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FRESHWATER MUSSELS OF THE POWELL RIVER, VIRGINIA AND TENNESSEE: ABUNDANCE AND DISTRIBUTION IN A BIODIVERSITY HOTSPOT

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ABSTRACT

The Powell River, located in southwestern Virginia and northeastern Tennessee, is a tributary of the Clinch River in the headwaters of the Tennessee River system. Historically, the Powell River had a diverse freshwater mussel fauna of 46 species. Various surveys conducted over the past century have recorded a decline in mussel densities and diversity throughout much of the river, due to historical and on-going anthropogenic impacts. In 2008 and 2009, random timed-search, systematic search, and quadrat sampling of 21 sites were completed to document species richness, relative abundance, density, and size-class structure of resident mussel populations. During the random timed search (10 sites) and systematic search (10 additional sites) portions of the survey (n=1,399 person-h), surveyors collected 15,084 mussels of 29 species. Catch-per-unit-effort ranged from 0.33 to 22.12 mussels/person-h. We observed living individuals (n = 412) of 9 of the 17 federally endangered species previously reported in the river (*Dromus dromas*, *Epioblasma brevidens*, *E. triquetra*, *Fusconaia cor*, *Lemiox rimosus*, *Plethobasus cyphyus*, *Quadrula cylindrica strigillata*, *Q. intermedia*, and *Q. sparsa*) and two candidate species for federal protection (*Pleuronaia dolabelloides* and *Ptychobranchus subtentum*). We recorded 19 species from 18 sites, including 5 endangered species during quadrat sampling efforts. Mean densities ranged from 0.00 to 2.25 mussels/m^2 among sites sampled. Relatively recent recruitment was also evident for 16 of 29 species; including 4 endangered species (*D. dromas*, *E. brevidens*, *Q. intermedia*, and *Q. sparsa*). The mussel fauna of the lower Powell River continues to represent one of the most diverse in the United States. Outside of the Powell River, only 2 or 3 populations remain for most of the listed species extant in the river. Given these qualities, the Powell River deserves recognition as a location for focused conservation efforts to protect its diverse mussel assemblage.

KEY WORDS Freshwater mussels, Powell River, Survey, Endangered Species, Biodiversity

INTRODUCTION

The freshwater mussel fauna of the Powell River is one of the most diverse in the United States. Historically, the river was inhabited by 46 species of mussels (Table 1). Various factors account for this diversity, such as the river valley not being glaciated during the Pleistocene epoch, a carbonate-rich lithology draining the Valley and Ridge Physiographic Province, diverse and favorable habitat types, and low level of development.

Several researchers over the past century, beginning with Ortmann (1918), have sampled mussels in much of the river (Ahlstedt, 1986, 1991a; Ahlstedt & Brown, 1979; Dennis, 1981; Ahlstedt & Jenkinson,

1987; Jenkinson & Ahlstedt, 1988; Hubbs et al., 1991; Wolcott & Neves, 1994; Ahlstedt & Tuberville, 1997; Eckert et al., 2004; Ahlstedt et al., 2005). Most recently, Ahlstedt et al. (2005) documented 36 extant species in the drainage from samples taken over a 30 y period. They reported 13 of the 17 species known from the drainage that are listed under the Endangered Species Act.

The river's mussel fauna was already experiencing a noticeable decline from anthropogenic impacts reported by Ortmann (1918). Ortmann noted that a large portion of the mussel fauna in the upper river had already been decimated downstream of a wood extraction plant located in Big Stone Gap, Virginia. Between the 1960s and 1990s, mussels in other portions of the river became increasingly rare (Dennis, 1981; Ahlstedt & Jenkinson, 1987; Jenkinson & Ahlstedt, 1988; Hubbs et al., 1991; Wolcott & Neves, 1994; Ahlstedt & Tuberville, 1997; Eckert et al., 2004; Ahlstedt et al., 2005). According to Ahlstedt et al. (2005), D. H. Stansbery used sampling data collected between 1963 and 1971 to confirm his initial observations that the mussel fauna had declined substantially in the half century since Ortmann's collections. Subsequent sampling has confirmed this decline (e.g., Wolcott & Neves, 1994; Ahlstedt et al., 2005).

Mussel declines in the Powell River have largely been attributed to habitat degradation caused by agricultural practices, urban development, and coal mining (Dennis, 1981; Ahlstedt & Tuberville, 1997; Diamond et al., 2002; Ahlstedt et al. 2005). Ahlstedt et al. (2005) considered mussel distributions and abundances to be heavily influenced by the location of mined lands in the watershed. Additional studies have shown that runoff of sediments contaminated with by-products from coal mining activities is a potential factor leading to mussel declines (McCann & Neves, 1992). Black-water events (coal fines released into the river from processing activities) have occurred frequently over the last 100 y in this watershed (Ahlstedt et al., 2005). Following a period in the early 1980s, when the entire river was known to occasionally run black with coal fines (Ahlstedt, 1986), a mussel die-off was observed in 1983 between Powell River kilometer (PRKM) 230.9 and 104.8 (Ahlstedt & Jenkinson, 1987; Jenkinson & Ahlstedt, 1988). In order to understand the effects these anthropogenic events have had on the river's diverse mussel fauna, we collected current data on species presence and abundances, distribution, and size-class structure. To that end, we utilized three different sampling techniques to assess demography, distribution, and abundance of freshwater mussels at 21 sites in the Powell River. We are providing this information so that future conservation efforts can better protect the threatened mussel fauna in the Powell River.

METHODS

Study Area

The Powell River originates near Norton in Wise County, Virginia, flows in a southwesterly direction, and enters Norris Reservoir, an impoundment of the Clinch River [at CRKM 127] (Fig. 1). The watershed drains an area of approximately 2,453 km2, and is wholly contained within the Valley and Ridge Physiographic Province. Numerous parallel ridges and subterranean drainages define the Powell River watershed (Tennessee Department of Environment and Conservation 2007). Prominent land cover includes forest (58.7%), agricultural lands (29.8%), developed, mined and barren lands (9.7%), and open water and wetlands (1.8%) (2006 NASA Landsat Data Collection [U.S. Geological Survey 2011], which were extracted via ArcMap version 9.2 using USGS 8-digit HUC [Steeves & Nebert 1994]). The mainstem of the river is characterized by long pools interrupted by periodic shallow shoals where substrate consists predominantly of a heterogeneous mix of sand, gravel and cobble.

Twenty-one sites were selected for sampling (Table 2; Fig. 1). Eighteen sites were selected based on the locations of previously documented living mussel assemblages (Dennis, 1981; Ahlstedt, 1991a; Wolcott & Neves, 1994; Ahlstedt et al., 2005; Eckert et al., 2004). Three additional sites, previously un-surveyed, were also selected because they contained accessible reaches that met the following criteria: (1) suitable shoal habitat present, and (2) where results of cursory visual and tactile survey (using snorkel gear) showed that mussels were present. We conducted these surveys in suitable habitat, which we defined as riffles and runs consisting of a stable heterogeneous mix of sand, gravel, and cobble.

Survey Approach

We employed three different survey strategies to obtain species richness, relative abundance, density estimates, and evidence of recruitment. To quantify species richness and relative abundance, one of two survey strategies was used. Based on previously obtained data (Ahlstedt et al., 2005; Wolcott & Neves, 1994; J.W. Jones, USFWS, unpublished data), if federally listed species were not likely to occur at a site, a random timed search (RTS; defined below) was used to maximize search area with minimal search time. Conversely, if federally listed species were likely to occur at the site, a systematic search (SS; defined below) was used to maximize detection. Quadrat sampling was performed at all sites to quantify density estimates and

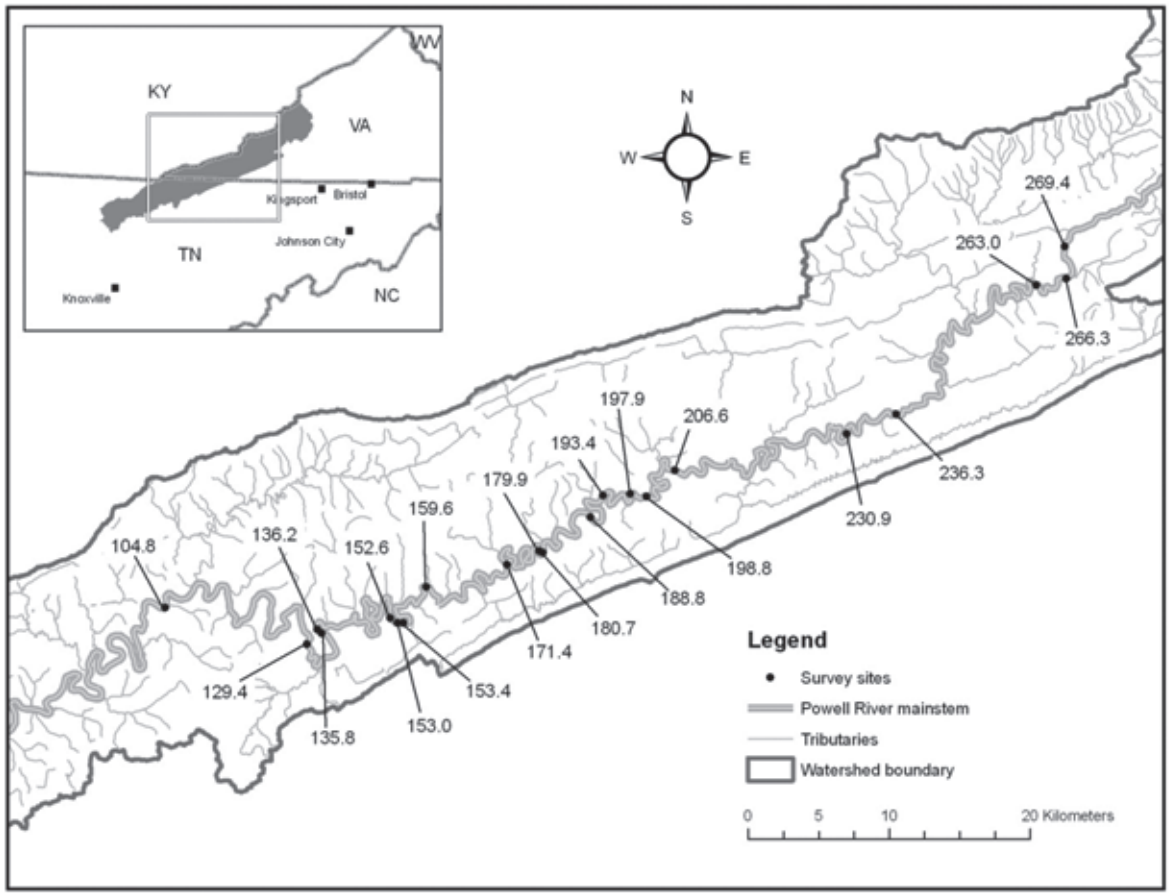

FIGURE 1
Sites surveyed using random timed search, systematic search, and quadrat sampling methods in the Powell River.

increase the probability of detecting recent recruitment.

For all survey methods, we utilized visual and tactile search methods with mask and snorkel to collect mussels. A core crew of three surveyors conducted all types of surveys; however, additional assistance was required at several sites and varied up to 20 people.

Random Timed Searches—We performed RTS at a total of 10 sites (Table 2). Surveyors initiated sampling at the downstream boundary using a series of lateral sweeps to cover as much habitat as possible within the entire delineated site, typically between 100 and 200 m. This method enabled surveyors to maximize search area while minimizing search time.

During RTS, we only collected mussels that were visible at the substrate surface and did not excavate to search for mussels. Surveyors attempted to sample the entire wetted-width of the river at each site. As mussels were found, surveyors left them undisturbed in the substrate, and marked their location with a wired florescent flag. A separate crew followed the snorkelers to remove flagged mussels and record data. Collected mussels were counted, identified to species and/or sub-species level, measured for maximum shell length (mm, anterior to posterior margin), sexed (if possible), and returned to their locations of collection. Catch-per-unit-effort (CPUE) was calculated as total number of mussels divided by the amount of time spent surveying per person, expressed hereafter as person-hours (p-h). For medium to large sized (e.g., 70 - 140 mm) mussels, we assumed mussels < 40 mm in shell length were approximately 2 to 4 y old, and that the presence of mussels below this threshold showed recent recruitment (e.g., Ahlstedt et al., 2005). For smaller species (e.g. < 70 mm), we assumed mussels < 30 mm were evidence of recent recruitment.

Systematic Searches—We conducted a SS at 10 sites (Table 2) based on likely occurrences of federally listed mussels. An eleventh site, PRKM 136.2, met the criteria for this mode of sampling; however, scheduling conflicts prevented a “SS” from being conducted at this site.

For each site, we partitioned the entire shoal into 1.5 m-wide by 50 m-long sampling lanes oriented parallel to water flow using twisted masonry nylon twine stretched between two rebar stakes (1.2 m long x 1.25 cm diameter) that were pounded into the stream bottom with a drilling hammer. The number of lanes used during sampling corresponded to the width and length of suitable habitat within the river reach. A surveyor was assigned to each lane and visually searched the substrate surface of the entire area within each lane in an upstream direction. Similar to the RTS method, surveyors minimized displacement of substrate material. Mussels were marked with flags and processed as previously described under the RTS survey technique.

