *Adetognathus* biofacies on the basis of its Pa elements being approximately 10 percent or more of the total Pas, all but one of our samples belong in that biofacies. *Adetognathus* increases in abundance upward from the base of the black shale overlying the coal to the top of the black shale in Locations A and B and increases to the middle of the black shale in Location C. We interpret this to indicate progressively harsher environmental conditions, perhaps with progressively greater freshwater influence.

*Adetognathus* continues to increase in abundance in the gray shale overlying the black shale in Location A. This distribution supports our view that the gray shale at this location generally represents a shallow-water, harsh euryhaline environment, perhaps a lagoon or a small embayment.

Also suggesting a shallow-water, restricted environment, such as a lagoonal setting, is the high ratio of juvenile to adult specimens of *Idiognathodus* in all samples. Juvenile idiognathodids comprise 88 percent to 100 percent of *Idiognathodus* Pas. This ratio suggests that the paleoenvironment was harsh and resulted in a high juvenile mortality rate. But our previous studies (for example, Brown and others, 1991) suggest that juveniles prefer shallow, quiet water. Based on that observance, we attribute the higher proportion of juveniles relative to adults in this area to quiet water rather than to harsh conditions.

The rare *Idioprioniodus* elements further suggest low-energy, reducing conditions (Merrill and von Bitter, 1976). But the fragmentation and rarity of *Hindeodus* and *Neognathodus*, both of which are representative of normal, nearshore, open water conditions (Merrill and von Bitter, 1976), may indicate introduction of these genera into the depositional site by irregular storm events.

## SUMMARY

In summary, we collected conodonts from a geographically restricted gray and black shale marine lens that was lying directly above the Bucktown Coal Member of the Dugger Formation in a Solar Sources pit 3 km south of Petersburg in Pike County, Indiana. Pa elements of *Idiognathodus* dominated the collection and we were able to document an excellent example of ontogenetic growth stages of this genus. We do not recognize *Streptognathodus* in the Desmoinesian. Conodont distribution in numbers and in communal diversity is not uniform either vertically or laterally. Thus we conclude that environmental conditions were varied. We interpret the geographically restricted black and gray shales overlying the Bucktown Coal in this locality to represent a very nearshore, restricted, deltaically influenced

coastal plain environment. We further interpret the basal black shales overlying the coal to be of flotant marsh origin and to represent a localized flooding event. Distributary channels associated with a deltaic system subsequently delivered fresh water that resulted in very localized deposition of brackish water gray shales.

Restricted bays and possibly lagoons developed post-coal black and gray shales as the deltaically influenced depositional environments shifted from swamp to shallow marine settings. We think that migrations of delta lobes allowed local incursions of euryhaline marine waters sporadically and that this conodont community distribution generally indicates a nearshore deltaically influenced coastal plain environment rather than a normal middle to outer shelf open marine one. The evidence supports a model of localized control on sedimentation rather than sedimentation controlled by glacially influenced eustatic sea level change and we think that glacially controlled sea level changes were not a contributing factor to post-Bucktown Coal sedimentation patterns.

## ACKNOWLEDGEMENTS

The authors thank Dr. G. K. Merrill, University of Houston – Downtown, for his continuing efforts in helping us to understand and interpret Pennsylvanian conodont taxonomy and paleoecology. Drs. Derek Wright and David Myton of Lake Superior State University provided the expertise for SEM photography. Publication is authorized by the State Geologist, Indiana Geological Survey.

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*Manuscript received 19 April 2012, revised 29 October 2013.*

2013. Proceedings of the Indiana Academy of Science 122(1):35–39

# ANALYSIS OF IRON AND CALCIUM IN A GEOTHERMAL SYSTEM OUTFLOW STREAM

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**ABSTRACT.** A newly constructed building with an open-loop geothermal system outflow stream on campus provides a powerful context for student driven experimentation in an environmental chemistry course. In less than a year of operation, the rocks toward the frontend of the stream have already begun to turn orange (rusty) which has become a point of curiosity among the students. As a result, iron and calcium concentrations were monitored by atomic absorption spectroscopy along the stream in order to study the metal deposition process. The iron oxide deposition on the rocks in the stream, in-stream iron and calcium concentrations, and temperature were analyzed along the stream. As expected, the in-stream iron and calcium concentrations decreased down the stream, with a particularly larger drop in concentration following a small decorative waterfall. The concentration of iron oxide deposited on the rocks also decreased down the stream at a similar rate to the in-stream dissolved iron decline, strongly suggesting that the deposition on the rocks is the primary mode of iron removal. At less than a year in operation, the iron and calcium concentrations begin declining immediately upon entering the stream, indicating that the frontend of the stream has not yet become saturated. The environmental chemistry course plans to repeat these studies in subsequent years to monitor if/when the frontend becomes saturated and the deposition process begins moving farther downstream.

**Keywords:** Geothermal, AAS, Stream, Deposition, Iron

## INTRODUCTION

Taylor University recently completed (Fall, 2012) the construction of a new science building (the Euler Science Complex), which employs many sustainable features including wind turbines, solar panels, and a geothermal cooling system. Geothermal heating/cooling systems are generally considered energy efficient and environmentally friendly design strategies in new construction (L'Ecuyer et al. 1993). In fact, it is estimated that more than half a million homes in the United States now use geothermal systems (DTE Energy 2013). Open-loop geothermal systems use relatively constant temperature ground (well) water to absorb heat from the building during the summer and to input heat to the building during the winter. After the heat exchange process, the warmed (summer) or cooled (winter) well water is released to the environment as surface water. The geothermal discharge will differ from natural surface water in temperature and high mineral content. Open-loop designs often incorporate intentionally designed streams to provide time and opportunity for the mineral rich ground water used in the system to be naturally softened before significantly impacting the chemistry of nearby surface water bodies. A significant rise in mineral content or change in lake temperature can alter the balance within a native ecosystem (USGS 2012). Rocky streambeds can be used to encourage temperature acclimation and mineral deposition before reaching a surface water body. The Spring, 2013 Environmental Chemistry class (CHE330/530) chose to evaluate the mineral deposition within this newly created geothermal outflow streambed as a way of learning several analytical laboratory methods in a meaningful context that might also be beneficial to the University.

One of the first things students noticed while looking at the streambed and brainstorming experiments to be performed was the obvious rust color on the rocks. Clearly the rocks near the beginning of the stream were being coated by iron oxide, and yet the geothermal system had only been operating for six months. The color became lighter farther from the start of the stream, and by approximately 100 meters the rocks no longer appeared to be discolored. As in a typical water treatment facility iron is

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primary precipitated as iron oxide through a process of aeration (Droste 2008). This reaction is encouraged in the streambed design by using rocks and even small, esthetic waterfalls. The class decided to monitor this successful deposition of iron by analyzing the stream iron concentration and extractions of iron oxide deposits from the surface of rocks by atomic absorption spectroscopy (AAS; Lee et al. 2007; Sidhu et al. 1981). Whether or not there exists a saturation limit for iron oxide on the rocks became a principle question the students were interested in exploring.

In addition to monitoring the iron deposition, students were eager to measure the calcium concentration in the stream, knowing that calcium is the principle hardness ion. Unless the stream was unusually acidic, calcium should primarily precipitate as an insoluble carbonate or bicarbonate, both white solids. Upon initial visual inspection these white precipitates were not observed on the rocks like the iron oxide, which led students to predict that either it is precipitating in the soil or not precipitating at all. Again, students decided to use AAS to monitor stream calcium concentration and extractions of calcium deposition from rock surfaces.

