Variation in glycogen concentrations within mantle and foot tissue in *Amblema plicata plicata*: Implications for tissue biopsy sampling

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Abstract: With the development of techniques to non-lethally biopsy tissue from unionids, a new method is available to measure changes in biochemical, contaminant, and genetic constituents in this imperiled faunal group. However, before its widespread application, information on the variability of biochemical components within and among tissues needs to be evaluated. We measured glycogen concentrations in foot and mantle tissue in *Amblema plicata plicata* (Say, 1817) to determine if glycogen was evenly distributed within and between tissues and to determine which tissue might be more responsive to the stress associated with relocating mussels. Glycogen was measured in two groups of mussels: those sampled from their native environment (undisturbed mussels) and quickly frozen for analysis and those relocated into an artificial pond (relocated mussels) for 24 months before analysis. In both undisturbed and relocated mussels, glycogen concentrations were evenly distributed within foot, but not within mantle tissue. In mantle tissue, concentrations of glycogen varied about 2-fold among sections. In addition, glycogen varied significantly between tissues in undisturbed mussels, but not in relocated mussels. Twenty-four months after relocation, glycogen concentrations had declined by 80% in mantle tissue and by 56% in foot tissue relative to the undisturbed mussels. These data indicate that representative biopsy samples can be obtained from foot tissue, but not mantle tissue. We hypothesize that mantle tissue could be more responsive to the stress of relocation due to its high metabolic activity associated with shell formation.

Key Words: glycogen, tissue variation, *Amblema plicata*, biopsy, relocation

With the development of techniques to obtain biopsy samples from mantle (Berg *et al.*, 1995; Byrne and Vesk, 1997) and foot tissue (Naimo *et al.*, 1998) in unionids, a new tool has emerged to measure the biochemical, contaminant, and genetic constituents in this imperiled faunal group. These techniques permit the measurement of indices of an organism's physiological condition without adversely affecting survival. Recently, biopsy samples have been removed from four unionid species (Berg *et al.*, 1995; Byrne and Vesk, 1997; Naimo *et al.*, 1998) and survival of biopsied and non-biopsied mussels has been similar for up to 19 months following biopsy. Furthermore, because of their nondestructive nature, these techniques have potential for use on endangered, threatened, or otherwise sensitive populations. However, possible variation in concentration of a given constituent within a tissue type is unknown. Because only a small mass of biopsied tissue is taken (about 10 to 40 mg wet weight), the representativeness of this sample must be determined.

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In the past few years, considerable effort has been invested into the relocation of unionids as a conservation strategy, principally in response to the threat of the exotic zebra mussel (Cummings *et al.*, 1997). As a consequence, numerous researchers are developing methods for relocation and for evaluating the success of relocations (Dunn and Layzer, 1997; Dunn and Sietman, 1997; Patterson *et al.*, 1997). However, because of the long life span of mussels, traditional measures of condition, such as changes in shell length or tissue weight, are often inappropriate for short-term observations. Thus, the identification of sublethal indicators of stress in unionids could provide valuable data on physiological condition that precedes measurable changes in survival.

We measured glycogen, because it is the principal storage form of carbohydrates in many aquatic invertebrates (Stetten and Stetten, 1960; De Zwaan and Zandee, 1972; Hummel *et al.*, 1989) and it has been used as an indicator of the energetic status of individual mussels (Holopainen, 1987; Hemelraad *et al.*, 1990). Alterations in glycogen concentrations in unionids can be observed long before changes in either growth or survival are observed. For

example, 3-month exposure to zebra mussels significantly reduced glycogen concentrations in *Amblema plicata* but did not adversely affect survival (Haag *et al.*, 1993). Furthermore, measurement of glycogen concentration is a good indicator of nutritional stress in unionids during quarantine periods before relocation (Patterson *et al.*, 1997).

Our goal was to determine the representativeness of biopsied tissue for the analysis of glycogen concentrations in foot and mantle tissue in *Amblema plicata plicata* (Say, 1817). Our objectives were (1) to determine if glycogen concentrations were evenly distributed within mantle and foot tissue; (2) to determine if glycogen concentrations varied between tissues; and (3) to qualitatively determine which tissue might be more responsive to the stress associated with relocating mussels from their native environment into an artificial environment.