Quadrat Sampling—To obtain density estimates of the mussel aggregations and to determine the occurrence of recent recruitment, we excavated multiple defined quadrat areas using a systematic sampling design that incorporated a single random start adapted from Strayer and Smith (2003). We established transects that were perpendicular to flow, and were evenly spaced across the full length of each survey site. Following the selection of a starting point from the random number table, approximately ten 0.25 m2 quadrats were placed at evenly spaced intervals (2 to 5 m) along each transect. Quadrats were placed along transects in alternating directions; i.e., placed from right ascending bank to left ascending bank on first transect, followed by left ascending bank to right ascending bank on second transect. If insufficient space existed between the final quadrat on a transect and the riverbank, the difference between the remaining distance, and distance to the riverbank would be continued on the following transect, and quadrat sampling would resume. For example, if quadrats were evenly spaced at 5 m apart, and only 3 m remained between the final quadrat and the riverbank, the first quadrat on the following transect would be placed 2 m from the riverbank.

One hundred to 200 quadrats were sampled at each site. The number of quadrats sampled at each site was primarily dictated by available resources, including time and personnel. Generally, more quadrats were taken at sites deemed to have a greater likelihood of federally endangered species and allowed us to more intensively focus our quantitative effort on areas that were most important for imperiled species within the river.

Quadrat samples were taken by placing a 0.5 m x 0.5 m square constructed of 1.25 cm diameter rebar over the area to be sampled. The area within the quadrat was then excavated by hand and visually examined to a depth of 15 cm or until hardpan (a compacted layer of substrate that could not be excavated by hand) or bedrock was reached. In each quadrat, all mussels were collected, identified, sexed, measured, and denoted as visible on the surface of the substrate or undetectable at the surface. Mussels were then returned to the substrate directly adjacent to the quadrat, and substrate that was excavated from the quadrat was returned. During the quadrat survey, mussels with any portion of their shell above the substrate were noted as “exposed”, and mussels in view during excavation were noted as “buried”. Quadrat data were used to estimate mussel density for each site. The precision of each density estimate was calculated post hoc using the formula: $CV = \sqrt[1.82]{(nm^{.51})/2.6}$, where, n = number of quadrats sampled, m = mean number of mussels per quadrat, and CV = precision (Strayer & Smith, 2003).

Data Analysis

All summary statistics of mussel lengths and total mussel densities were calculated using Minitab 16 (Minitab, Inc., State College, Pennsylvania). Simple linear regression of total mussel densities and PRKM also was performed using Minitab 16. P-values < 0.05 were considered significant. Tables and figures were created using Excel 2007 (Microsoft, Inc., Redmond, Washington). The site map was produced using ArcMap 9.2 (Environmental Systems Resource Institute (ESRI), Redlands, California).

RESULTS

Mussel Surveys

Based on RTS and SS, a total of 15,084 mussels representing 29 species were collected among the 21 sites surveyed (Tables 3 and 4). Species richness ranged from 1 to 23 species per site (x±SE; 13.8 ± 1.58), with the highest number at PRKM 152.6 and the fewest at PRKM 263.0 (Table 3; Fig. 2). Total live mussels ranged from 1 (PRKM 263.0) to 4,297 (PRKM 152.6) mussels (754 ± 240) per site (Table 3). Total CPUE ranged from 0.33 (PRKM 263.0) to 22.12 (PRKM 152.6) mussels/p-h (8.68 ± 1.68; Table 3). Evidence of relatively recent recruitment was observed for 16 of 29 species collected live among nine sites (Table 4). Of the 15,084 mussels collected during RTS and SS sampling, 74 (0.5%) were considered recent recruits (Table 4).

Quadrat sampling (n = 2,580) yielded 580 mussels of 19 species from 18 of 21 sites (Table 5). Mean densities ranged from 0.00 (PRKMs 269.4, 266.3, and 263.0.) to 2.25 (PRKM 135.8) mussels/m2 among sites (0.88 ± 0.144) (Table 6). A significant linear relationship was shown between mussel density and PRKM (r2 = 0.295, F = 7.94, df = 20, P = 0.011; Fig. 3). Similarly, a significant relationship occurred between PRKM and the number of species collected during quadrat sampling (r2 = 0.655, F = 36.10, df = 17, P < 0.001; Fig. 2). Pre-

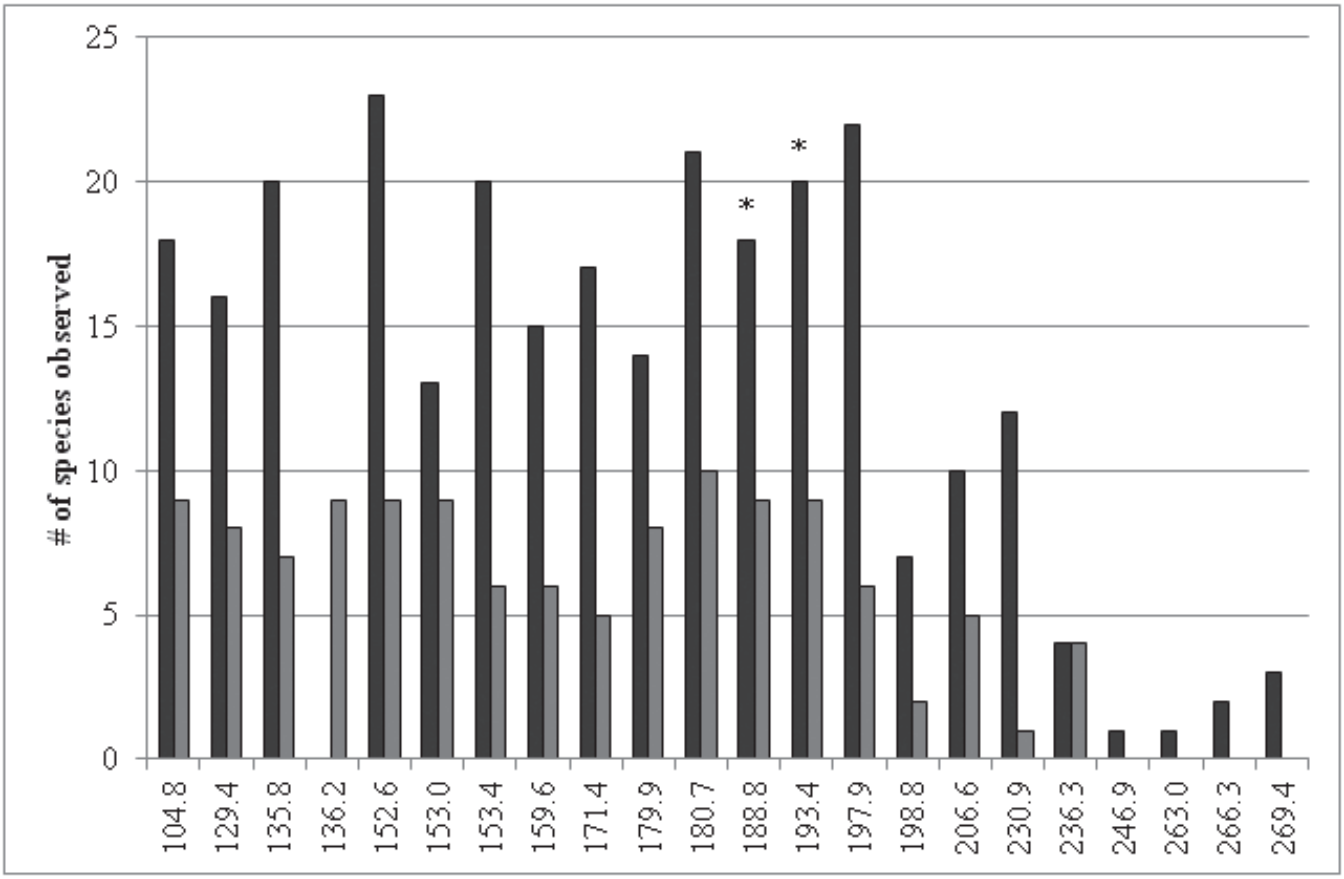

FIGURE 2

Species observed at selected sites in the Powell River during random timed search, systematic search, and 0.25 m2 quadrat sampling. Systematic sampling was not conducted at PRKM 136.2 due to resource constraints. Dark bars: Number of species collected during random timed search and systematic search; Light bars: Number of species collected during quadrat sampling. Statistically significant linear relationship between number of species collected during quadrat sampling and PRKM: r2 = 0.655, F = 36.10, df = 17, P < 0.001; * = site where propagated juveniles have been released.

cision of density estimates ranged from 0.09 to 0.22. Species richness among sites ranged from 0 (PRKMs 269.4, 266.3, and 263.0) to 10 (PRKM 180.7) species (5.81 ± 0.75).

Of 580 mussels collected from quadrats, 21 (3.6%) individuals were deemed to be relatively recent recruits among six species (*A. pectorosa, E. dilatata, E. brevidens, L. ovata, M. conradicus*, and *V. iris*) over nine sites (Table 5). For species that were sexually dimorphic, all but two species *(L. ovata* and *V. iris*) were represented by both male and female individuals.

DISCUSSION

The results of this survey show that a speciose mussel fauna still inhabits the lower Powell River, including at least 11 federally endangered and candidate species. For example, the presence of relatively recent recruits of the critically imperiled *Quadrula intermedia* and *Quadrula sparsa* illustrates the importance of continued conservation efforts in the basin. However, despite the presence of diverse, recently recruiting populations, the fauna has likely lost one-third of its species since Ortmann (1918) (from 46 species historically to 29 current species) (Table 1).

Although not collected during this survey, *Cumberlandia monodonta*, *Fusconaia cuneolus* and *Pleurobema oviforme* could still inhabit the river at undetectable levels. While live individuals of *C. monodonta* were not collected, fresh-dead specimens indicated that the species probably persist in the Powell River. Both *F. cuneolus* and *P. oviforme* may also inhabit the river in very low densities, because both having been collected in recent decades (Eckert et al., 2004). In addition, only a few older

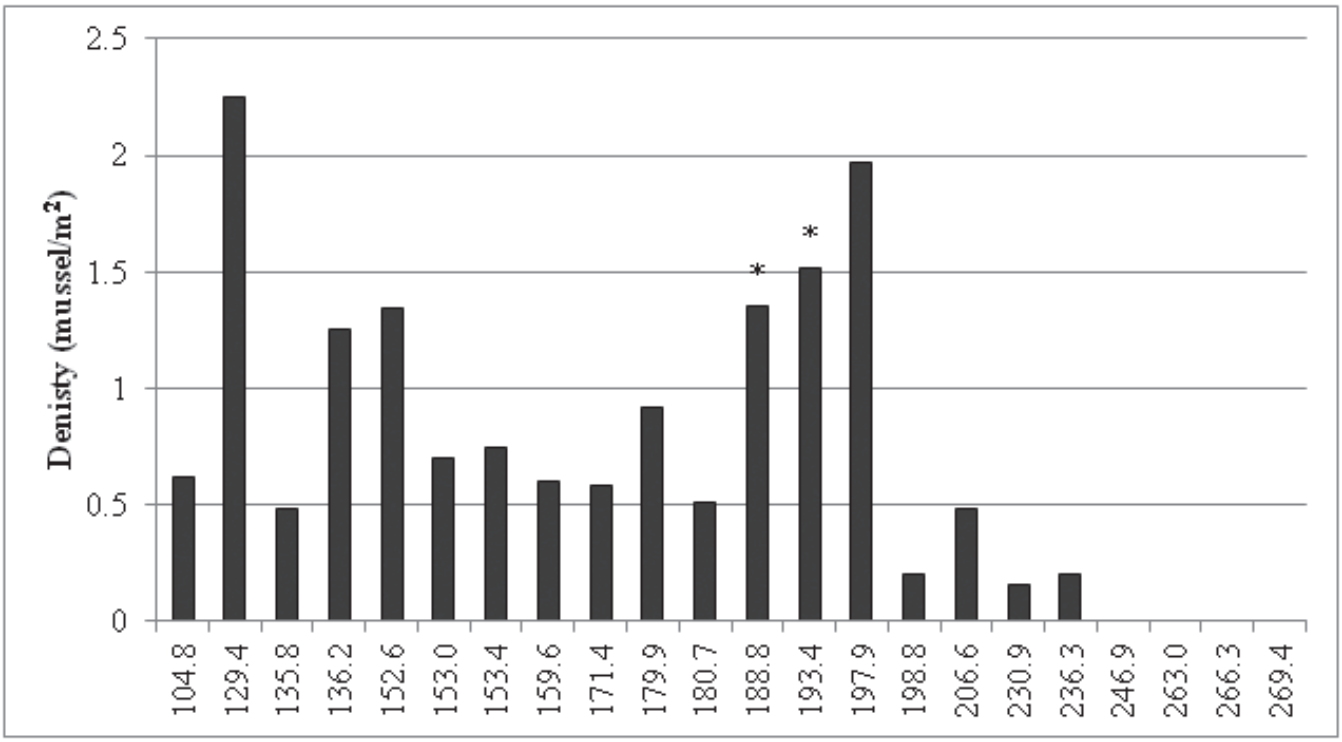

FIGURE 3

Estimated mussel densities at selected sites in the Powell River, utilizing 0.25 m2 quadrat sampling. Statistically significant linear relationship between estimated mussel density and PRKM: r2 = 0.295, F = 7.94, df = 20, P = 0.011; * = site where propagated juveniles have been released.

individuals of *Potamilus alatus* and *Q. pustulosa* were collected. However, *P. alatus* is probably more common than our sampling indicated, because slackwater, soft substrate habitat was not adequately surveyed using our site selection criteria targeting shoals.