Students also elected to monitor temperature and conductivity to complete the study. Temperature is one of the primary variables of concerns for open loop geothermal systems. If there is not sufficient time and distance for the stream to reach typical surface water temperatures before reaching a meaningful surface water body, there is potential for a gradual increase in temperature for the water body in the warm summer months and a gradual decrease in temperature for the water body in the cold winter months. Additionally, conductivity was monitored to investigate any trends among total dissolved ions in the stream.

## METHODS

The newly opened geothermal outflow stream on campus provides a rich and meaningful context for student laboratory exploration. The students' goals of measuring stream water iron and calcium concentration, deposited minerals on rocks, and stream water temperature and conductivity exposed them to the use of a state-of-the-art atomic absorption spectrometer, chemical extraction methods, and portable handheld data collection devices, respectively. Upon initial inspection of the stream, the rust colored coating on the rocks in the streambed faded away by about 100m from the stream source. Wanting to increase the level of student ownership of the project, this critical section of the stream was divided into fourteen sampling sites, one for each student in the class. Sampling sites were marked with flags throughout the study and for future follow-up work, at 6m intervals starting at the source. The would-be site at 24m is within a small culvert under a sidewalk and was not included as a sample site.

**Stream iron and calcium monitoring by AAS.**—Stream dissolved iron and calcium concentrations were analyzed using an iCE 3000 Series AAS (Thermo Scientific) equipped with an ASX-520 autosampler (CETAC). Three 300mL samples were collected at each sampling location. Samples were collected at 6 meter increments from 0 to 90m. 10mL of each sample were transferred into autosampler tubes for analysis. The instrument was calibrated using stock 1000ppm standard iron and calcium solutions from Sigma-Aldrich. 0.1 mg/L, 1.0 mg/L, 5.0 mg/L, 10.0 mg/L, and 30.0 mg/L calibration standards of iron and of calcium were prepared for quantitation.

**Rock surface deposition extraction.**—Average sized rocks, approximately the size of a baseball, were collected from the middle of the streambed at each of the 14 sample sites. In every case the top surface of the rocks were at least slightly discolored with the iron oxide. A 2cm by 2cm square was drawn with a Sharpie marker on the surface of the rock from where the surface mineral deposition would be extracted. Cotton swabs soaked in 0.1 M HCl were used to clean the marked $4cm^2$ sampling area.(Lee et al. 2007; Sidhu et al. 1981) In most cases several cotton swabs were needed for each rock. The used cotton swabs were placed in a beaker containing 50mL of 0.1 M HCl to dissolve the extracted minerals from the swabs. During this step, the solutions turn a typical yellow color indicating the presence of iron. The extract solutions were then transferred to autosampler tubes for analysis of iron and calcium. The determined extract concentrations (mg/L) are multiplied by the extract volume (0.05L) and divided by the sampling surface area ($4cm^2$) to produce a surface deposition concentration value in terms of mass of mineral per $cm^2$.

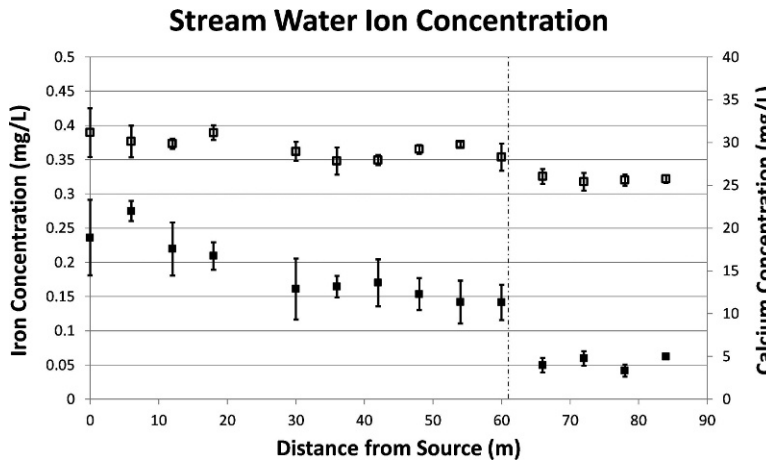


Figure 1.—In-stream dissolved iron (left axis, ■) and calcium (right axis, □) are shown versus distance from the stream source. Significant decreases in ion concentration occur following a small waterfall (61m) indicated with a dashed vertical line.

**In-stream measurements of temperature and conductivity.**—Temperature and conductivity measurements were made using respective probes for an Xplorer portable/handheld data collection device from Pasco Scientific. The temperature and conductivity measurements were recorded at each of the 14 sampling sites on five different days. The air temperature on each day was also recorded.

## RESULTS AND DISCUSSION

**Stream iron and calcium monitoring by AAS.**—The average of the triplicate samples for dissolved iron and calcium by AAS show a decrease in concentration with distance from the stream source as seen in Figure 1 ($Iron_{(aq)}$: linear $R^2 = 0.954$, with a total drop in concentration of −0.19mg/L which is much greater than the linear regression standard deviation of 0.014mg/L; $Calcium_{(aq)}$: linear $R^2 = 0.954$, with a total drop in concentration of −4.78mg/L which is much greater than the linear regression standard deviation of 0.70mg/L). Interestingly, iron and calcium share the same pattern of decrease in concentration. The set of iron and calcium concentration data pass the paired t-test after setting the expected mean difference equal to the difference between the observed mean values for all sites ($p = 0.721$; t-calc. = 0.37, t-crit. = 2.23). It is important to notice that although the iron concentration is significantly less than calcium, by 90m the iron concentration has decreased by approximately 85%. Calcium, which is much higher in concentration, has only decreased by approximately 16%. Despite the nearly complete depletion of iron, the small decrease in calcium concentration corresponds to a much larger total amount of calcium deposition than iron. Also, it is interesting to note the significant decrease in stream concentrations of both ions following the 60m sample. At approximately 61m, the stream has a small 1m aesthetic waterfall which may be contributing to oxidative deposition through aeration.

**Rock surface deposition extraction.**—The concentration of iron on the rock surfaces decreases with distance from the stream source until approaching an apparent minimum at around 66m and is displayed in Figure 2 (linear $R^2 = 0.963$, with a total drop in concentration of −1.66mg/L which is much greater than the linear regression standard deviation of 0.10mg/L). The surface iron concentration decreases by approximately 85% and at a very similar rate to the decline in dissolved iron concentration. The change in concentration for the two sets of iron data (deposition and dissolved) pass the paired t-test after setting the expected mean difference equal to the difference between the observed mean values for all sites ($p = 0.9999$; t-calc. = $3.3\times10^{-5}$, t-crit. = 2.2). There is a slight rise in iron deposition following the waterfall, which may be a result of increased aeration. One of our primary questions regarding the rock deposition is if there is a saturation point at which no more iron will collect on the rocks. Because the concentration at the 2nd site is