METHODS

TISSUE SAMPLING

To estimate the distribution of glycogen in foot and mantle tissue in *Amblema plicata plicata*, we relocated individuals from Pool 9 of the Upper Mississippi River in May 1995, and placed them into mesh bags in a 0.04 hectare earthen pond. The pond was filled with well water (retention time of about 1 week) and was continuously aerated. No supplemental food was added to the pond during the study. After 24 months (June 1997), 5 individuals were removed, and mantle and foot tissue were dissected and subsequently frozen at -84°C until analysis for glycogen. To determine if the relocation altered the distribution of glycogen within and between tissues, we also determined the distribution of glycogen in undisturbed *Amblema plicata plicata* that were removed directly from the Upper Mississippi River in May 1997, but not subjected to relocation.

In both groups (relocated and undisturbed), we sacrificed 5 individuals (shell length 80-90 mm) for determination of glycogen. All unionids were free of zebra mussels. In each individual, the mantle and foot tissue were dissected into three sections. In mantle, the three 5-mm sections extended ventral (section A) to dorsal (section C) and in foot tissue, the three 15-mm sections extended anterior (section A) to posterior (section C; Fig. 1). All foot sections were removed from the ventral margin to exclude reproductive tissue from the sample. Within each section, we randomly removed five 5-mm^2 samples, mean 10.1 ± 0.1 mg wet weight in both foot and mantle tissue.

Because glycogen is frequently reported on a wet-weight basis, spatial differences in water content within a given tissue may bias estimates of glycogen concentrations. To determine if water content varied within a tissue, we measured the percent water in each section of foot and