Neither live individuals nor shell material of *Epioblasma capsaeformis* or *Hemistena lata* were collected during this survey. The last evidence of *E. capsaeformis* comes from the collection of a single individual at PRKM 193.4 in the late 1980s (Wolcott & Neves, 1994). The last evidence of *H. lata* was a single shell collected from PRKM 179.9 in the late 1990's by J. Jones (unpublished data). Given the short life spans of these species (< 15 y) (Watters et al., 2009; Jones & Neves, 2011), any remnant individuals have likely been extirpated from the river. However, *H. lata* may still reside in the river because it is difficult to detect (individuals burrow deeply (10 to 15 cm) in the substrate (Ahlstedt, 1991b), and old shell material degrades quickly. *Alasmidonta marginata, A. viridis, Pegias fabula, Strophitus undulatus, Toxolasma lividum, Truncilla truncata, Villosa fabalis*, and *Villosa perpurpurea* have not been collected in the past several decades and are very likely extirpated from the river. These species are considered headwater forms and have likely been impacted by upstream pollution (Ahlstedt & Brown, 1979; Dennis, 1981). *Epioblasma torulosa gubernaculum*, once documented as inhabiting the Powell River, is believed extinct (Williams et al., 1993). Although *Lasmigona holstonia* has been seemingly extirpated from the mainstem of the Powell River, the species is still extant in Beaverdam Creek, a tributary of the South Fork Powell River, upstream of Big Stone Gap, Virginia (The Catena Group, 2008).

As documented in previous surveys (Ahlstedt & Brown, 1979; Dennis, 1981; Wolcott & Neves, 1994), a significant decline in both species diversity and mussel abundance was observed, particularly in an upstream direction and above the island at Snodgrass Ford. This decline has been attributed primarily to coal mining, but also to agriculture, and effects from urban areas have been implicated (Ahlstedt & Tuberville, 1997; Wolcott & Neves, 1994; Ahlstedt et al., 2005). These activities will likely continue in the upper Powell River watershed over

time. In addition, natural gas extraction is expanding throughout the Appalachian region and may become a factor in the future (Zoback et al., 2010; Osborn et al., 2011). During our survey, large amounts of sediment were evident in both the water column and covering the substrate surface at the most upstream sites (e.g., above PRKM 130.9). It has been suggested that sedimentation can lead to reduced reproductive success in some mussel species (Brim Box & Mossa, 1999). It is generally believed that the major decline in mussels of the Powell River headwaters is attributable to coal mining activities, and associated contaminants (e.g., McCann & Neves, 1992). The role of stressors on the mussel fauna, particularly in the upstream portions of the Powell River (upstream of PRKM 206.6), needs further study to determine their effects on all life-history stages.

Dam construction in the upper Tennessee River system will continue to have a legacy effect on Powell River mussels. Low abundance and large size indicates a long-term lack of recruitment for *Elliptio crassidens, Ligumia recta*, and *Truncilla truncata* (Table 4). This may be caused by a virtual loss of their primary host fishes, skipjack herring (*Alosa chrysochloris*) and sauger (*Sander canadense*). This loss of host fish is due to downstream dams blocking their spawning runs. As a result, extirpations of species like *Elliptio crassidens* and *Ligumia recta* can be expected due to the extinction debt caused by habitat fragmentation (Tilman et al., 1994).

Evidence of recent recruitment is an indicator of population viability. It is important to note that sub-adults of multiple species were collected during this survey, albeit in low numbers (3.1% of total abundance in quadrats), including several endangered species (*Epioblasma brevidens, Lemiox rimosus, Pleuronaia dolabelloides, Q. intermedia*, and *Q. sparsa*). Nonetheless, this is evidence that portions of the lower Powell River continue to support recruiting populations of federally endangered species and that the differences between these reaches and reaches that do not support recruitment should be studied further.

Due to the presence of several recruiting federally endangered species (e.g., *E. brevidens, Q. intermedia*, and *Q. sparsa*), the section between PRKMs 153.4 and 152.6 is perhaps the most productive reach in the river. Based on our search methods, 7 of the 8 endangered species found at PRKM 152.6 had their greatest abundance at that site (161 individuals), representing 39.1% of the total. The greatest abundance (28.5% of total abundance) and the highest CPUE (22.1 mussels/p-h) were also found at this site. This is significant because despite the presence of the *Quadrula* species in other sections of the river, young individuals were not collected outside of this reach. In addition, only one other recruiting population of *Q. intermedia* is known to exist (Duck River of central Tennessee), and no additional recruiting populations of *Q. sparsa* are known to occur elsewhere (Parmalee & Bogan, 1998). For these reasons, it is important that this reach of the river be protected.

In addition to the river section between PRKMs 153.4 and 152.6, the river section between PRKMs 197.9 and 188.8 is also of particular interest for future conservation efforts. The sites at PRKMs 193.4 and 188.8 have been release sites for propagated juveniles of both common and threatened species (Eckert et al., 2004). Densities at these sites were among the highest of sites sampled, which may be in part due to these juvenile releases. The mussel densities at PRKM 197.9 are also among the highest of the sites sampled during this study. The mussel aggregations at this site have not been frequently sampled like some adjacent sites (Eckert et al., 2004; Ahlstedt et al., 2006), so declines at this site have not been as thoroughly monitored. Additional sampling should be conducted near this site to determine why densities at this site have not declined to the extent that they have both upstream and downstream of this reach at un-augmented sites.

The mussel fauna of the Powell River continues to be threatened by numerous anthropogenic activities. Despite these impacts, the river still contains one of the most diverse mussel faunas in the United States. Among national rivers, only the Clinch River harbors more extant populations of naturally occurring federally endangered mussels. Although low, there was evidence of recruitment at a number of our sample sites, indicating that the Powell River, if managed correctly, has the potential to rebound from ongoing and historical anthropogenic impacts. It is imperative that research, habitat and population restoration, and monitoring efforts continue in this river to conserve its speciose mussel fauna.

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TABLE 1

Conservation status for mussel species known from the Powell River. American Fisheries Society (AFS) status from Williams et al. (1993). Conservation Status: CS = currently stable, E = endangered, FE = federal endangered, FC = federal candidate, PE = federal proposed endangered, T = threatened, V = vulnerable or special concern, X = possibly extinct, - = no status and √ = considered extant based on current study.

Scientific Name		Common Name	Federal Status	AFS Status
Actinonaias ligamentina (Lamarck 1819)	√	mucket	-	CS
Actinonaias pectorosa (Conrad 1834)	√	pheasantshell	-	V
Alasmidonta marginata (Say 1818)	-	elktoe	-	V
Alasmidonta viridis (Rafinesque 1820)	*	slippershell mussel	-	V
Amblema plicata (Say 1817)	√	threeridge	-	CS
Cumberlandia monodonta (Say 1829)	√	spectaclecase	FE	T
Cyclonaias tuberculata (Rafinesque 1820)	√	purple wartyback	-	V
Dromus dromas (Lea 1834)	√	dromedary pearlymussel	FE	E
Elliptio crassidens (Lamarck 1819)	√	elephantear	-	CS
Elliptio dilatata (Rafinesque 1820)	√	spike	-	CS
Epioblasma brevidens (Lea 1831)	√	Cumberlandian combshell	FE	E
Epioblasma capsaeformis (Lea 1834)	-	oystermussel	FE	E
Epioblasma haysiana (Lea 1834)	-	acornshell	-	X
Epioblasma lewisii (Walker 1910)	-	forkshell	-	X
Epioblasma torulosa gubernaculum (Reeve 1865)	-	green blossom	FE	X
Epioblasma triquetra (Rafinesque 1820)	√	snuffbox	PE	T
Fusconaia cor (Conrad 1834)	√	shiny pigtoe	FE	E
Fusconaia cuneolus (Lea 1840)	-	finerayed pigtoe	FE	E
Fusconaia subrotunda (Lea 1831)	√	longsolid	-	V
Hemistena lata (Rafinesque 1820)	-	cracking pearlymussel	FE	E
Lampsilis fasciola (Rafinesque 1820)	√	wavyrayed lampmussel	-	CS
Lampsilis ovata (Say 1817)	√	pocketbook	-	V
Lasmigona costata (Rafinesque 1820)	√	flutedshell	-	CS
Lasmigona holstonia (Lea 1838)	√	Tennessee heelsplitter	-	V
Lemiox rimosus (Rafinesque 1831)	√	birdwing pearlymussel	FE	E
Leptodea fragilis (Rafinesque 1820)	-	fragile papershell	-	CS
Ligumia recta (Lamarck 1819)	√	black sandshell	-	V
Medionidus conradicus (Lea 1834)	√	Cumberland moccasinshell	-	V
Pegias fabula (Lea 1838)	-	littlewing pearlymussel	FE	E
Plethobasus cyphyus (Rafinesque 1820)	√	sheepnose	FE	T
Pleurobema oviforme (Conrad 1834)	-	Tennessee clubshell	-	V
Pleuronaia barnesiana (Lea 1838)	√	Tennessee pigtoe	-	V
Pleuronaia dolabelloides (Lea 1840)	√	slabside pearlymussel	FC	T
Potamilus alatus (Say 1817)	√	pink heelsplitter	-	CS
Ptychobranchus fasciolaris (Rafinesque 1820)	√	kidneyshell	-	CS
Ptychobranchus subtentum (Say 1825)	√	fluted kidneyshell	FC	V
Quadrula cylindrica strigillata (Wright 1898)	√	rough rabbitsfoot	FE	E
Quadrula intermedia (Conrad 1836)	√	Cumberland monkeyface	FE	E
Quadrula pustulosa (Lea 1831)	√	pimpleback	-	CS
Quadrula sparsa (Lea 1841)	√	Appalachian monkeyface	FE	E
Strophitus undulatus (Say 1817)	-	creeper	-	CS
Toxolasma lividum (Rafinesque 1831)	-	purple lilliput	-	V
Truncilla truncata (Rafinesque 1820)	-	deertoe	-	CS
Villosa fabalis (Lea 1831)	-	rayed bean	FE	V
Villosa iris (Lea 1829)	√	rainbow mussel	-	CS
Villosa perpurpurea (Lea 1861)	-	purple bean	FE	E
Villosa vanuxemensis (Lea 1838)	√	mountain creekshell	-	V

*Known historically from a tributary of the Powell River but not from the mainstem.

TABLE 2

Site locations, site numbers, site names and survey methods used in the Powell River.

Site Number	PRKM	State	Site Name	Random Timed Search	Systematic Search	Quadrat Sampling
1	269.4	VA	Dryden	X	-	X
2	266.3	VA	State Rte. 619 Bridge	X	-	X
3	263.0	VA	Swimming Hole	X	-	X
4	236.3	VA	Cheekspring Ford	X	-	X
5	230.9	VA	Sewell Bridge	X	-	X
6	206.6	VA	Hall Ford	X	-	X
7	198.8	VA	Snodgrass Ford	X	-	X
8	197.9	VA	Island below Snodgrass	X	-	X
9	193.4	VA	State Rte. 833 Bridge	X	-	X
10	188.8	VA	Fletcher Ford	X	-	X
11	180.7	TN	Bales Ford	-	X	X
12	179.9	TN	Fugate Ford	-	X	X
13	171.4	TN	McDowell Shoal	-	X	X
14	159.6	TN	Buchanan Ford	-	X	X
15	153.4	TN	Bar above Brooks Bridge	-	X	X
16	153.0	TN	Brooks Bridge	-	X	X
17	152.6	TN	Bar below Brooks Bridge	-	X	X
18	136.2	TN	Yellow Shoals	-	-	X
19	135.8	TN	Below Yellow Shoals	-	X	X
20	129.4	TN	Double S Bend	-	X	X
21	104.8	TN	Above U.S. Rte. 25E Bridge	-	X	X

TABLE 3

Numbers and relative abundances of each species collected during random timed search and systematic search at selected sites in the Powell River. Total numbers of mussels collected, catch-per-unit-effort (CPUE), and total species collected are also provided for each site.