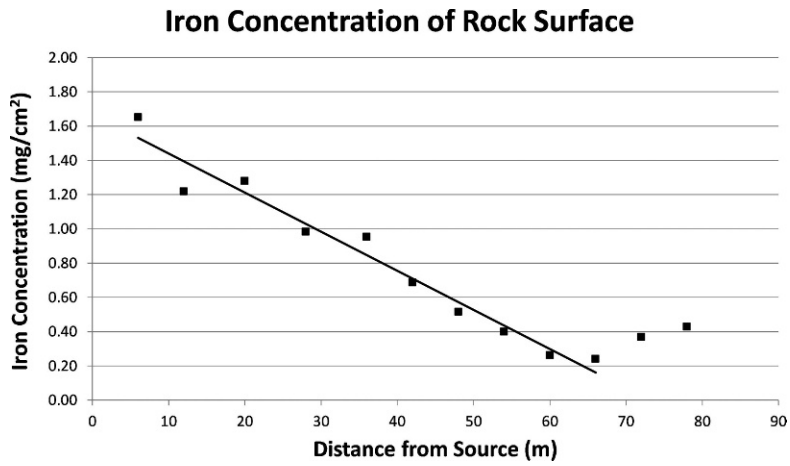


Figure 2.—A three point running average of the rock surface iron deposition concentration is shown versus the distance from the stream source. The deposition concentration decreases somewhat linearly ($R^2 = 0.963$) until reaching a minimum at 66m. The rate of decay (slope = $-0.023$ mg/cm$^2$ per m) will be compared to that observed in subsequent years.

significantly lower than that at the 1st site (drop in concentration from 1st to 2nd = $-0.433$mg/L which is much greater than the linear regression standard deviation of 0.10mg/L), the data reveals an immediate decline in deposition from the source suggesting that such a saturation has not occurred, yet. This type of study may be repeated in subsequent years, to specifically monitor the potential saturation at the beginning of the stream. If saturation occurs, it is expected that this deposition range will begin migrating downstream.

Calcium deposition on the rocks was below the detection limit and considered negligible. In every case the extraction concentrations of calcium were below 0.1mg/L (the smallest prepared standard) and are therefore not reported. The dissolved calcium concentration was observed to decrease significantly, and it is therefore concluded that most of the calcium deposition must be occurring with/on the streambed soil.

**In-stream measurements of temperature and conductivity.**—Stream conductivities at all sites

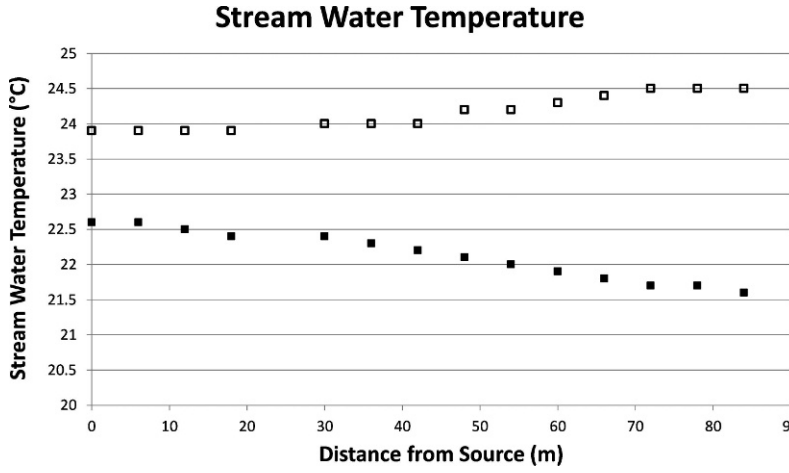


Figure 3.—The stream temperature versus distance from the source is shown. The warmest day, 23.0°C, is indicated with open squares, and the coolest day, 14.3°C, is indicated by the solid squares.

and on all days were in the range of 800–1000 μS but no discernible pattern of increase, decrease, or homeostasis could be determined. Plots of conductivity versus distance for source for each day of the five days, yielded least squares fit line with $R^2$ values ranging from 0.0063–0.862 and an average $R^2$ of 0.272. Temperature data from the warmest and coldest days are shown in Figure 3. On all five days, the temperatures at the outflow source were similar, ranging from 22.6 and 23.9°C and all within two standard deviations of the mean (22.26–24.24°C). However, on coolest day the temperature decreased gradually from the source (linear $R^2 = 0.977$, with a total $\Delta T = -1.0°C >>$ than the linear regression standard deviation = 0.06°C) and on warmest day the temperature increased gradually from the source (linear $R^2 = 0.913$, with a total $\Delta T = +0.6°C >>$ than the linear regression standard deviation = 0.07°C. Despite the fact that it is difficult to predict what stream temperatures ought to be, it can be generalized that in the warm spring/summer months (when this data was collected) the optimal scenario would be that the stream temperature is primarily governed by atmospheric temperature and not by the geothermal system (initial temperature at source). This small collection of data may suggest that the atmospheric conditions are capable of manipulating the stream water temperatures. Although there are challenges in accounting for variation in shade, flow rates, and surface area, future studies may include monitoring a natural stream nearby to provide statistical comparison with the geothermal stream and to determine if the geothermal stream temperatures eventually reach that of the natural stream.

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*Manuscript received 15 July 2013, revised 3 November 2013.*

2013. Proceedings of the Indiana Academy of Science 122(1):40–43

# A COMPARISON OF *CHIONASPIS SALICIS* INFESTATION INTENSITY UNDER ARTIFICIALLY ELEVATED $CO_2$ AND $O_3$

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**ABSTRACT.** Elevated greenhouse gases have significant impacts on forest communities at multiple trophic levels. To understand the effect of greenhouse gases on the phytophagous *Chionaspis salicis* bi-weekly observations were made at the Aspen Free-air Carbon Enrichment site in northern Wisconsin. These observations show that although the presence versus absence of scale was not related to greenhouse gas treatment, the intensity of scale infestation is related to greenhouse gas treatment.

**Keywords:** *Chionaspis salicis*, greenhouse gases

## INTRODUCTION

Atmospheric carbon dioxide ($CO_2$) levels are increasing with the current average concentration of $CO_2$ being approximately 360 parts per million (ppm) and the concentration is expected to increase to 550 ppm by 2050 (IPCC 2007). Troposopheric ozone ($O_3$) levels have also increased by 40% (IPCC 2007). Given these increases it is important to understand how changes in greenhouse gases affect forest ecosystems and at what trophic level these gases are most influential. Plants grown under elevated $CO_2$ have lower concentrations of foliar nitrogen compared to plants grown under ambient concentrations of $CO_2$ (Mattson 1980; Lincoln et al. 1986; Fajer et al. 1989). Decreases in foliar nitrogen concentration alter feeding behavior of herbivorous insects and result in the consumption of more plant material (Lincoln et al. 1986; Fajer 1989). Other consequences of feeding on plants grown under enriched $CO_2$ include increased mortality, decreased mass, and longer periods of time in larval stages (Price et al. 1980; Osbrink et al. 1987; Akey & Kimball 1989; Fajer et al. 1989, 1991).

*Chionaspis salicis* (Walsh 1868, Hemiptera: Diaspididae) is a forest insect pest (Miller & Davidson 1990; Miller et al. 2005) distributed throughout the United States, Ontario, and Mexico (Nakahara, 1982; Kosztarab 1996). *Chionaspis salicis* has been collected from bark and leaves of several ornamental trees including Canadian serviceberry (*Amelanchier canadensis* L.), red osier dogwood (*Cornus sericea*), eastern roughleaf dogwood (*C. florida*), white ash (*Fraxinus americana* L.), balsam poplar (*Populus balsamifera*), eastern cottonwood (*P. deltoides*), quaking aspen (*P. grandidentata*), sandbar willow (*Salix exigua*), and black willow (*S. nigra*; Kosztarab 1963; Dekle 1976). Infestation by *C. salicis* can completely cover twigs and branches of trees. Sap extraction by *C. salicis* can result in decreased tree vigor, dieback, stunting, and eventual death (Ulgenturk & Canakeloglu 2004). Scale insects are rare in forest habitats. But when present damaging infestations have been observed in managed ecosystems with low levels of plant diversity and structural complexity (Langford 1926; Johnson & Lyon 1988). In northern Wisconsin (where this study was conducted) *C. salicis* becomes active in late May. Two generations of *C. salicis* emerge each year (pers. obsv.).