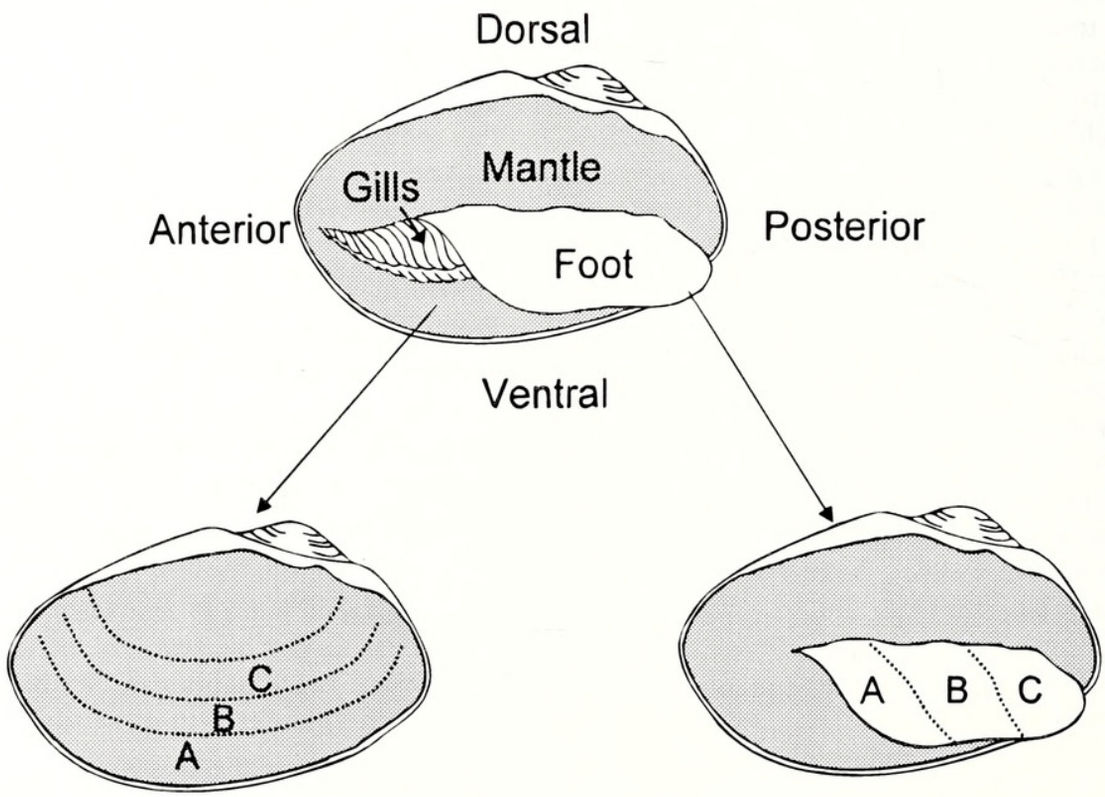

Fig. 1. Sample locations for foot and mantle tissue in *Amblema plicata plicata*. In the mantle, the three 5-mm sections extended ventral (section A) to dorsal (section C) and in the foot, the three 15-mm sections extended from anterior (section A) to posterior (section C).

(Hemminga *et al.*, 1985). Similarly, in the marine mussel *Mytilus edulis* (Linnaeus, 1758), the hepatopancreas and the mantle have been identified as the primary storage organs for glycogen (Bayne, 1973a, b; Zaba *et al.*, 1981). However, comparisons of glycogen concentrations between mantle tissue in freshwater and marine mussels should be made cautiously because mantle tissue in *Mytilus* is also the site for gonad development (Zaba *et al.*, 1981). Although we observed elevated concentrations of glycogen in mantle tissue, relative to foot tissue, in undisturbed *Amblema plicata plicata* this does not necessarily suggest that mantle tissue is a storage site for glycogen. Because the mass of foot tissue likely exceeds that of mantle tissue, the mass of glycogen in the foot could easily surpass that in the mantle.

The consistent reduction in glycogen concentrations in the relocated mussels, relative to the undisturbed mussels, suggests that the relocated mussels were not obtaining adequate nutritional requirements in this artificial environment. For example, mean concentrations of chlorophyll *a* (± 1 SEM) in the pond averaged 11 ± 4 µg/L (n=11) during April through November, whereas concentrations in the river averaged 51 ± 9 µg/L (n=15) during this same period. Furthermore, the magnitude of the reduction in glycogen was substantial--80% in mantle tissue and 56% in foot tissue. Similarly, Patterson *et al.* (1997) reported a 67% reduction in glycogen concentrations in mantle tissue in *Amblema plicata* from a site in the Ohio River heavily infested by zebra mussels (3,600 zebra mussels/m^2) relative to glycogen concentrations in mussels from a lightly infested site (0.3 zebra mussel/m^2). These data suggest that the relocation of unionids into refugia could be unsuccessful if the new environment does not contain adequate nutritional resources. In addition, even if adequate food resources are available, mussels must be physiologically-capable of obtaining and processing the available food.

Our criteria for selecting which tissue to biopsy for biochemical analysis were (1) within section variation in glycogen was minimal; (2) glycogen concentrations were similar across sections; and (3) it would be responsive to stress (in this case, relocation to an artificial pond). Using these criteria, the measurement of glycogen in foot tissue meets all three criteria. While the magnitude of the responsiveness to stress was greater in mantle tissue than in foot tissue, glycogen concentrations were not evenly distributed and were highly variable in mantle tissue. However, this should not preclude the use of mantle tissue, it just suggests a need for consistency in the location of the biopsied sample. In addition, the water content in a given section should be determined and used when reporting glycogen on a dry-weight basis in mantle tissue. If the mantle tissue is sampled, we recommend sampling the 5-mm section closest to the shell margin (section A) because it may be the most metabolically active and the easiest section to biopsy.

Although this study was conducted on a few individuals of *Amblema plicata plicata*, our preliminary data suggest that glycogen concentrations vary between and within certain tissues, and between relocated and undisturbed mussels. However, the applicability of these data to other species at other times of the year remains unknown. Future studies need to examine the influence of confounding factors, such as reproductive status, seasonal trends, and differences between sexes, on the distribution and utilization of glycogen in several unionid species.