	Powell River Site (PRKM and Site Number)									
	269.4	266.3	263.0	236.3	230.9	206.6	198.8	197.9	193.4	188.8
Species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Actinonaias ligamentina	-	-	-	7	11	23	9	609	192	235
Actinonaias pectorosa	1	-	-	28	53	34	9	1573	1287	443
Amblema plicata	-	-	-	-	2	3	1	8	11	34
Cyclonaias tuberculata	-	-	-	-	2	4	1	9	9	24
Dromus dromas	-	-	-	-	-	-	-	2	7	6
Elliptio crassidens	-	-	-	-	-	-	-	-	-	-
Elliptio dilatata	-	1	-	2	3	7	-	246	137	56
Epioblasma brevidens	-	-	-	-	-	-	-	-	1	6
Epioblasma triquetra	-	-	-	-	-	-	-	-	-	-
Fusconaia cor	-	-	-	-	-	-	-	12	4	-
Fusconaia subrotunda	-	-	-	1	6	2	-	7	9	3
Lampsilis fasciola	1	-	-	-	1	2	-	1	11	5
Lampsilis ovata	-	-	-	-	1	-	-	1	2	12
Lasmigona costata	-	-	-	-	-	1	-	1	-	-
Lemiox rimosus	-	-	-	-	-	-	-	3	2	4
Ligumia recta	-	-	-	-	-	-	-	-	-	-
Medionidus conradicus	-	-	-	-	-	1	1	49	33	63
Plethobasus cyphyus	-	-	-	-	-	-	1	5	1	6
Pleuronaia barnesiana	-	-	-	-	-	-	-	3	-	-
Pleuronaia dolabelloides	-	-	-	-	-	-	-	3	1	-
Potamilus alatus	-	-	-	-	-	-	-	-	-	-
Ptychobranchus fasciolaris	2	1	1	-	-	2	-	8	14	23
Ptychobranchus subtentum	-	-	-	-	-	-	-	26	1	-
Quadrula c. strigillata	-	-	-	-	3	-	-	-	-	-
Quadrula intermedia	-	-	-	-	1	-	-	7	6	6
Quadrula pustulosa	-	-	-	-	-	-	-	2	-	-
Quadrula sparsa	-	-	-	-	1	-	-	-	1	2
Villosa iris	-	-	-	-	1	-	3	3	9	14
Villosa vanuxemensis	-	-	-	-	-	-	-	1	-	6
Total Number	**4**	**2**	**1**	**38**	**85**	**79**	**25**	**2579**	**1738**	**948**
CPUE (mussels/p-h)	**1.33**	**0.67**	**0.33**	**5.07**	**4.53**	**9.03**	**2.78**	**20.97**	**7.79**	**5.02**
Total Species	**3**	**2**	**1**	**4**	**12**	**10**	**7**	**22**	**20**	**18**

TABLE 3
(Continued)

	Powell River Site (PRKM and Site Number)									
	180.7	179.9	171.4	159.6	153.4	153.0	152.6	135.8	129.4	104.8
Species	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**19**	**20**	**21**
Actinonaias ligamentina	291	57	88	145	388	132	1717	153	80	113
Actinonaias pectorosa	64	59	74	270	485	17	1776	159	646	235
Amblema plicata	42	11	35	15	26	35	194	66	12	48
Cyclonaias tuberculata	17	7	14	7	48	53	146	53	29	45
Dromus dromas	6	7	5	8	10	2	61	2	-	1
Elliptio crassidens	-	-	2	-	-	-	2	1	-	-
Elliptio dilatata	22	17	12	10	4	-	52	17	59	24
Epioblasma brevidens	1	-	1	1	4	2	26	5	12	1
Epioblasma triquetra	-	-	-	-	2	-	4	-	1	-
Fusconaia cor	-	1	-	-	-	-	1	1	-	-
Fusconaia subrotunda	-	-	-	-	2	-	1	1	-	-
Lampsilis fasciola	5	2	1	1	6	3	9	5	34	5
Lampsilis ovata	9	-	4	4	15	-	31	5	10	1
Lasmigona costata	3	-	-	4	-	1	12	2	-	5
Lemiox rimosus	2	-	-	-	-	-	4	-	-	-
Ligumia recta	1	-	2	-	1	3	10	2	1	1
Medionidus conradicus	16	8	24	38	58	9	83	60	358	69
Plethobasus cyphyus	32	3	7	-	3	4	33	3	2	2
Pleuronaia barnesiana	-	-	-	-	-	-	-	1	-	2
Pleuronaia dolabelloides	-	-	-	-	-	-	-	-	-	-
Potamilus alatus	1	-	-	-	-	-	-	-	-	-
Ptychobranchus fasciolaris	12	1	5	4	23	2	96	12	38	25
Ptychobranchus subtentum	-	-	-	-	3	-	4	-	1	-
Quadrula c. strigillata	1	-	-	-	1	-	-	1	-	2
Quadrula intermedia	3	3	8	3	3	4	23	-	1	-
Quadrula pustulosa	-	-	-	-	-	-	3	-	-	-
Quadrula sparsa	1	-	-	1	1	-	9	-	-	-
Villosa iris	7	1	3	2	-	-	-	4	-	1
Villosa vanuxemensis	4	1	1	-	1	-	-	-	2	1
Total Number	**540**	**178**	**286**	**513**	**1084**	**267**	**4297**	**553**	**1286**	**581**
CPUE (mussels/p-h)	**8.64**	**2.28**	**2.83**	**4.35**	**18.69**	**4.02**	**22.12**	**17.28**	**20.91**	**14.90**
Total Species	**21**	**14**	**17**	**15**	**20**	**13**	**23**	**20**	**16**	**18**

TABLE 4

Summary statistics of abundance, length, and recruitment for mussel species collected in the Powell River, during random timed search and systematic search. F = female, M = Male, U = Sex Undetermined.

Species	Total Number	No. Sites	Percent Relative Abundance	Length Range and Mean ± SE (mm)	No. Recent Recruits	No. Sites with Recruits	F	M	U	F:M Ratio
Actinonaias ligamentina	4250	17	28.18	16-137 (94.32 ± 0.19)	4	3	-	-	4250	-
Actinonaias pectorosa	7213	18	47.82	13-132 (95.39 ± 0.14)	10	5	-	-	7213	-
Amblema plicata	543	16	3.60	25-143 (95.62 ± 0.67)	2	2	-	-	543	-
Cyclonaias tuberculata	468	16	3.10	15-131 (85.41 ± 0.74)	2	2	-	-	468	-
Dromus dromas	117	12	0.78	46-103 (67.67 ± 0.88)	0	0	-	-	117	-
Elliptio crassidens	5	3	0.03	103-135 (117.20 ± 6.21)	0	0	-	-	5	-
Elliptio dilatata	669	16	4.44	35-110 (69.60) ± 0.40	1	1	-	-	669	-
Epioblasma brevidens	60	11	0.40	33-81 (50.65 ± 1.27)	3	1	18	28	14	0.65:1
Epioblasma triquetra	7	3	0.05	40-59 (47.00 ± 2.38)	0	0	-	-	7	-
Fusconaia cor	19	5	0.13	50-96 (98.95 ± 2.55)	0	0	-	-	19	-
Fusconaia subrotunda	32	9	0.21	56-102 (75.41 ± 2.00)	0	0	-	-	32	-
Lampsilis fasciola	92	16	0.61	22-89 (56.52 ± 1.02)	2	2	32	52	8	0.61:1
Lampsilis ovata	95	12	0.63	18-121 (80.45 ± 2.23)	7	1	4	9	82	0.44:1
Lasmigona costata	29	8	0.19	50-103 (77.31 ± 2.33)	0	0	-	-	29	-
Lemiox rimosus	15	5	0.10	35-44 (39.90 ± 0.88)	6	4	1	3	11	0.33:1
Ligumia recta	21	8	0.14	89-135 (114.81 ± 2.79)	0	0	3	8	10	0.38:1
Medionidus conradicus	870	15	5.77	21-65 (43.61 ± 0.22)	22	6	269	526	75	0.5:1
Plethobasus cyphyus	102	13	0.68	58-103 (81.97 ± 0.92)	0	0	-	-	102	-
Pleuronaia barnesiana	6	3	0.04	45-79 (63.00 ± 5.47)	0	0	-	-	63	-
Pleuronaia dolabelloides	4	2	0.03	68-76 (71.50 ± 2.06)	0	0	-	-	4	-
Potamilus alatus	1	1	0.01	119	0	0	-	-	1	-
Ptychobranchus fasciolaris	269	17	1.78	33-131 (85.61 ± 0.80)	1	1	-	-	269	-
Ptychobranchus subtentum	35	5	0.23	39-104 (79.11 ± 1.78)	1	1	-	-	35	-
Quadrula c. strigillata	8	5	0.05	58-89 (72.50 ± 4.37)	0	0	-	-	8	-
Quadrula intermedia	68	12	0.45	39-68 (57.01 ± 0.83)	2	2	-	-	68	-
Quadrula pustulosa	5	2	0.03	52-61 (56.50 ± 4.50)	0	0	-	-	2	-
Quadrula sparsa	16	7	0.11	44-80 (62.29 ± 2.94)	1	1	-	-	16	-
Villosa iris	48	11	0.32	26-72 (48.65 ± 1.53)	8	4	4	5	39	0.80:1
Villosa vanuxemensis	17	8	0.11	34-65 (47.06 ± 2.24)	2	2	4	10	3	0.40:1
Total	**15,084**	**21**			**74**	**9**				

TABLE 5

Summary statistics of abundance, length, and recruitment for mussel species collected in the Powell River, during 0.25 m2 quadrat sampling. S = # of mussels found on substrate surface, B = # of mussels found buried in substrate, F = female, M = Male, U = Sex Undetermined.

Species	Total Number	No. Sites	% Relative Abundance	Length Range and Mean ± SE (mm)	No. Recent Recruits	No. Sites with Recruits	S	B	F	M	U	F:M Ratio
Actinonaias ligamentina	104	17	17.93	36-127 (91.48 ± 1.44)	0	0	70	34	-	-	104	-
Actinonaias pectorosa	242	17	41.72	38-124 (86.56 ± 1.01)	1	1	123	119	-	-	242	-
Amblema plicata	14	7	2.41	76-107 (93.71 ± 2.38)	0	0	12	2	-	-	14	-
Cyclonaias tuberculata	16	9	2.76	46-109 (84.25 ± 4.05)	0	0	12	4	-	-	16	-
Dromus dromas	3	3	0.52	47-72 (63.67 ± 8.33)	0	0	3	0	-	-	3	-
Elliptio crassidens	1	1	0.17	105	0	0	1	0	-	-	1	-
Elliptio dilatata	47	12	8.10	28-91 (64.62 ± 2.02)	1	1	26	21	-	-	47	-
Epioblasma brevidens	6	6	1.03	22-71 (48.33 ± 6.65)	1	1	4	2	1	3	2	0.33:1
Fusconaia subrotunda	5	4	0.86	44-77 (65.40 ± 6.58)	0	0	4	1	-	-	5	-
Lampsilis fasciola	16	9	2.76	21-73 (50.00 ± 3.95)	3	1	6	10	6	7	3	0.86:1
Lampsilis ovata	2	2	0.34	95-97 (96.00 ± 1.00)	0	0	0	2	0	0	2	-
Lasmigona costata	1	1	0.17	76	0	0	1	0	-	-	1	-
Lemiox rimosus	1	1	0.17	42	0	0	1	0	-	-	1	-
Medionidus conradicus	82	12	14.14	23-55 (40.72 ± 0.77)	8	4	28	54	20	33	29	0.61:1
Plethobasus cyphyus	2	2	0.34	79	0	0	1	1	-	-	2	-
Ptychobranchus fasciolaris	15	10	2.59	62-118 (87.33 ± 4.33)	0	0	7	8	-	-	15	-
Quadrula c. strigillata	1	1	0.17	70	0	0	1	0	-	-	8	-
Villosa iris	18	7	3.10	13-51 (35.56 ± 2.87)	7	4	3	15	0	0	18	-
Villosa vanuxemensis	4	3	0.69	41-47 (44.33 ± 1.47)	0	0	2	2	3	1	0	3:1
Total	**580**	**18**			**21**	**9**	**305**	**275**				

TABLE 6

Estimated densities (mussels/m2) of each species at each site during 0.25 m2 quadrat sampling in the Powell River. Total density estimates and standard errors (SE), density estimate precision, and total species collected are also provided for each site. * = site where propagated juveniles have been released.