Elevated greenhouse gasses have been shown to act on multiple trophic levels, however the majority of this research has utilized leaf chewing insects (Fajer et al. 1989, 1991). Insect damage can also occur when the insect feeds on plant sap, rather than leaves, and therefore may be less affected by elevated greenhouse gases. The goal of this research is to examine the effect of greenhouses gases ($CO_2$ and $O_3$) on the frequency and intensity of scale infestation of quaking aspen (*P. tremuloides*).

## METHODS

**The Aspen FACE site.**—The Aspen Free-air Carbon Enrichment (FACE) site in Harshaw,

Table 1.—The intensity of infestation of *Chionaspis salicis* on aspen tree clone 216 grown under one of four conditions. The data analysis was conducted on trees showing infestation only.

| | No infestation | Low infestation | Moderate infestation | Heavy infestation |
|---|---|---|---|---|
| Control | 390 | 6 | 0 | 0 |
| Elevated CO2 | 382 | 2 | 4 | 8 |
| Elevated O3 | 379 | 1 | 7 | 9 |
| Elevated CO2 + O3 | 385 | 8 | 3 | 0 |
| Total | 1536 | 17 | 14 | 17 |

WI, located in north-central Wisconsin, U.S.A. (89.7° W, 45.7° N). A complete description of experimental design, set-up, and operation of the FACE site is described in Dickson et al. (2001). In brief, the FACE site (32 ha) is comprised of 12 rings (30-m diameter), each of which is divided into three sections: the eastern half is made up of mixed aspen genotypes of five clones. The southwestern quarter is alternately planted with aspen clone 216 and paper birch (*Betula papyrifera*). Finally, the northwestern quarter is alternately planted with aspen clone 216 and sugar maple (*Acer saccharum*). The trees are exposed to one of four treatments: (1) control, (2) elevated $CO_2$, (3) elevated $O_3$, or (4) elevated $CO_2$ and $O_3$. Elevated $CO_2$ concentrations are based on levels predicted for 2060. Elevated $O_3$ levels are approximately 1.5 times that of background concentrations which replicates levels of moderately polluted locations in the western Great Lakes region (Pinkerton & Lefohn 1987). Ambient air is blown into the rings for the control treatment. The gasses were blown into the rings during daylight hours of the growing season. At the time of this study the trees were 6-years old.

***Chionaspis salicis* Assessment.**—The current generation of *C. salicis* (as evidenced by intact and pure white cover of the test) were examined on quaking aspen (*P. tremuloides*) clone 216 under field conditions. There are 132 clone 216 in each of the 12 rings. The intensity of scale infestation from the current generation was recorded between 20 May and 25 September, 2003 twice a week. To relate the presence of the white test (a waxy secretion that covers the body) to the number of scale on a tree, trees were recorded as having none, low, moderate, or heavy scale infestations (Van Driesche et al. 1998; Matadha et al. 2003). No infestation occurred when, upon close inspection of trees, there was no current generation as evidenced by the presence of the white test. Low infestation occurred when the trees examined from 1 m away appear uninfested, but upon close inspection revealed the presence of scattered scale. Moderate infestation occurred when the tree was visibly infested, but scale did not encrust stems and dieback of branches was not apparent. Heavy infestation occurred when the infestation was immediately visible from a distance, scales encrusted stems and dieback of branches was apparent.

**Statistical Analysis.**—The number of trees infested shown to have some scale present was low (Table 1) as was observed in other research (Langford 1926; Johnson & Lyon 1988). To understand the effect of elevated greenhouse gases on scale infestation the data analyzed includes only those trees that are infested. A chi-squared statistical test was used to assess the distribution of scale infestation in the four treatments.

## RESULTS

Biweekly surveys showed that scale infestations were present early in the growing season (mid-May) and scale were present throughout the growing season. Scale infestation in this experimental forest was low (48 trees out of 1584). The presence of scale was not related to greenhouse gas treatment ($X^2 = 5.793$; df = 3; $p = 0.1221$; Table 1). However, the intensity of scale infestation was related to the greenhouse gas treatment ($X^2 = 22.067$; df = 6; $p = 0.0012$). All trees ($n=6$) with scale infestation in control rings had an infestation categorized as low. Trees grown under elevated $CO_2$ or $O_3$ had more severe infestations (Table 1).

## DISCUSSION

Increases in greenhouse gases have been shown to influence tree growth, physiology, and phytochemistry (Poorter et al. 1997; Penuelas & Estiarte 1998; Isebrands et al.

2001; Percy et al. 2002; Veteli et al. 2002). Elevated $CO_2$ can decrease herbivore growth (Zvereva & Kozlov 2006) and elevated $O_3$ has been shown to increase insect growth (Valkama et al. 2007). Generally, in natural ecosystems, sap-sucking insects are found at low infestations as was observed in this study. Although there were scale present on trees under all three elevated gas treatments the severity of infestation was greatest under elevated $CO_2$ or $O_3$ and least under the CO2 + O3 rings and control rings. There could be several possible explanations for this pattern. First, the infestation could have been related to tree health rather than the greenhouse gas treatment. The severity of infestation was the greatest in the CO2 and O3 rings and if the infestation is related to tree health or vigor the growth and survivorship of trees in these treatments should be similar. This is not the case. Trees growth and survivorship in the CO2 rings was higher compared to the other treatments (Kubiske et al. 2007). Conversely, tree growth and survivorship in the O3 rings was lower compared to the other treatments (Kubiske et al. 2007).

Second, greenhouse gases could affect population dynamics of predators and prey. An increase in the number of aphids found under elevated $O_3$ and this is coincident with a decrease in the number of natural enemies (Percy et al. 2002). Thus, the increase in $O_3$ has affected predator prey dynamics of aphids. Therefore, it is possible greenhouse gases affect population dynamics of natural predators of scale insects similar to that seen in aphids.

Third, plants produce secondary metabolites important in defense against plant herbivory. It has been shown that changes in these secondary metabolites in response to atmospheric changes are more likely to occur in juvenile trees. However, phloem is generally lacking in these secondary metabolites (Douglas 2013). It is unclear how elevated atmospheric gases affect the presence or absence of secondary metabolites in phloem.

In summary, the presence of *C. salicis* on quaking aspen is independent of the greenhouse gas condition. However, the intensity of infestation is greater under elevated CO2 or $O_3$. Understanding these effects is important to better manage these habitats as greenhouse gases continue to increase.