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Recruitment in a freshwater unionid (Mollusca: Bivalvia) community downstream of Cave Run Lake in the Licking River, Kentucky

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Abstract: Unionids, fish, and glochidia were collected to determine why recruitment had ceased or had been dramatically decreased in a speciose unionid community in the Licking River at Moores Ferry, Kentucky, 35.4 km downstream of Cave Run Lake. Only six unionid glochidia were collected with drift nets, and only six fish collected had infestations of glochidia. A small percentage (10.1%) of the unionids observed had their gills modified as marsupia. An analysis of water temperature and discharge indicated no significant difference in average monthly discharge ($p > 0.05$) and a significant decrease in temperature ($p < 0.05$) between pre- and post-impoundment periods. Average monthly discharge and temperature may not be as biologically important as the spikes of discharge and corresponding sudden decreases in temperature that are caused by releases of hypolimnionic water from the reservoir.

Key Words: Unionidae, recruitment, gametogenesis, impoundments, Kentucky

North America has the richest unionoidean (mussel) (Bivalvia: Unionidae) fauna in the world. This fauna has disproportionately more endangered, threatened, and special concern taxa than all the groups of terrestrial organisms. Only 70 of the 297 taxa known from the United States are considered stable (Williams *et al.*, 1993). Of the freshwater unionid taxa recognized from the United States and Canada, 35% (103 taxa) are known to occur, or have occurred in Kentucky, which ranks this state third in faunal richness behind Tennessee and Alabama (Cicerello *et al.*, 1991). Human activities in the Commonwealth have severely impacted unionid populations during the last 200 years, making this group of organisms the most endangered in the state (Cicerello *et al.*, 1991).

Freshwater mussels are impacted by anthropogenic factors such as the additions of toxic substances into aquatic systems, sedimentation, habitat destruction, loss of their host fish(es), introduced species, and commercial harvesting (van der Schalie and van der Schalie, 1950; Fuller, 1980; Bogan, 1993; Williams *et al.*, 1993). The construction of dams along the course of several rivers in North America has resulted not only in the loss of taxa, but also of whole beds (*e. g.*, van der Schalie and van der Schalie, 1950; Bates, 1962; Miller *et al.*, 1984; Miller *et al.*, 1992; Williams *et al.*, 1992; Layzer *et al.*, 1993; Sickel and Chandler, 1996). One of the most severe and perplexing problems facing freshwater mussels is the documented loss of recent recruitment (reproduction) in unionid communities that were previously thought to be healthy.

Recent research hypothesized that recruitment had ceased or had been dramatically decreased in a diverse unionid community in the Licking River at Moores Ferry, Kentucky (Kane, 1990; Smathers, 1990). The present study was an attempt to determine if recruitment had ceased or had been decreased in this diverse unionid community, and if so, to determine where in the life cycle reproduction was breaking down. This study was also an attempt to determine what effects, if any, the hypolimnionic discharges from Cave Run Lake were having on the mussels in the bed at Moores Ferry. It was hypothesized that the loss of recruitment in this bed was either directly or indirectly the result of the dam, located approximately 35.4 km upstream, which has altered both the natural temperature and flow regimes of the river.

METHODOLOGY

STUDY AREA

The Licking River is a sixth order tributary to the Ohio River, originating on the Unglaciated Allegheny Plateau in the Appalachian Province of eastern Kentucky. The river flows through an extremely variable topography in a northwesterly direction through the Blue Grass region

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of the Commonwealth for 496 km until its confluence with the Ohio River near Covington, Kentucky, at Ohio River km 757.2 (Harker *et al.*, 1979, Hannan, *et al.*, 1982, Burr and Warren, 1986). This drainage encompasses approximately 10% of the Commonwealth (9601 km^2) and covers all or a portion of 21 counties (Harker *et al.*, 1979).

In 1974 the Licking River was impounded to form Cave Run Lake, a warm oligotrophic lake (Clinger, 1974). The lake has a surface area of 3347 ha that inundates 61 km of the mainstem and the lower reaches of several small tributaries (Burr and Warren, 1986). The Licking River drainage has a speciose unionid fauna with 53 taxa, over half of the state's native mussel fauna, historically existing in the drainage (Cicerello *et al.*, 1991). A recent survey below the reservoir indicated that 50 taxa still reside in that portion of the river (Laudermilk, 1993).