	Powell River Sites (PRKM and Site Number)									
	269.4	266.3	263.0	236.3	230.9	206.6	198.8	197.9	*193.4	*188.8
Species	1	2	3	4	5	6	7	8	9	10
Actinonaias ligamentina	-	-	-	0.04	-	0.12	0.12	0.27	0.06	0.31
Actinonaias pectorosa	-	-	-	0.04	0.16	0.20	-	1.14	0.94	0.50
Amblema plicata	-	-	-	-	-	0.04	-	-	-	-
Cyclonaias tuberculata	-	-	-	-	-	-	-	0.04	-	0.03
Dromus dromas	-	-	-	-	-	-	-	-	0.02	-
Elliptio crassidens	-	-	-	-	-	-	-	-	-	-
Elliptio dilatata	-	-	-	0.04	-	0.08	-	0.40	0.19	0.13
Epioblasma brevidens	-	-	-	-	-	-	-	-	-	0.03
Fusconaia subrotunda	-	-	-	-	-	-	-	0.08	0.02	-
Lampsilis fasciola	-	-	-	-	-	-	0.08	-	-	0.08
Lampsilis ovata	-	-	-	-	-	-	-	-	0.02	-
Lasmigona costata	-	-	-	-	-	0.04	-	-	-	-
Lemiox rimosus	-	-	-	-	-	-	-	-	0.02	-
Pleuronaia dolabelloides	-	-	-	-	-	-	-	-	0.17	-
Medionidus conradicus	-	-	-	-	-	-	-	-	-	0.16
Plethobasus cyphyus	-	-	-	-	-	-	-	-	-	-
Ptychobranchus fasciolaris	-	-	-	0.08	-	-	-	0.04	-	0.03
Quadrula c. strigillata	-	-	-	-	-	-	-	-	-	-
Villosa iris	-	-	-	-	-	-	-	-	0.08	0.08
Villosa vanuxemensis	-	-	-	-	-	-	-	-	-	-
Total Density Estimate:	**0.00**	**0.00**	**0.00**	**0.20**	**0.16**	**0.48**	**0.20**	**1.97**	**1.52**	**1.35**
Total Density Estimate SE:	**0.00**	**0.00**	**0.00**	**0.10**	**0.08**	**.017**	**0.10**	**0.42**	**0.21**	**0.20**
Precision:	**-**	**-**	**-**	**0.31**	**0.33**	**0.24**	**0.31**	**0.16**	**0.12**	**0.15**
Total Species:	**0**	**0**	**0**	**4**	**1**	**5**	**2**	**6**	**9**	**9**

	Powell River Site (PRKM and Site Number)										
	180.7	179.9	171.4	159.6	153.4	153.0	152.6	136.2	135.8	129.4	104.8
Species	11	12	13	14	15	16	17	18	19	20	21
Actinonaias ligamentina	0.16	0.12	0.14	0.19	0.16	0.24	0.48	0.27	0.05	0.11	0.05
Actinonaias pectorosa	0.03	0.37	0.20	0.27	0.40	0.05	0.53	0.37	0.11	1.12	0.24
Amblema plicata	0.03	-	-	-	0.03	0.13	0.11	-	0.03	-	0.03
Cyclonaias tuberculata	-	0.07	-	-	-	0.11	0.05	0.05	0.03	0.05	0.03
Dromus dromas	-	0.03	-	-	-	-	0.03	-	-	-	-
Elliptio crassidens	-	-	-	-	-	-	-	-	0.03	-	-
Elliptio dilatata	0.03	0.12	0.08	0.03	-	-	-	0.11	0.05	0.11	-
Epioblasma brevidens	-	-	-	0.03	-	-	0.03	0.03	-	0.03	0.03
Fusconaia subrotunda	0.03	-	-	-	0.03	-	-	-	-	-	-
Lampsilis fasciola	0.03	0.03	-	-	0.03	-	0.03	-	0.05	0.08	0.05
Lampsilis ovata	-	-	-	0.03	-	-	-	-	-	-	-
Lasmigona costata	-	-	-	-	-	-	-	-	-	-	-
Lemiox rimosus	-	-	-	-	-	-	-	-	-	-	-
Pleuronaia dolabelloides	-	-	-	-	-	-	-	-	-	-	-
Medionidus conradicus	-	0.12	0.14	0.05	0.05	0.03	0.05	0.45	0.08	0.67	0.13
Plethobasus cyphyus	0.03	-	-	-	-	0.03	-	-	-	-	-
Ptychobranchus fasciolaris	0.03	-	-	-	0.05	0.03	0.03	-	0.05	0.08	0.03
Quadrula c. strigillata	0.03	-	-	-	-	-	-	-	-	-	-
Villosa iris	0.08	0.06	0.02	-	-	0.05	-	0.08	-	-	-
Villosa vanuxemensis	0.03	-	-	-	-	0.03	-	-	-	-	0.03
Total Density Estimate:	**0.51**	**0.92**	**0.58**	**0.60**	**0.75**	**0.70**	**1.34**	**1.36**	**0.48**	**2.25**	**0.62**
Total Density Estimate SE:	**0.13**	**0.18**	**0.11**	**0.12**	**0.17**	**0.15**	**0.18**	**0.59**	**0.13**	**0.35**	**0.15**
Precision:	**0.19**	**0.18**	**0.16**	**0.18**	**0.17**	**0.18**	**0.15**	**0.15**	**0.20**	**0.13**	**0.18**
Total Species:	**11**	**8**	**5**	**6**	**7**	**9**	**9**	**7**	**9**	**8**	**9**

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EARLY LIFE HISTORY AND CONSERVATION STATUS OF THE MONKEYFACE, *THELIDERMA METANEVRA* (MOLLUSCA: BIVALVIA) IN MINNESOTA AND WISCONSIN

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ABSTRACT

Conservation and restoration of freshwater mussel species requires an understanding of current and historical distributions as well as key aspects of life history. Most freshwater mussels (Unionoida) depend on particular species of host fish for the development and dispersal of the parasitic glochidia larvae. The degree of host specificity varies and is not well known for many mussel species. We tested 90 fish species in 18 families as potential hosts for the Monkeyface mussel (*Theliderma metanevra*), determined its brooding period, and assessed its distribution and current status in Minnesota and Wisconsin. *Theliderma metanevra* brood embryos and glochidia from late April-early August in the St. Croix River. In laboratory experiments, glochidia metamorphosed on 21 cyprinid species (11 genera) but not on other taxa, confirming the host association between *Theliderma* spp. and minnows. The historical and recent distribution of *T. metanevra* in the upper Midwest reflects geological dispersal barriers as well as its apparent sensitivity to a range of human disturbances. These results contribute to an understanding of the evolutionary diversification of the tribe Quadrulini and inform efforts to conserve this regionally threatened species.

KEY WORDS *Quadrula metanevra*, freshwater mussels, host fish, minnows, distribution, brooding

INTRODUCTION

In recent decades there has been a surge in the study of freshwater mussels (Unionoida) spurred by the recognition that many taxa have become extinct and many more are at risk (Bogan, 1993; Ricciardi & Rasmussen, 1999; Lydeard et al., 2004). Particular interest has focused on the brief period during which the larvae (glochidia) are obligate parasites on fish (Zale & Neves, 1982; Kat, 1984; Parmalee & Bogan, 1998) and on adaptations that facilitate this process (Haag & Warren, 2003; Barnhart et al., 2008). Glochidia must be encysted on the gills, fins, or skin of their host in order to complete metamorphosis into juveniles (Rogers-Lowery & Dimock, 2006). For many mussel species, fish hosts are unknown or reported hosts are based on potentially erroneous identifications (Haag & Warren, 2003).

Conservation and restoration of any freshwater mussel species requires an understanding of its current and historical distribution as well as key aspects of its life history, such as glochidia brooding period and

host use (National Native Mussel Conservation Committee, 1998). The Monkeyface mussel (*Theliderma metanevra*) (Rafinesque, 1820) (formerly *Quadrula*) is a thick-shelled, commercially valuable species that is broadly distributed in medium-sized and large rivers of the Mississippi and Mobile river basins, but it has declined in recent decades in many areas (Cummings & Mayer, 1992; Oesch, 1995; Parmalee & Bogan, 1998; Williams et al., 2008). In Minnesota and Wisconsin, *T. metanevra* is classified as threatened species (Natural Heritage & Nongame Research Program, 1996; Wisconsin Department of Natural Resources, 2004).

Host fish associations vary among genera in the tribe Quadrulini (sensu Graf & Cummings, 2007). Catfishes (Ictaluridae) are the primary hosts for *Amphinaias* (formerly within the *Quadrula pustulosa* species group), *Quadrula* (including *Q. fragosa* and *Q. quadrula*), *Cyclonaias tuberculata*, and *Tritogonia verrucosa* (Coker et al., 1921; Howells et al., 1996; Hove et al., 1997; Howells, 1997; Haag & Warren, 2003; Steingraeber et al., 2007; Hove et al., 2011; Hove et al., 2012), whereas *Theliderma intermedia* and *T. cylindrica* (formerly within the *Quadrula metanevra* species group) transform most robustly or only on minnows (Cyprinidae) (Yeager & Neves, 1986; Yeager & Saylor, 1995; Fobian, 2007). Earlier studies suggested that hosts for *T. metanevra* were Bluegill (*Lepomis macrochirus*), Green Sunfish (*L. cyanellus*) and Sauger (*Sander canadensis*) (Surber, 1912a; Howard, 1914; Wilson, 1916). These host determinations were based on the occurrence of natural glochidial infestations on fishes, but encysted glochidia can be difficult to identify, and transformation to the juvenile stage was not observed. Given results from laboratory host studies on other species of *Theliderma*, transformation of *T. metanevra* glochidia on minnows seems more likely than on either sunfishes or perches. In addition to host information, accurate knowledge of the glochidia brooding period is necessary for future host work and potential propagation efforts.

Our objectives for this study were to 1) describe the glochidia brooding period in the northern range of *T. metanevra*, 2) identify host fish suitability in laboratory trials, and 3) determine the historical distribution and current status of *T. metanevra* in Minnesota and Wisconsin, and discuss how its current status may be influenced by host use.

METHODS

Brooding and host suitability

We studied *Theliderma metanevra* at two sites in the St. Croix River: Interstate State Park (45°23’36”N, 92°39’47”W) and Franconia access (45°22’03”N, 92°41’21”W), Minnesota and Wisconsin. This is the approximate northern limit for *T. metanevra* and the sites support diverse mussel assemblages (Hornbach et al., 1996), including several regionally and globally imperiled species. To document the brooding season we collected at least 20 *T. metanevra* from the Interstate site biweekly from May to November 1997 and April to October 1998, but we were not able to sample in June 1998. We examined the gills of each individual by opening the shells slightly with modified O-ring pliers or a flathead screwdriver. Brooding females were identified as those with swollen gills. We were not able to distinguish males from non-brooding females by this method.

Host suitability was examined using standard methods of artificially inoculating fishes with glochidia and monitoring the success of these infections (e.g., Neves et al., 1985). Host trials were conducted from May to August, 2006-2009, at the University of Minnesota Wet Laboratory or Minnesota Pollution Control Agency Biomonitoring Laboratory. During this period, 15 separate trials were conducted with glochidia from a total of 30 female mussels and using a total of 90 fish species, with special emphasis on minnows (Cyprinidae). Most fishes used in host trials were collected with a seine from rivers and lakes in Minnesota. When possible, we collected fishes from water bodies without *T. metanevra* populations to minimize use of fishes with acquired immunity caused by previous glochidial exposure (Reuling, 1919). For those few fishes that were collected near *T. metanevra* populations, we assumed those fishes had at most only partial immunity and would still produce some juveniles even if overall metamorphosis success was reduced (Dodd et al., 2005, 2006). Some fishes were collected from the Saline or Black rivers in Arkansas, or the Black, Little, St. Francis, or Whitewater rivers in southeastern Missouri, and others were obtained from hatcheries. Fish were held in the laboratory for at least two weeks or were inspected for pre-existing glochidia infections prior to being inoculated with glochidia.

Gravid mussels often spontaneously released glochidia during transport or soon after returning to the laboratory. For host trials, we used glochidia that were recently released by females, or we removed glochidia by puncturing the gravid gill and flushing the contents with a syringe. Prior to inoculating fishes, a sample of glochidia from each female mussel was tested for viability by salt exposure (Coker et al., 1921). If >30% of glochidia were unresponsive or showed only a weak shell closure response, glochidia from that female were not used for inoculation. After glochidia were obtained, adult mussels were returned to their collection site.

We inoculated fish in a vigorously aerated wa-

ter bath (1-7 L) containing several hundred to several thousand glochidia. Multiple fish were inoculated in the same bath, and each bath contained glochidia from multiple female mussels. After exposure, the number of attached glochidia was assessed by examining fish under a dissecting microscope while another person applied a gentle stream of water over the gills to keep them wet and separate the gill filaments. After approximately 10-20 glochidia had attached to fishes 2-10 cm in length, or 50-100 glochidia to fishes >10 cm (Hove et al., 2000), they were removed from the glochidial suspension and placed in community holding tanks. Water temperature of the holding tanks was 19-25°C.

Three to four days after inoculation, each fish was re-examined for encysted glochidia. If glochidia were no longer present on any individuals, the trial for that fish species was ended. If encysted glochidia remained on the gills after 3-4 days, all individuals of that fish species were placed together in a separate aquarium for additional monitoring. Subsequently, water from the aquarium floor was siphoned every 3-4 days and washed across two sieves with 1 mm and 125 µm mesh openings, respectively. Particulates from the 125 µm filter were placed in gridded Petri dishes and examined under a dissecting microscope. Transformed juveniles were distinguished from glochidia by the presence of a foot and movement of the valves. A sample of transformed juveniles from each trial was preserved in 95% ethanol. A trial was terminated after three consecutive periods of siphoning without finding a juvenile.