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*Manuscript received 13 August 2013, revised 18 October 2013.*

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# EFFECTS OF BETAINE ON THE ULTRASTRUCTURE OF SALT-TREATED *ESCHERICHIA COLI*

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**ABSTRACT.** The ultrastructural changes have been studied in *Escherichia coli* 1130B-2ATCC (*E. coli*) grown on nutrient agar medium, medium + 0.8 M NaCl and medium + 0.8 M NaCl + 0.001 M betaine. Each dish was inoculated with $10^{-7}$ bacteria and allowed to grow for 72 hours. Colony counts in the media was, medium alone (83), medium + NaCl was (18) and medium + NaCl + betaine was (48), indicating that nearly three times as many colonies grew in the presence of betaine in the NaCl medium, than those grown in the NaCl medium alone. Electron micrographs of sectioned *E. coli* cells grown on normal medium displayed a cell wall composed of outer membrane and cytoplasmic membrane enclosing a periplasmic space. The nucleoid was centrally located and contained fine DNA fibrils. Numerous ribosomes were present in the electron-dense cytoplasm. The cell division was achieved by septum formation. Sectioned cells grown on NaCl medium displayed many vesicles being pinched off from the cell surface. The outer membrane and the cytoplasmic membrane were disrupted at several sites, resulting in loss of cellular contents. The cytoplasm in some sections became electron-lucent, devoid of ribosome and contained dense bodies and membrane whorls. In other cells the cytoplasm appeared fragmented into small masses. In longitudinal sections of the cells the nucleoid material appeared in thick DNA fibrils, which in transverse sections was seen as a dark, round clump. The cell division was arrested, non-dividing cells were elongated and displayed aberrant mesosomes, vesicles and bulges. Sectioned cells grown on NaCl-betaine medium appeared normal. Their outer and cytoplasm membranes were intact and enclosed a periplasmic space. The nucleoid material appeared as fine DNA fibrils. The cytoplasm was electron-dense and rich in ribosomes. This study suggests that betaine acts as an effective osmoprotectant for *E. coli* offsetting NaCl-induced osmotic stress by maintaining the integrity and stability of its cell constituents.

**Keywords:** *Escherichia coli*, outer membrane, cytoplasmic membrane, periplasmic space, nucleoid, ribosomes, vesicles, aberrant mesosomes, betaine

All organisms depend upon maintaining the consistency of their internal environment in different environmental conditions. Organisms have devised different mechanisms to cope with changes in their internal water, which is essential for survival (Yancey, 2005).

It is well know that high concentrations of sodium salts in the external medium exert toxic effects on microorganisms, plants and animals, retarding their growth and respiration, which can cause death (Heilbrunn, 1952; Lanyi, 1979; Yancey, *et-al.*, 1982). Organic substances such as free amino acids, sugars, methylamines and urea act as osmolytes in salt-stressed bacteria, plants and animals exposed to high salinity (Rudulier *et al.*, 1984; Yancey, 1982; 1985).

Certain species of non-halophilic bacteria exposed to high salt concentration in growth medium, respond by increasing intracellular concentration of amino acids like free proline or glutamate. In the presence of exogenously added amino acids to the salty growth-medium, accumulation of these metabolites is elevated, stimulating bacterial growth and respiration (Britten & McClure, 1962; Brown & Stanley, 1972; Measures, 1975; Csonka, 1979). Recently it has been shown that accumulation of trehalose is induced in NaCl-treated water-stressed cyanobacterium, *Nostoc punctiforme* (Yoshida & Sakamoto, 2009).

The aim of the present study was to investigate ultrastructural changes in the NaCl-treated *E. coli* and to demonstrate if exogenous betaine added to the NaCl medium counteracts deleterious effects

* Our esteemed colleague and co-investigator Dr. Duncan T. Kennedy passed away after completion of this study.

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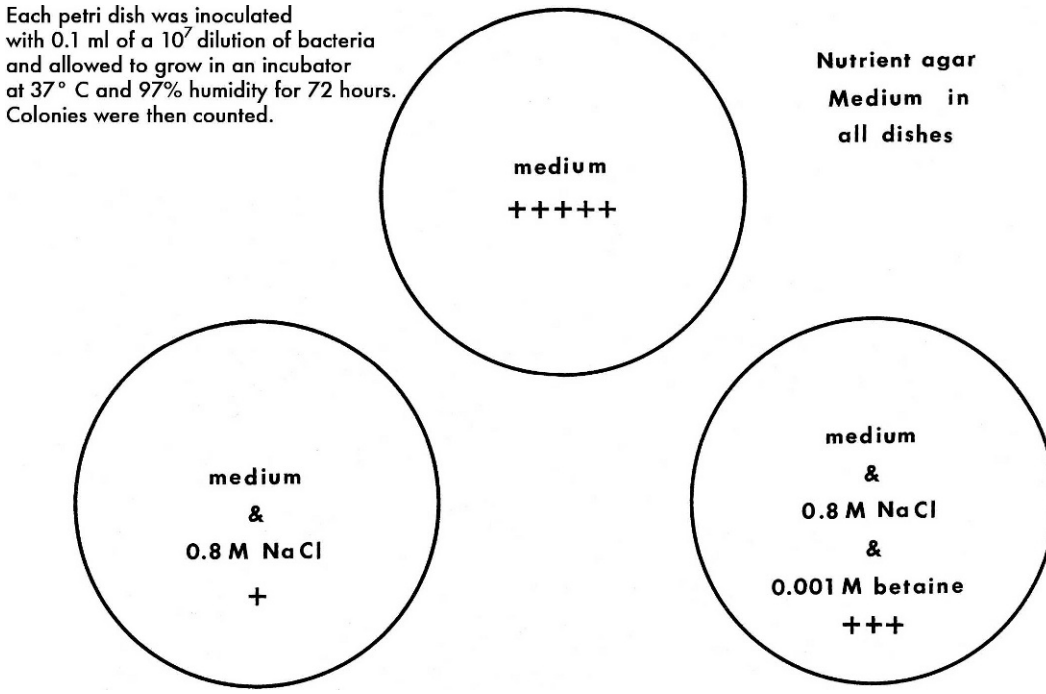


Figure 1.—A diagrammatic representation of numbers (+) of *E. coli* colonies grown in petri dishes on normal growth medium, medium + NaCl and medium + NaCl + betaine.

of high-salt medium and stabilizes the cellular constituents.

## METHODS

*Escherichia coli* 1130B-2ATCC was grown on nutrient agar medium (Difco Labs, Detroit, MI, USA), medium + 0.8 M NaCl and medium + 0.8 M NaCl + 0.001 M betaine (glycine betaine) (Sigma Chemical Company, St. Louis, MO. USA). Each petri dish was inoculated with $10^7$ bacteria and allowed to grow in an incubator at 37° C and 97% humidity for 72 hours to achieve equilibrium with the media. Colonies were counted.

For electron microscopic study, *E. coli* colonies were fixed for 15 minutes in the following mixture: 1part 8% glutaraldehyde, 1 part 4% osmic acid and 2 parts 0.1 M phosphate buffer (pH 7.2). The material was washed in several changes of phosphate buffer, dehydrated in an ethanol series to propylene oxide and embedded in Poly/Bed 812 resin (Polysciences, Warrington, PA, USA). Polymerization was carried out at 60° C overnight. Thin sections were cut on a Porter-Blum MT-2 ultra-microtome (ThermoFisher Scientific, Waltham, MA, USA). Sections were stained with uranyl acetate and lead citrate and examined with a Hitachi transmission electron microscope (Hitachi, Tokyo, Japan).

## RESULTS

**Growth of E. coli in media.**—The number of *E. coli* colonies grown on normal growth medium was five times the number of colonies that grew on the medium containing 0.8 M NaCl. Colony count (mean of two replicates) in the media was medium alone (83), medium + NaCl (18) and medium + NaCl + betaine (48), indicating that nearly three times as many colonies grew on the medium containing 0.8 M NaCl and 0.001 M betaine than those grown on the medium containing 0.8 M NaCl alone (Fig. 1).

**Electron microscopic observations.**—Electron micrographs of sectioned *E. coli* cells grown on normal medium measured about 3.0 um in length and 0.7 um in width. The cell wall was composed of an outer membrane, a distinct cytoplasmic (inner) membrane enclosing a periplasmic space.