Moores Ferry, Kentucky, is a ford approximately 35.4 km downstream from Cave Run Lake (Fig. 1). The watershed at this site is utilized mainly for agriculture. Discharge at this site is affected by releases of water from Cave Run Lake, which at times causes drastic water level fluctuations. Substrata consisted mainly of cobble, gravel, rubble, and some boulders with intermixed sandy areas (Smathers, 1990). The unionid community at this site had a rich assemblage of unionid species, with 35 known present or historical taxa (Smathers, 1990; Laudermilk, 1993).

SAMPLING AND LABORATORY PROCEDURES

Five collections of glochidia, fish, and unionids were made from July through October 1995. High water conditions prevented collections during Spring 1996. For each collection period a drift net was haphazardly placed in the bed to collect glochidia. After one hour, the contents of the drift net were removed and preserved in 70% ethanol and returned to the laboratory. Drift net collections were examined using cross-polarized light microscopy (Johnson, 1995), with a portion of the sample being delivered into a watch-glass with a gridded bottom. Each square in the grid was then systematically searched under cross-polarized light (10-20X) for glochidia, which were counted and removed along with any juvenile *Corbicula fluminea* (Müller, 1774).

Fish were collected for one hour using a minnow seine. All fish retained were initially preserved in a 10% formalin solution and then transferred to 70% ethanol in the laboratory for final preservation. Following sorting and identification, the fins and scales of each individual were examined under a dissecting microscope (10-30X) for attached glochidia. The opercular flaps were removed, and each gill arch was carefully examined under a dissecting microscope for attached glochidia (Bruenderman and Neves, 1993).

Unionids were collected by hand for one hour by snorkeling, or by wading with the use of water scopes. After identification, the shell of each unionid was carefully opened with a small screwdriver, and the gills examined for signs of gravidity. The species name was recorded and notes were made on the condition of the gills. Except for individuals of two target species retained for histological examination, all unionids were returned to the river. Three to five individuals of the two most common species in the bed at Moores Ferry (Smathers, 1990), *Actinonaias ligamentina* (Lamarck, 1819) and *Elliptio dilatata* (Rafinesque, 1820), were chosen for histological examination from each collecting period. These species represented both of the breeding regimes of freshwater mussels, they were both commonly encountered throughout their respective ranges (Oesch, 1995), and neither had any federal or state protection status in Kentucky (Kentucky State Nature Preserves Commission [KSNPC], 1996).

Individuals were prepared for histological examination by placing them into a 10% formalin solution, then transferring them to 70% ethanol in the laboratory. The valves were opened by cutting the adductor muscles and portions of the gonadal and gill tissues were removed and placed into either 70% ethanol or Bouins fixative. These were then dehydrated through a series of alcohols and embedded in paraffin (Humason, 1967). Sections were made at a thickness of 10 μm using an American Optical 820 Microtome. Slides were stained with Ehrlich's hematoxylin and eosin was used as the counterstain (Humason, 1967). Sections were mounted with Permount. The sections were then examined under a compound microscope (400-430X) to determine a sex ratio for both species; to determine if gametogenesis was occurring, and, if so, to try to quantify it; and to determine the contents of the marsupia. All drift net, fish, and unionid collections were deposited in the Branley A. Branson Museum of Zoology, Eastern Kentucky University (EKU).

Five cell types of spermatogenesis (Garner, 1993) were used to determine the stage of gametogenesis in males. Stage 1 males were those that had only spermatogonia present in their acini, and Stage 5 males had mature spermatozoa present. Stages 2, 3, and 4 corresponded respectively to sperm morulae, spermatocytes, and spermatids being present in the acini. Three cell types of oogenesis (McMurray, 1997) were used to determine the stage of gametogenesis in female *Elliptio dilatata*. Stage 1 females were those with oogonia as the dominant cell type in their alveoli, Stage 2 were those with oocytes dominant, and Stage 3 were those with mature ova dominant.

Marsupia were classified according to their contents as being empty (EM), or containing mature glochidia (MG), early embryos (EE), or advanced embryos (AE) (Garner, 1993). In the case of known females that did not have their gill tissues examined, the marsupia were consid-