Distribution and status

We compared the recent and historical distribution of *T. metanevra* in Minnesota and Wisconsin to evaluate its status. Most data used to determine recent distribution of live individuals were from Minnesota and Wisconsin departments of natural resources (DNR) surveys completed from 1999 to 2010 and 1985 to 2008, respectively. Surveys in Minnesota were based on qualitative methods (i.e., timed searches; Allen et al., 2007). Methods for Wisconsin surveys were similar, except some sites were also quantitatively sampled using quadrats (Piette, 2005). Border waters (Mississippi and St. Croix rivers) were sampled both by MN DNR and WI DNR. We also included information from post-1985 surveys on the Cannon (Davis, 1987), Zumbro (Bright et al., 1988) and Minnesota (Bright et al., 1990) rivers (Minnesota), Chippewa River (Wisconsin) (Balding, 1992; Balding & Balding, 1996) and Mississippi (Hornbach et al., 1992) and St. Croix (Hornbach, 2001) rivers. In total, these studies represent a comprehensive survey of our study area. We treated live individuals collected within the last 25 years as recent records, which is likely within the lifespan of *T. metanevra* based on longevity estimates for other quadruline species (Haag & Rypel, 2011).

To determine *T. metanevra*'s historical distribution, we gathered data from several sources, including 1) relic shells found during recent surveys listed above, 2) museum specimens housed at the University of Minnesota's James Ford Bell Museum of Natural History, Milwaukee Public Museum, Ohio State University Museum of Biological Diversity, and Illinois Natural History Survey Mollusk Collection, and 3) literature pertaining to the region (Grier, 1922; Baker, 1928; Dawley, 1944; van der Schalie & van der Schalie, 1950; Finke, 1966; Havlik & Stansbery, 1978; Mathiak, 1979; Fuller, 1980; Thiel, 1981; Havlik, 1983; Theler, 1993; Theler, 2000). Recent surveys included most areas sampled in these earlier studies, except that Mississippi River navigation pools 9 and 11 were not sampled as thoroughly as other pools in recent surveys.

RESULTS

Brooding and host suitability

Females brooded glochidia in all four demibranchs, and the brooding period was similar in both years. Gravid female *T. metanevra* were found from 7 May to 29 July in 1997, and from 28 April to 3 August in 1998 (Fig. 1). Brooding females were absent before and after this period. The proportion of gravid mussels varied among sample dates, with 37% being the highest recorded.

Of the 90 fish species in 18 families tested, glochidia metamorphosed on 21 of 40 minnow species but not on any other fishes (Table 1). *Cyprinella spiloptera* and *Macrhybopsis storeriana* produced the greatest number of juveniles per individual, but *Campostoma anomalum, Clinostomus elongatus, Cyprinella lutrensis, Luxilus chrysocephalus* , and *L. zonatus* each produced >25 juveniles per individual in some trials. However, juvenile mussel production was highly variable among trials for these species, and production also varied among congeneric species. For example, although *Cyprinella spiloptera* produced large numbers of glochidia in some trials, other trials produced none, and other species of *Cyprinella* produced few juveniles (*C. whipplei, C. venusta*). *Nocomis* and *Pimephales* produced moderate but variable numbers of juveniles, and *Hybognathus, Margariscus, Rhinichthys*, and *Semotilus* produced consistently low numbers. Within the Cyprinidae, ten *Notropis* species and 9 other species were tested and none proved to be acceptable hosts. None of the previously reported hosts (*Lepomis macrochirus, L. cyanellus, Sander canadensis*) or their congeners produced juveniles, and most sloughed glochidia in < 8 days. The duration of the parasitic period on suitable hosts varied with water temperature and ranged from 7-46 days, but

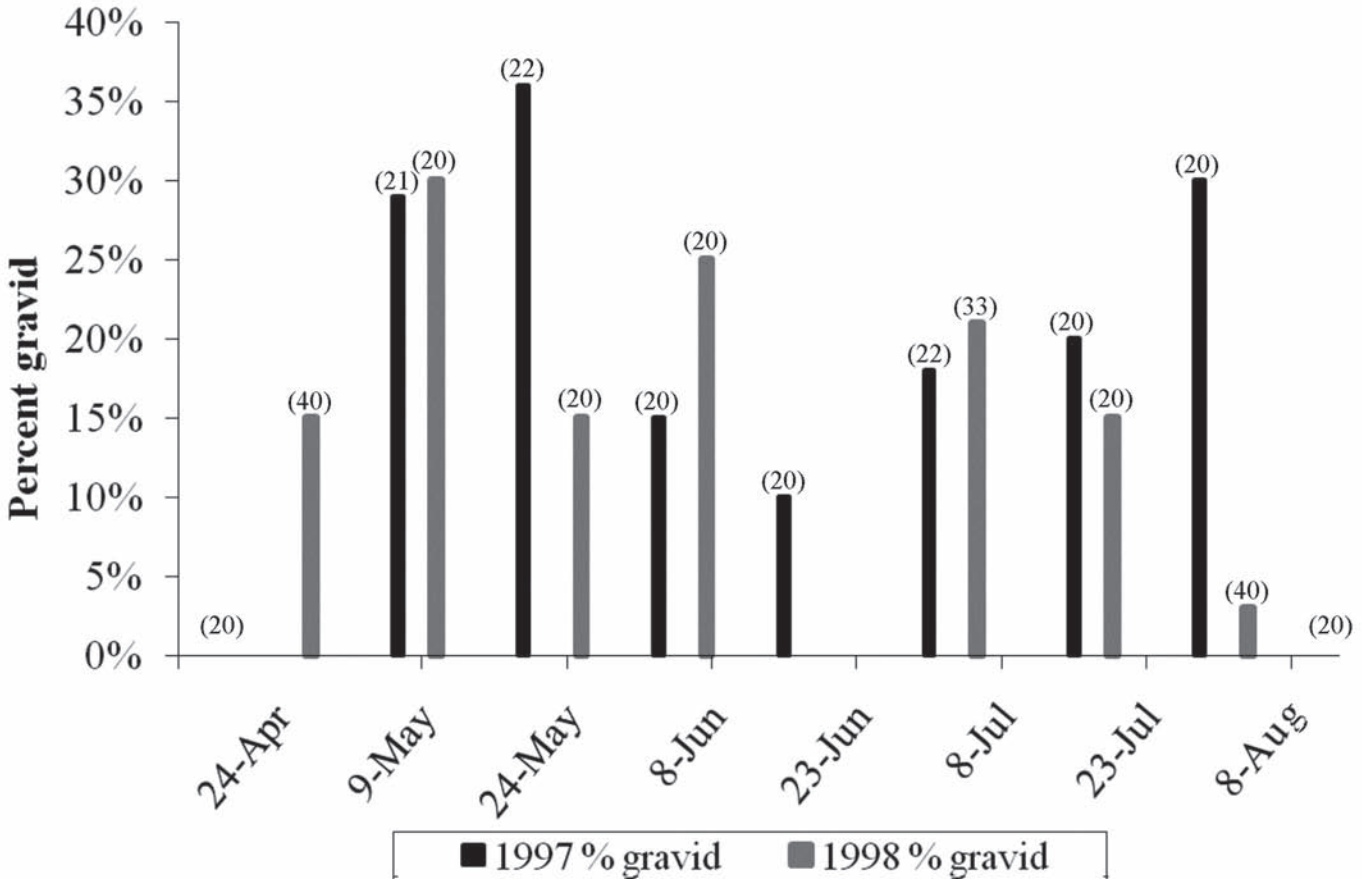

FIGURE 1

Theliderma metanevra brooding periods in the St. Croix River during 1997 and 1998. Number of animals observed is in parentheses.

most juvenile mussels metamorphosed between 13-25 days post inoculation.

Distribution and status

Historically, *T. metanevra* occurred throughout much of the main stems of the upper Mississippi, Minnesota, and Wisconsin rivers, and in the lower reaches of some larger Mississippi River tributaries (Fig. 2). In the last 25 years, a total of 2,182 live individuals were collected in the St. Croix (1,377), Wisconsin (569), Mississippi (225) and Chippewa (11) rivers. *Theliderma metanevra* is now apparently extirpated from interior Minnesota, including 391 km of the Minnesota River and from 376 km of the Wisconsin River above Prairie du Sac Dam. Empty, weathered valves were collected at single sites in the Des Moines and Cedar rivers (Minnesota), and the Black River and Mill Creek (Wisconsin). *Theliderma metanevra*'s range has apparently decreased in the lower Chippewa River, Wisconsin, and in portions of the Mississippi River, where populations are disjunct (Fig. 2). On the basis of the presence of juvenile individuals, reproducing populations are present in the Mississippi, St. Croix, and lower Wisconsin rivers; no evidence of recent reproduction has been documented in the Chippewa River.

DISCUSSION

Brooding and host suitability

Early studies describe *T. metanevra* as tachytictic (short-term brooder), bearing glochidia from May to July (Lefevre & Curtis, 1910; Ortmann, 1911; Utterback, 1915; Surber, 1912b; Baker, 1928), and our study confirms this. In a more southerly population in the Tennessee River, *T. metanevra* was gravid from late March to July (Garner et al., 1999). The brooding period in the St. Croix River (late April to early August), near the northern limit of the species' range, was about a month behind this southern population. The brooding period at our study site also corresponds to the time during which gravid females display their mantle lure (Sietman et al., 2012). Most other quadruline species are short-term spring-summer brooders (Howard, 1914; Coker

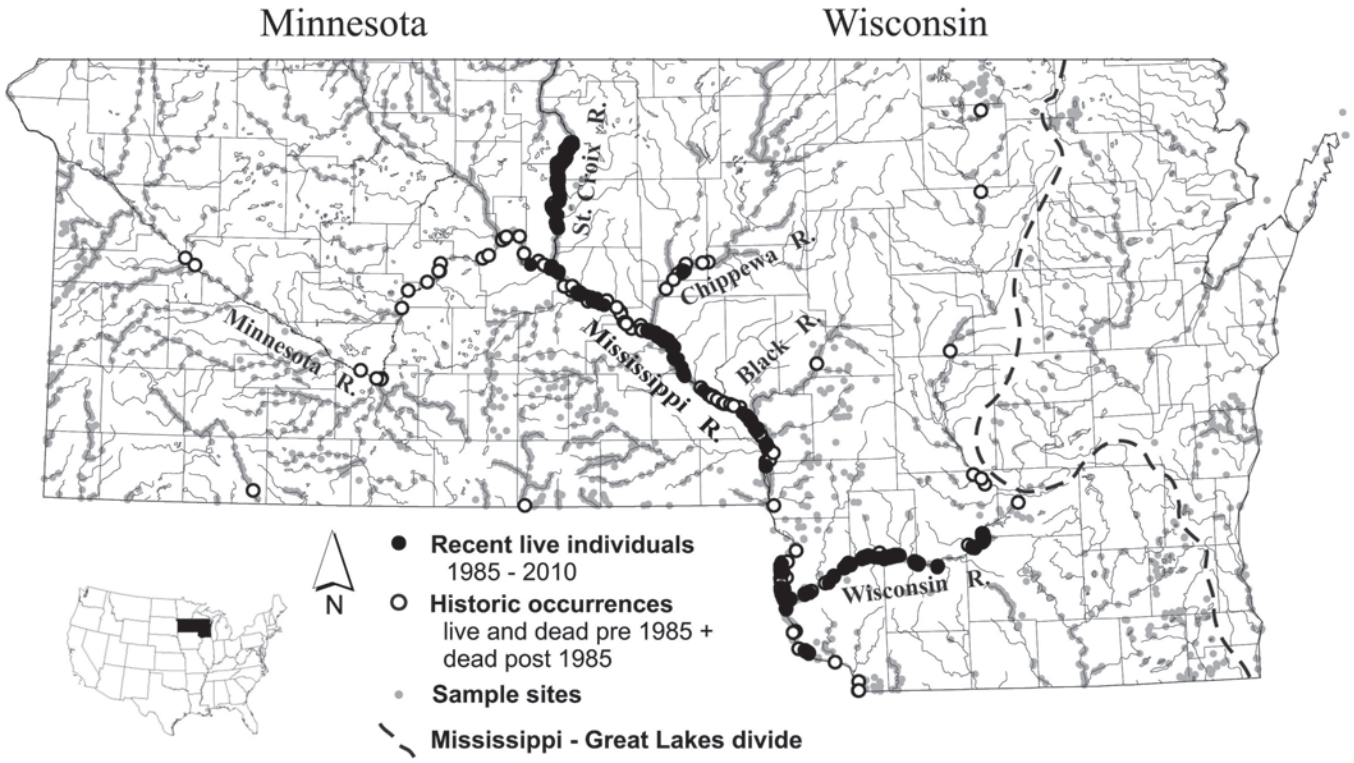

FIGURE 2
Recent and historical distribution of *Theliderma metanevra* in Minnesota and Wisconsin.

et al., 1921; van der Schalie, 1936; Yeager & Neves, 1986; Yeager & Saylor, 1995; Howells, 2000), except for winged mapleleaf (*Quadrula fragosa*) (Sietman et al., 2012; Hove et al., 2012) and washboard (*Megalonaias nervosa*) (Woody & Holland-Bartels, 1993), which brood glochidia for a brief time in the fall.