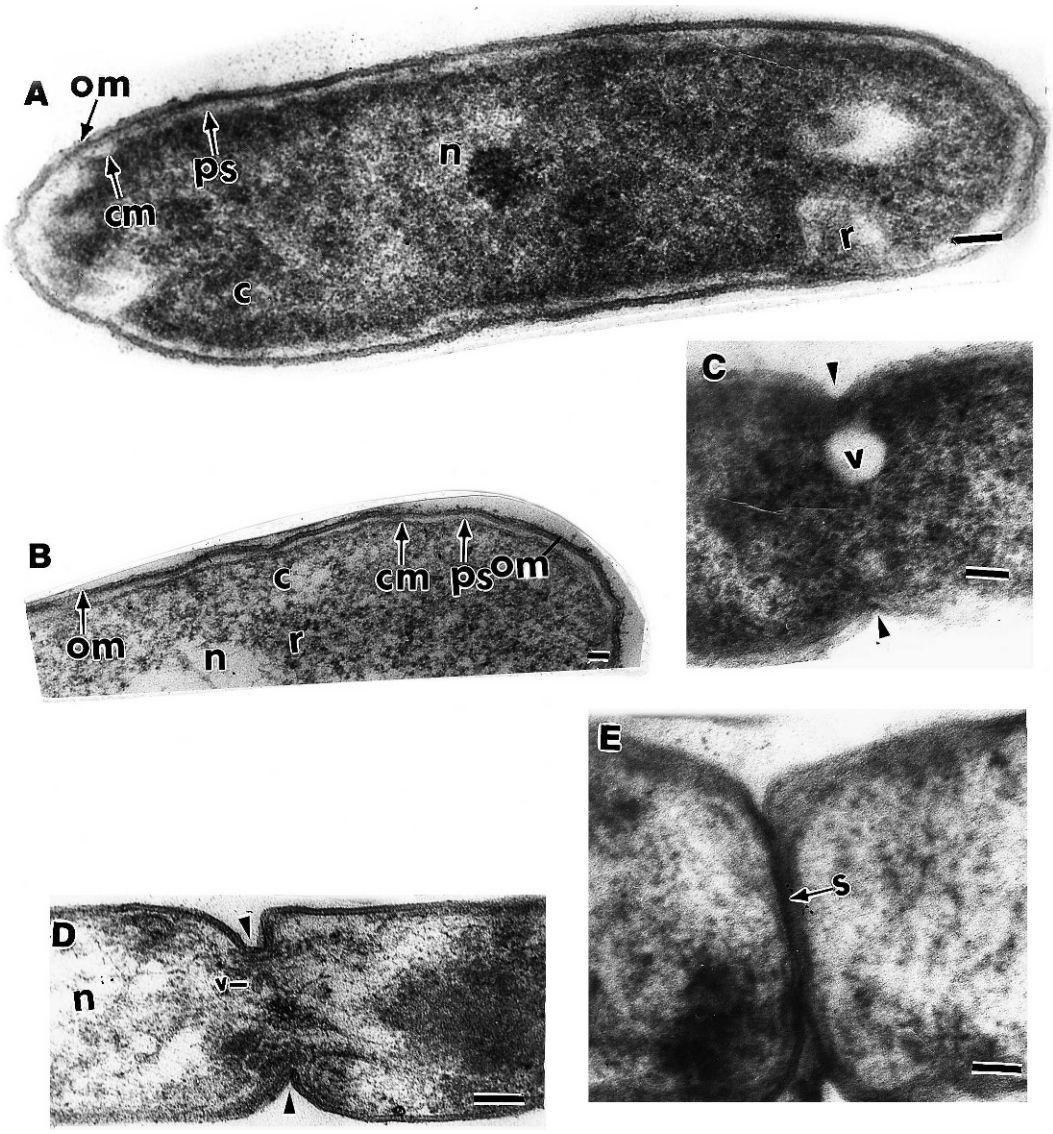


Figure 2.—Electron micrographs of sectioned *E. coli* in normal medium. A and B represent longitudinal sections of the rods displaying outer membrane (om), periplasmic space (ps) and cytoplasmic membrane (cm) constituents of the cell wall, nucleoid (n) and electron-dense cytoplasm (c) containing numerous ribosomes (r). C, D and E show progression (arrowheads) of cell division. n, nucleoid; s, septum; v, vesicles. Bar A–E, 0.1 μm.

The periplasmic space contained fine filamentous material, which probably represent peptidoglycan layer. The nucleoid displaying fine DNA fibrils occupied the central zone of the cells. The cytoplasm appeared electron-dense and contained numerous ribosomes (Figs. 2A, 2B).

The cell division was achieved by septum formation initiated by a bilateral shallow invagination in the middle of the cell. The invagination became progressively deeper toward the center of the cell and vesicles appeared at the division site (Figs. 2C, 2D). The septum was apparently formed by inward invagination of the cytoplasmic membrane and peptidoglycan layer with later contribution from the outer membrane (Fig. 2E).

Electron micrographs of sectioned *E. coli* cells grown on NaCl containing medium revealed drastic changes in all cell constituents. The nucleoid in longitudinal sections displayed thick