Gravid female *T. metanevra* display a diminutive mantle lure to attract hosts, and glochidia are ejected in a loose mass when the lure is attacked by fishes or otherwise disturbed (Sietman et al., 2012), a behavior Barnhart et al. (2008) defined as reflexive release (see display photos and video footage online at http://www.dnr.state.mn.us/mussels/quadrula). Many unionid species release conglutinates (aggregates of glochidia) to attract host fishes (Haag & Warren, 2003; Barnhart et al., 2008; White et al., 2008), but we did not find evidence for this in *T. metanevra*. Individuals in the laboratory occasionally released puerile conglutinates composed of immature glochidia or eggs, and this type of premature abortion of the brood is a common response to stress in quadruline mussels (Lefevre & Curtis, 1912); however, mature glochidia were never released in conglutinates. These findings indicate that *T. metanevra* does not use conglutinates as a host infection strategy.

A wide variety of minnow species in several genera were suitable hosts for *T. metanevra*, similar to host use of *T. cylindrica*, which metamorphosed robustly on 8 minnow species in 3 genera, and marginally on several non-cyprinid species (Yeager & Neves, 1986; Fobian, 2007). In contrast, *T. intermedia* metamorphosed only on 2 minnows, *Erimystax dissimilis* and *E. insignis* (Yeager & Saylor, 1995), showing the wide range in host specificity in this genus. The other species of *Theliderma* are either presumed extinct (*T. stapes*) or hosts have not been identified *(T. sparsa*). After multiple laboratory trials, our findings did not corroborate previous reports of *Lepomis cyanellus*, *L. macrochirus* and *Sander canadensis* as hosts for *T. metanevra* (Surber, 1912a; Howard, 1914; Wilson, 1916), results that have been repeated in the literature for nearly a century (e.g., Fuller, 1974; Parmalee & Bogan, 1998). Although controlled, replicated host trials can show the potential suitability of fishes as hosts, it is also necessary to examine patterns of naturally occurring infections and to consider other ecological factors that may determine which host species are most important in the wild.

Of the suitable minnow hosts we identified, *Cyprinella spiloptera* is likely an important natural host for *T. metanevra* in our study region because it produced

the strongest metamorphosis, and it is widespread and abundant in rivers where *T. metanevra* occurs (Becker, 1983; Dieterman, 2008). Other co-occurring minnows that are less abundant or more localized in our study area, but are potentially important hosts include *Luxilus cornutus, Macrhybopsis storeriana, Pimephales notatus*, and *Pimephales promelas*. It is likely that several fish species we identified as suitable hosts in the lab rarely, if ever, serve as natural hosts in our study region because their primary habitats do not overlap with those of *T. metanevra*. Fishes such as *Semotilus, Rhinichthys, Campostoma, Clinostomus*, and *Nocomis* are found primarily in smaller tributaries (Becker, 1983) and probably are rarely exposed to glochidia of *T. metanevra* in the wild.

The use of minnows as glochidial hosts by *Theliderma* contrasts with other quadruline genera, all of which use catfishes (Ictaluridae) (Coker et al., 1921; Howells et al., 1996; Howells, 1997; Haag & Warren, 2003; Steingraeber et al., 2007; Barnhart et al., 2008). *Amphinaias asperata* glochidia transformed only on *Ictalurus punctatus*, but not on 15 cyprinids or additional fish species from other families (Haag & Warren, 2003). Similarly, *Cyclonaias tuberculata, Tritogonia verrucosa*, and *Quadrula fragosa* transformed only on species in the Ictaluridae, and not on a wide variety of minnows or other fishes (Hove et al., 1997, 2011, 2012; Steingraeber et al., 2007). The use of cyprinids as hosts may be a primitive trait among the Quadrulini. Molecular phylogenies of the North American Ambleminae place Quadrulini as sister to the rest of the subfamily (including the tribes Amblemini, Lampsilini, and Pleurobemini; sensu Serb et al., 2003; Campbell et al., 2005). Specialization on catfishes is not reported for any other unionid clade, but use of Cyprinidae is shared with many species in the Pleurobemini (Bruenderman & Neves, 1993; Haag & Warren, 2003; White et al., 2008). However, it is equally likely that use of minnows arose independently in the Pleurobemini and *Theliderma*. Nevertheless, within the Quadrulini, *Theliderma* is sister to a larger clade including *Amphinaias, Cyclonaias, Quadrula*, and *Tritogonia* (Serb et al., 2003; Campbell et al., 2005); specialization on catfishes supports the inclusiveness of this latter group and the evolutionary distinctiveness of *Theliderma*.

Transformation of juveniles was inconsistent among trials for several fish species, with some trials producing large number of juveniles and others producing few or none (e.g., *Cyprinella, Luxilus*, and *Pimephales* spp.). We were unable to document the cause for these inconsistencies but they could have been related to water quality issues, unhealthy glochidia, or predation of newly transformed juveniles by the host fish. We recommend holding small fishes and catostomids in suspended nets or using a false bottom tank or a modified recirculating aquatic housing aquarium system when testing host suitability. Aquatic housing units (e.g., Aquatic Habitats, Aquatic Ecosystems, Inc.) are multiple tank flow-through systems that allow researchers to hold fish individually and collect sloughed glochidia and transformed juveniles with a filter cup placed under the outfall of each tank. These measures can help protect juveniles from possible predation by fishes within experimental chambers. The potential for inconsistent results among trials due to numerous, external factors underscores the value of replication in laboratory host trials. We further recommend that host trials include as a positive control species that are known hosts when such information exists. Inclusion of controls can aid in assessing when other factors may have influenced results of host trials (e.g., poor glochidial health, water quality issues, cross contamination of siphonate).

Distribution and status

Theliderma metanevra is a species of large and medium sized rivers (Cummings & Mayer, 1992), and in the upper Midwest it occurred historically only in portions of the Mississippi River and its larger tributaries. Barrier waterfalls on the Mississippi River at Minneapolis-St. Paul, and a 10 km reach of steep rapids on the St. Croix River at Taylors Falls, Minnesota, further limited the post-glacial upstream dispersal of *T. metanevra* and other aquatic organisms (Underhill, 1957; Graf, 1997; Hornbach, 2001). Because of its large number of suitable hosts that together occur across a range of stream sizes and habitats, unknown factors other than host fish limitation are probably responsible for the restriction of *T. metanevra* to large rivers.

The recent decline of *T. metanevra* suggests it is sensitive to human disturbance. Rivers where it has been extirpated from large areas are, or have been, heavily affected by dams, wetland drainage, or water quality degradation associated with agricultural and urban land development; these areas include the Minnesota River (Lundeen & Koschak, 2011), Wisconsin River (Wisconsin State Board of Health, 1927; Mathiak, 1979), and the Mississippi River below Minneapolis-St. Paul (Scarpino, 1985). The St. Croix River apparently supports the largest remaining population of *T. metanevra* in our study area, as well as several other rare mussel species (Hornbach, 2001), likely because it has largely escaped these impacts (Fago & Hatch, 1993; Wenger et al., 2000).

Prior to impoundment of the Mississippi River for navigation, *T. metanevra* was locally abundant even after intense exploitation by the button industry (Grier, 1922), but the species declined considerably after

impoundment (Finke, 1966; Fuller, 1980; Thiel, 1981). Even though populations persist in portions of the Mississippi River, they are sparse and disjunct. Minnow populations in the Mississippi River also appear to have declined after impoundment, or their distribution within the stream channel changed, with many species now being restricted to channel margins or backwaters (Winston et al., 1991; Dettmers et al., 2001). Consequently, the decline of *T. metanevra* may be due to loss of host fishes or habitat changes that limit their occurrence near main-channel mussel beds.

Because minnows are less vagile than larger fishes such as catfishes (Hill & Grossman, 1987; Pellett et al., 1998; Daugherty & Sutton, 2005), *T. metanevra* may not recolonize formerly inhabited areas as readily as other quadrulines. We see evidence of this in the Minneapolis-St. Paul region of the Mississippi River where populations of *Amphinaias pustulosa*, *A. nodulata*, and *Quadrula quadrula*, species which use catfishes as host, were extirpated (Fuller, 1980) but have since recolonized this reach; in contrast, *T. metanevra* remains absent in the area even though it occurred there historically. Consequently, reintroduction of captively propagated juveniles or translocated adults of *Theliderma* may be necessary to recover populations, whereas it may be less necessary for other quadruline species, at least in areas where host fish movement is not restricted.

Our study reveals key aspects of the life history and status of *T. metanevra* which will benefit efforts to conserve this regionally threatened species and contribute to an understanding of the evolutionary diversification of the Quadrulini. Identifying suitable hosts in the laboratory is an important step in understanding unionid life histories but it is also essential to identify hosts used in the wild. Further early life history research should be directed toward recovering juvenile *T. metanevra* from naturally infested fishes (Boyer et al. 2011). The current distribution of *T. metanevra* in the upper Midwest is reduced, and this species may not be able to readily recolonize areas where it has been extirpated. Invasive bivalves (i.e., *Dreissena* sp.) within most of its current range are also a constant threat (Schloesser et al., 1996). Therefore, we agree with the current threatened status of this species in Minnesota and Wisconsin. For reintroduction efforts that involve culturing juvenile mussels, we recommend the use of species within the genera *Cyprinella* and *Luxilus* due to their high juvenile production rates and co-occurrence with *T. metanevra*. *Pimephales* may also be a useful host due to the ease of obtaining large numbers of these species from hatcheries or bait dealers.

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TABLE 1

Fishes identified as suitable hosts for *Theliderma metanevra* glochidia in the laboratory[a]. Recovery period is the number of days post-infection during which juvenile mussels were observed or, for trials that produced no juveniles, the number of days until individuals ceased to carry glochidial infections. Location of fish collections if other than Minnesota are: MO = Missouri, AR = Arkansas, HR = hatchery raised. †Juveniles observed during first siphonate check. *Number of fish equals the average between the number of fish infested and survivors. Fish nomenclature follows Nelson et al. (2004), except for taxonomic revisions in Wood et al. (2002), Blum et al. (2008) and Strange & Mayden (2009).

Species	Trial	Water temp. range(C)	No. fish infested (survivors)	Recovery period (d)	No. juveniles recovered	Mean no. juveniles/fish*
Campostoma anomalum	May-07	19-20	3 (2)	15-30	158	63.2
	Jun-07	19-20	10 (2)	15-23	10	1.7
Campostoma oligolepis (MO)	May-07	19-20	3 (1)	15-25	8	2.0
	Jun-07	19-20	7 (0)	15-23	4	1.1
Clinostomus elongatus	Jun-08	22-24	14 (0)	8-16	80	12.3
	Jun-08	22-24	2 (0)	13-21	48	48.0
Cyprinella galactura (MO)	Jun-08	22-24	16 (16)	7-21	234	14.6
	May-09	22-24	3 (1)	13-21	24	12.0
Cyprinella lutrensis	Jul-06	21-22	1 (0)	21	0	0
	May-07	19-20	6 (2)	15-25	142	35.5
	Jun-07	19-20	10 (9)	16-25	143	15.1
Cyprinella spiloptera	Jun-06	21-22	18 (9)	13-21	8	0.6
	Jul-06	21-22	21 (8)	13-27	77	5.3
	Jun-07	19-20	11 (8)	7	0	0
	Jun-07	19-20	9 (6)	28	0	0
	Aug-07	23-25	9 (9)	10†	7	0.8
	Jun-08	22-24	5 (4)	9-28†	1275	283.3
	Jun-08	22-24	8 (7)	7-25	474	63.2
Cyprinella venusta (AR)	May-07	19-20	8 (8)	15-22	70	8.8
	Jun-07	19-20	10 (10)	3	0	0
	Jun-07	19-20	12 (6)	16-23	55	6.1
Cyprinella whipplei (MO)	Jun-07	19-20	11 (11)	3	0	0
	Jun-07	19-20	14 (14)	15-18	9	0.6
Hybognathus hankinsoni	May-06	19-20	1 (1)	11	0	0
	May-07	19-20	3 (3)	15-25	12	4.0

TABLE 1
(Continued)