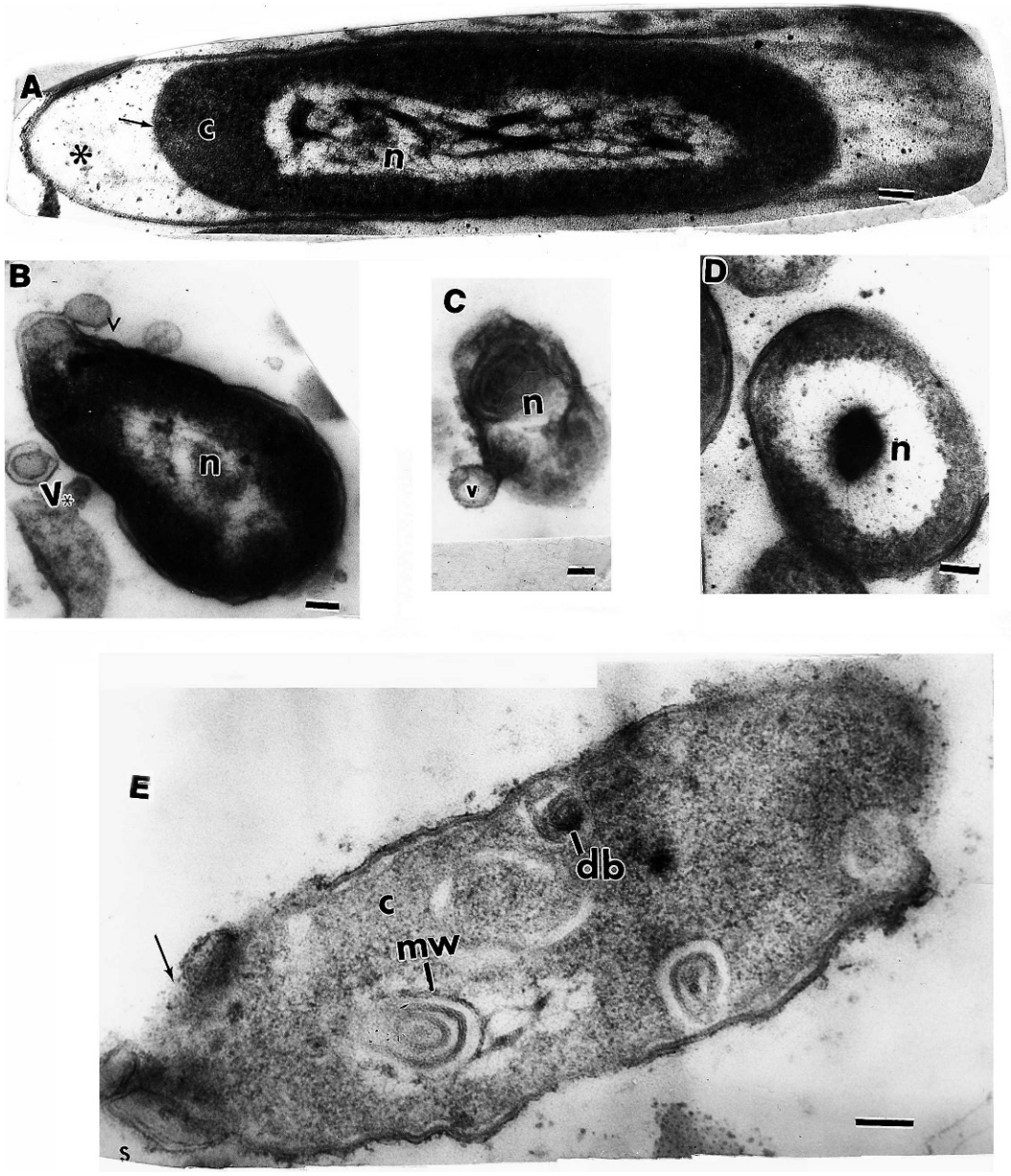


Figure 3.—Electron micrographs of sectioned *E. coli* in medium + NaCl. A represents a longitudinal section showing nucleoid (n) with thickened DNA fibrils and cytoplasm (c) separating from the cell wall (arrow) creating a clear space at the pole (*). B, C and D are transverse sections displaying dark, round nucleoid (n) and vesicles (v) extruding from the outer surface. Note the vesicle in Fig 3B is bounded by a double membrane (v*). E is a longitudinal section of an *E. coli* cell displaying a break in the cell wall (arrow) and electron-lucent cytoplasm (c) containing a dense body (db) and membrane whorls (mw). Bar A–E, 0.1 μm.

DNA fibrils (Fig. 3A), which in transverse sections appeared as a dark round clump (Figs. 3B, 3C, 3D). The cytoplasm was separated from the cell wall creating a clear space at the poles (Fig. 3A). In some cells the cytoplasm became electron-lucent devoid of ribosomes and displayed dense bodies and membrane whorls (Fig. 3E). In other cells the cytoplasm was broken into small masses and the cytoplasmic membrane was displaced toward the interior of the cell (Fig. 4A).


Figure 4.—Electron micrographs of *E. coli* cells in medium + NaCl *continued*. A represents a longitudinal section of a rod showing cytoplasm broken into dark masses (c). Displacement of the cell membrane (cm) into the cell interior is noteworthy. B represents transverse sections of empty looking rods with broken cell walls (arrows) and many vesicles (v) in close association with the external surface of the cells. C represents an elongated non-dividing cell showing vesicles (v) and bulges (b) at the external surface. m, aberrant mesosome; n, nucleoid. Bar A–C, 0.1 μm.

The cells exposed to NaCl containing medium displayed numerous vesicles with an average size of 100 nm, pinched off from the outer surface into the external space (Figs. 3B, 3C, 4B, 4C). In many cells the outer and cytoplasmic membranes appeared broken with the loss of cell contents into the external space (Figs. 3E, 4B). Cell division was arrested, non-dividing cells were elongated and displayed aberrant mesosomes and bulges near the center of the cells (Fig. 4C).

Most of the *E. coli* cells grown on medium containing 0.8 M NaCl + 0.001 M betaine appeared normal. They displayed intact outer membrane and cytoplasmic membrane enclosing a periplasmic space and centrally placed nucleoid containing fine DNA fibrils (Fig. 5A). The cytoplasm appeared electron-dense and contained numerous ribosomes (Figs. 5A, 5B, 5E). The increase in the number of *E. coli* colonies in the NaCl + betaine medium (Fig 1) apparently occurred due to division of rods by septum formation.

## DISCUSSION

This study has shown that the number of *E. coli* colonies grown on a growth medium containing 0.8 M NaCl and 0.001 M betaine were nearly three times more than the number of colonies grown on the medium containing 0.8 M NaCl alone. This observation suggests that betaine exerts a strong osmoprotecting effect, stimulating *E. coli* growth under salt-induced osmotic stress.

Electron micrographs of sectioned *E. coli* cells grown on normal medium showed the cell wall composed of outer and cytoplasm membranes enclosing a periplasmic space, the nucleoid containing fine DNA fibril and the cytoplasm containing numerous ribosomes. Similar ultrastructural features have been described in gram-negative bacteria by other investigators (Jensen & Park, 1967; Murray *et al.*, 2002). The cell division is achieved by septum formation. The septum is formed by a process apparently similar to that described by other authors (Burdett & Murray, 1974; Weigand, Vinci & Rothfielf, 1976). In the recent years it has been proposed that multi-protein Tol-Pal complex in gram-negative bacteria plays a physiological role in completion of cell division (Gerding *et al.*, 2007, Yeh *et al.*, 2010).

High NaCl concentrations in the external environment have been known to cause harmful effects on bacteria, plants and animals, retarding their growth and causing death (Heilbrunn 1952; Lanyi, 1979). Electron micrographs of sectioned *E. coli* cells grown on NaCl containing medium revealed deleterious changes in all cell constituents. The nucleoid displaying thickened DNA fibrils, which is seen as a dark clump in transverse sections, is apparently due to the osmotic loss of water from the cells. The drastic changes in the nucleoid and accompanying lack of ribosomes indicate a loss of protein synthesis. The presence of numerous vesicles at the external surface of NaCl- treated cells suggests changes in permeability of the outer and cytoplasmic membranes, possibly from alterations in the Tol-Pal protein complex. In the gram-negative bacteria, Tol-Pal protein complex is also implicated in maintaining outer membrane integrity (Cascales *et al.*, 2007; Yeh, 2010). In *E. coli* Tol-Pal mutants, electron micrographs clearly demonstrate the presence of vesicles at the cell surface, their formation has been attributed to a major defect in the outer membrane (Bernadac *et al.*, 1998).

In the present study of NaCl-treated cells failed to divide, the non-dividing cells were elongated and displayed aberrant mesosomes and bulges. Disruption of the outer and cytoplasmic membranes apparently has adverse effects on the framework of cell wall, transport of metabolites and energy production resulting in cell death.

Certain amino acids such a proline glutamate and betaine are known to protect cells against salt-induced osmotic stress and dehydration (Yancey *et al.*, 1982 Rudulier *et al.*, 1984). Britten & McClure, (1962), reported that in *E. coli* levels of intracellular proline were elevated in direct proportion to increases in osmolality of the medium in the presence of externally added proline. Exogenous proline has also been shown to offset the inhibitory effect of high NaCl in *Salmonella typhimurium* (Csonka, 1979). Furthermore, it has been demonstrated that proline transport into the cell-free membrane vesicles of *E. coli* is enhanced on exposure to media of high osmolality (Kaback & Deuel, 1969).

Glycine betaine, a small N-trimethylated amino acid, is widely distributed in nature and serves as an osmolyte, protecting cells from salt-induced osmotic stress (March, 1992; Rudulier *et al.*, 1984; Yancey, 2005). In marine invertebrates, betaine balances the osmotic pressure of the blood with that of the surrounding sea water (Baldwin, 1964). Halophilic plants growing in

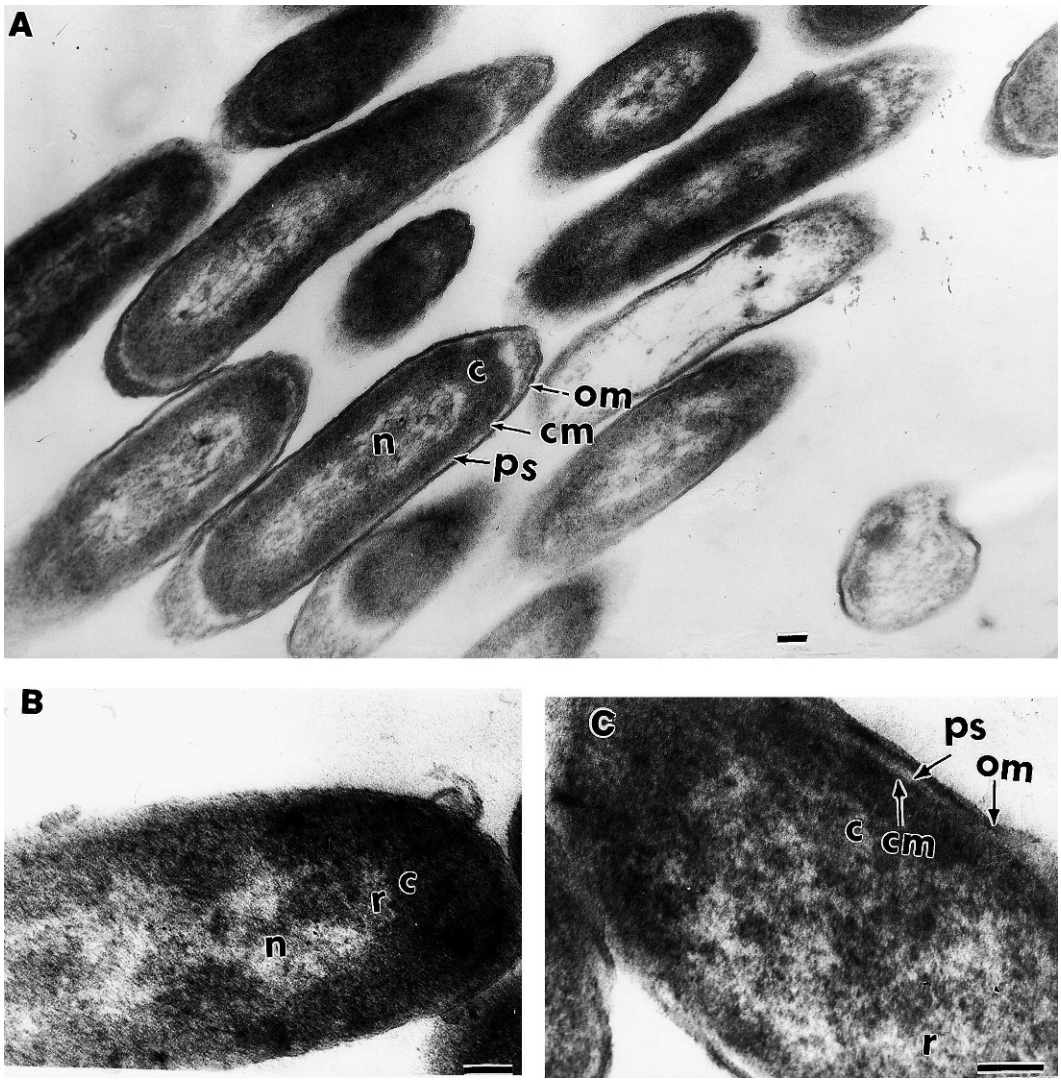


Figure 5.—Electron micrographs of *E. coli* sectioned cells in medium + NaCl + betaine. A represents a low magnification field showing normal looking rods. c, cytoplasm; cm, cytoplasmic membrane; om, outer membrane; n, nucleoid; and ps, periplasmic space. B and C are higher magnification images of parts of the cells, showing outer membrane (om), cytoplasmic membrane (cm), periplasmic space (ps) and numerous ribosomes (r) in the electron-dense cytoplasm (c) - n, nucleoid. Bar A–C 0.1 μm.

environments, in which the NaCl concentration fluctuates widely, increase synthesis of glycine betaine in response to salt stress, correlating with salt resistance (Rains & Valentine, 1979; Storey & Wyn Jones, 1975). Cyanobacteria strains with low salt tolerance synthesize and accumulate trehalose and or sucrose, and strains with the highest salt tolerance accumulate glycine betaine or glutamate betaine (Mackey et al., 1984; Yashide & Sakamoto, 2009).

In moderately halophilic bacterium $Ba_1$, the intracellular level of glycine betaine is directly proportional to salt-stress in salinities of 0.5 to 3.0 M NaCl, suggesting its role as a strong osmoregulatory solute (Risk, *et al.*, 1982). In addition Synechocystis DUN52, a halophilic cyanobactrium utilizes glycine betaine as a major osmolyte (Mohammed, 1983). Glycine betaine also stimulates the growth rate of enteric bacteria, *Klebsiella pneumoniae*, *Salmonella*

*typhimurium* & *E coli* in high-salt media and this stimulatory effect has been found to be far greater than that in proline, (Rudulier & Bouillard, 1983). Rudulier, *et al.*, (1984), have shown that growth of *E. coli* in NaCl medium is enhanced when supplemented with 0.001 M glycine betaine, which even in such a low concentration protects the bacterium from salt-stress. Furthermore, exogenous glycine betaine stimulates E. coli growth in the high-salt media by active transport into the cells driven by an electrochemical gradient, (Penroud & Rudulier, 1985).

In the present study, sectioned *E. coli* cells grown on NaCl-betaine containing medium appeared normal, displaying intact and distinct outer membrane and cytoplasmic membrane enclosing a periplasmic space. The centrally located nucleoid contained fine DNA fibrils. The electron-dense cytoplasm contained numerous ribosomes. These observations suggest that betaine acts as an effective osmoprotactant for NaCl-stressed *E. coli* by offsetting salt-induced osmotic stress. Organic molecules such as betaine are referred to as compatible solutes since they protect cells of organisms by counterbalancing perturbation of intracellular macromolecules (Yancey, 2005).

In conclusion this study has shown that *E. coli* growth is retarded in a NaCl containing medium, but its growth is stimulated in a NaCl-betaine containing medium. Ultrastructural features of normal *E. coli* cells including the outer and cytoplasmic membrane and enclosed periplasmic space, the nucleoid and ribosomes in the cytoplasm are severely altered and/or destroyed in a NaCl containing medium, but are restored to their normal form in a NaCl-betaine containing medium. The findings of this study suggest that yields of crops grown in soils with increasing salinity could be increased by adding small quantities of osmolytes like betaine to the irrigating water to protect soil microbes essential to plants, from salt-induced stress.

## ACKNOWLEDGEMENTS

This study was supported by a grant from the Indiana Academy of Science to M. S. Jarial. Technical support of Mrs. Deborah Brunner is gratefully acknowledged. The authors are thankful to Mr. Kevin Brooks, Science Librarian, Ball State University, Muncie, IN for his help in the literature search. We are also thankful to Ms. Michelle Jones for her help in preparing the figure plates.

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*Manuscript received 10 April 2013, revised 29 October 2013.*