	Jun-07	19-20	8 (2)	23-32	14	2.8
Hybognathus nuchalis (AR)	Jun-07	19-20	3 (2)	18-29	4	1.6
Hybognathus nuchalis (MO)	May-09	22-24	2 (0)	12-13	2	2.0
Luxilus chrysocephalus (MO)	May-07	19-20	4 (4)	15-25	379	94.8
	Aug-07	19-20	8 (8)	14-46	69	8.6
Luxilus cornutus	May-06	19-20	9 (2)	25	0	0
	Jul-06	22-23	12 (6)	10	0	0
	Jun-08	22-24	26 (26)	7-21	124	4.8
	Jul-09	22-24	4 (4)	13-21	61	15.3
Luxilus zonatus (MO)	May-07	19-20	4 (4)	15-30	104	26.0
	Aug-07	19-20	6 (5)	14-24	84	15.3
Macrhybopsis storeriana	May-07	19-20	1 (0)	16-26	159	159.0
	May-09	22-24	2 (2)	17-21	91	45.5
Margariscus margarita	May-07	19-20	1 (1)	9	0	0
	Jun-08	22-24	12 (3)	18-21	1	0.1
	May-09	22-24	4 (4)	9-24	32	8.0
Nocomis biguttatus	Jun-06	21-22	2 (1)	18	0	0
	Jun-07	19-20	9 (7)	4	0	0
	Aug-07	19-20	2 (2)	14-46	25	16.7
	Jun-08	22-24	21 (5)	11-14	1	0.1
Pimephales notatus	Jun-06	19-20	8 (1)	9	0	0
	Jul-06	21-22	3 (1)	13-22	2	1.0
	May-07	19-20	6 (5)	16-30	70	12.7
	Jun-07	19-20	9 (9)	3	0	0
	Jun-07	19-20	11 (10)	16-25	60	5.7
Pimephales promelas	May-07	19-20	6 (2)	16-22	20	5.0
	Jun-07	19-20	15 (4)	16-25	152	16.0
Rhinichthys cataractae	May-07	19-20	3 (2)	16-30	34	13.6
	Jun-07	19-20	10 (1)	23	0	0
	Jun-07	19-20	9 (8)	4	0	0
	Aug-07	19-20	9 (4)	20-34	14	2.2
Rhinichthys obtusus	Jun-06	21-22	6 (5)	16-24	3	0.5
	Jul-06	21-22	20 (20)	15-17	1	0.1
	May-07	19-20	2 (1)	23	0	0
	Jul-07	19-20	12 (2)	14	0	0
	Aug-07	19-20	24 (23)	18	0	0

TABLE 1
(Continued)

Semotilus atromaculatus	Jun-06	21-22	15 (11)	16-24	2	0.2
	Jul-06	22-23	20 (20)	10	0	0
	Jun-07	19-20	10 (10)	4	0	0
	Jun-07	19-20	29 (16)	10	0	0
	Jul-07	21-22	29 (12)	11	0	0

[a] Fish species that did not facilitate glochidia metamorphosis (number of trials, total number of fish tested, range of maximum number of days to glochidia rejection): *Acipenser fulvescens* (HR) (1, 2, 5), *Scaphirhynchus albus* (HR) (1, 2, 5), *Lepisosteus osseus* (1, 1, 4), *Lepisosteus platostomus* (1, 3, 4), *Chrosomus eos* (2, 29, 4-10), *Chrosomus erythrogaster* (2, 15, 10-12), *Cyprinus carpio* (1, 16, 4), *Hybopsis amblops* (MO) (2, 23, 8-11), *Lythrurus umbratilis* (1, 10, 11), *Macrhybopsis hyostoma* (1, 8, 3), *Notemigonus crysoleucas* (2, 9, 14-17), *Notropis atherinoides* (4, 31, 4-11), *Notropis blennius* (1, 6, 5), *Notropis buccatus* (MO) (1, 2, 5), *Notropis dorsalis* (2, 16, 5-8), *Notropis hudsonius* (2, 6, 5-14), *Notropis nubilus* (MO) (1, 3, 5), *Notropis percobromus* (2, 5, 5-9), *Notropis texanus* (1, 12, 8), *Notropis topeka* (HR) (2, 15, 4-8), *Notropis volucellus* (2, 29, 3-4), *Phenacobius mirabilis* (1, 14, 7), *Pimephales vigilax* (3, 28, 5-15), *Carpiodes cyprinus* (1, 2, 5), *Catostomus commersonii* (1, 10, 4), *Hypentelium nigricans* (1, 1, 5), *Ictiobus bubalus* (1, 1, 4), *Moxostoma duquesnei* (1, 5, 4), *Moxostoma macrolepidotum* (1, 2, 4), *Ameiurus melas* (4, 21, 4-7), *Ameiurus natalis* (1, 1, 4), *Ictalurus punctatus* (3, 12, 3-4), *Noturus exilis* (2, 7, 4-5), *Noturus flavus* (1, 4, 3), *Noturus gyrinus* (4, 11, 3-4), *Esox lucius* (1, 2, 4), *Umbra limi* (1, 1, 9), *Oncorhynchus mykiss* (HR) (1, 4, 4), *Percopsis omiscomaycus* (1, 1, 4), *Lota lota* (1, 2, 9), *Gambusia affinis* (MO) (2, 20, 1-17), *Fundulus catenatus* (MO) (1, 2, 11), *Fundulus diaphanus* (1, 3, 4), *Fundulus olivaceus* (MO) (1, 1, 11), *Culaea inconstans* (1, 9, 4), *Cottus bairdii* (1, 2, 18), *Morone chrysops* (1, 1, 5), *Ambloplites rupestris* (1, 3, 5), *Lepomis cyanellus* (4, 21, 3-10), *Lepomis gibbosus* (1, 3, 5), *Lepomis humilis* (1, 4, 4), *Lepomis macrochirus* (2, 12, 3-4), *Lepomis megalotis* (MO) (1, 3, 5), *Micropterus dolomieu* (2, 7, 3-4), *Micropterus salmoides* (1, 3, 4), *Pomoxis annularis* (1, 2, 5), *Pomoxis nigromaculatus* (2, 9, 3-4), *Etheostoma caeruleum* (1, 3, 5), *Etheostoma flabellare* (2, 7, 4-7), *Etheostoma nigrum* (1, 12, 4), *Etheostoma zonale* (1, 5, 7), *Perca flavescens* (1, 26, 8), *Percina caprodes* (1, 4, 4), *Percina maculata* (1, 11, 4), *Percina phoxocephala* (1, 3, 4), *Percina shumardi* (1, 4, 4), *Sander canadensis* (2, 22, 5-9), *Sander vitreus* (1, 1, 4), *Aplodinotus grunniens* (1, 1, 5).

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GENOTOXIC RESPONSE OF UNIONID MUSSEL HEMOLYMPH TO HYDROGEN PEROXIDE AND POLYCYCLIC AROMATIC HYDROCARBONS

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ABSTRACT

The single cell gel electrophoresis or comet assay is widely used to detect DNA damage in isolated cells following exposure to genotoxic compounds. This assay, although commonly used with marine bivalve tissue and circulatory fluid, has received little use or demonstration in freshwater mussels of the order Unionida. Because such a large proportion (>70%) of this faunal group is globally imperiled and is being adversely impacted by environmental contaminants, including many genotoxicants, the aim of this study was to assess the applicability of the comet assay in unionid mussel hemolymph sampled non-lethally with a reference genotoxicant, hydrogen peroxide (H_2O_2) and polycyclic aromatic hydrocarbons (PAHs), a class of common environmental pollutants of genotoxic action. DNA damage was evaluated in samples of hemolymph from *Elliptio complanata* in both *in vitro* and *in vivo* exposures and quantified using the endpoints % tail DNA, or the percentage of DNA in the comet tail and OTM or olive tail moment, the product of the fraction of DNA in the tail and tail length. Hemocytes were isolated and the comet assay was performed on control, 160 µM H_2O_2, and PAH treated cells. From the *in vitro* exposures, 160 µM H_2O_2, as well as the 50 and 100 µg/L total PAH treatments yielded statistically significant ($p < 0.05$) levels of DNA damage, with the H_2O_2 yielding an average of 39.7 % tail DNA and 13.3 OTM and the two PAH treatments yielding 40.7 % and 38.6 % tail DNA, and 12.4 and 11.0 OTM, respectively. An *in vivo* PAH exposure with adult *E. complanata* did not detect a similar genotoxic response to that detected with *in vitro* exposure, indicating that additional research and evaluation may be necessary before implementing the widespread use of a non-lethal, unionid mussel hemolymph based genotoxicity screening tool for environmental biomonitoring.

KEY WORDS Comet Assay, Freshwater Mussel, Unionid, Genotoxic, Hemolymph, PAH

INTRODUCTION

Freshwater ecosystems in North America are home to about 12,580 described species of invertebrates, of which 820 are mollusks (Bogan, 1993). There are eighteen families of bivalves (Phylum: Mollusca) that have resided in such freshwater ecosystems. One order in particular that has been the most successful in diversifying is Unionida, with Unionidae and Margaritiferidae being the two predominant families in North America, consisting of approximately 300 recognized species (Williams et al., 1993). However successful Unionida have been, they are also considered extremely sensitive to disturbances (e.g., contaminants) in freshwater ecosystems and are recognized as the most endangered group of mollusks in the world (Neves, 1999).

The cumulative effect of contaminant exposure on native freshwater bivalves is largely unknown during realistic exposure scenarios. Their sedentary, suspension and deposit feeding behaviors combined with a lifespan of 30-130 years (Bauer, 1992) provides numerous opportunities, potentially for an entire lifespan, for exposure and accumulation of anthropogenic contaminants within mussel tissues and circulatory fluid (Cope et al., 2008), including genotoxic compounds. Genotoxic chemicals have the potential of interacting with biologically important molecules and causing a damaging chain of events to DNA. Mollusks, and bivalves in particular, possess a wide range of defenses to mitigate the toxic effects of chemicals at the cellular level, including multi xenobiotic resistance proteins that actively reduce the cellular entrance of toxicants, detoxifying

enzymes, and DNA repair mechanisms (Rocher et al., 2006). In a variety of aquatic animals, DNA damage has been associated with reduced growth, abnormal development and reduced survival of embryos, larvae and adults (Lee & Steinert, 2003).

Hemolymph, the circulatory fluid of bivalves, contains hemocytes, which are collectively involved in a variety of physiological and pathological functions throughout the mussel body such as nutrient transport and digestion, wound and shell repair, internal defense, and exogenous and endogenous material excretion (Giamberini et al., 1996). An evaluation of a nonlethal sampling technique for hemolymph, withdrawn from the anterior adductor muscle sinus of *Elliptio complanata* (Mollusca: Unionidae), demonstrated the lack of negative impacts on survival or growth (Gustafson et al., 2005a). In addition, Rigonato et al. (2005) found hemolymph to be valuable due to the ease of manipulation and efficient response to DNA-stressing compounds in comparison to gill and digestive gland tissue for genotoxicity studies while researching the invasive, non-unionid Asian clam, *Corbicula fluminea*. Sampling hemolymph from native freshwater mussels of the family Unionidae has the potential to provide information pertinent to the health assessment of threatened or endangered individuals or populations without inflicting harm.

The single cell gel electrophoresis or comet assay is widely used to detect DNA damage in isolated cells following exposure to genotoxic compounds (Buschini et al., 2003; Hartl et al., 2004; Lee & Steinert, 2003; Rigonato et al., 2005; Rocher et al., 2006). This assay, although commonly used with marine bivalve tissues (Mitchelmore et al., 1998; Perez-Cadahia et al., 2004; Wessel et al., 2007; Wilson et al., 1998), has received less use or demonstration in unionid mussels (Conners & Black, 2004; Stambuk et al., 2008; 2009). Therefore, the aim of this study was to assess the applicability of the comet assay in hemolymph of the unionid mussel, *Elliptio complanata* sampled non-lethally with a reference genotoxicant (Lee & Steinert, 2003), hydrogen peroxide (H_2O_2), and in laboratory exposures with polycyclic aromatic hydrocarbons (PAHs), a class of common aquatic pollutants containing compounds of known genotoxic action (USEPA, 1986).

METHODS AND MATERIALS

Mussel Collection and Husbandry

For this study, *Elliptio complanata* or eastern elliptio, were collected from a relatively uncontaminated (USGS, 1999), rural forested segment of the Eno River that flows through Hillsborough in Orange County, North Carolina, USA. The Eno River has high biodiversity, an indication of good water and habitat quality (NCDENR, 2009), including the presence of 12 species of native freshwater mussels. Field collection events for this study involved the hand-collection of approximately 30 mussels per sampling trip, held in dive bags and transported (30 min trip) in coolers containing site water to the Aquatic Toxicology Laboratory on the campus of North Carolina State University in Raleigh, NC, USA. Once in the laboratory, the mussels were held within an aerated, recirculating living stream (Frigid Units Inc., Toledo, OH, USA) with reconstituted soft water (pH 7.2-7.6, hardness 40-48 mg CaCO3/L, and alkalinity 30-35 mg CaCO3/L) (ASTM, 2006) at temperatures consistent with river temperatures and fed a commercial mixture of nonviable microalgae prepared from Instant Algae® Shellfish Diet 1800 and *Nannochloropsis* (Nanno 3600) concentrate (Reed Mariculture, Campbell, CA, USA) on a weekly basis. The maximum length of time a group of mussels was held in the living stream and used for in vitro experimentation was two months.

In Vitro*: Hemolymph Collection*

At the time of hemolymph collection, mussels were randomly selected from the living stream and weight and length measurements recorded. To collect hemolymph, the mussel was gently pried open with a thin-blade knife just far enough to insert a 5 mm wide flat-end forceps to keep the shell open and expose the anterior adductor muscle, and a small sterile 25-gauge needle on a 1.0 mL syringe (PrecisionGlide™, Becton Dickinson and Company, Franklin Lakes, NJ, USA) was inserted into the anterior adductor muscle sinus. Up to 1 mL of hemolymph was extracted per mussel and expelled from the syringe (with the needle removed to prevent any potential physical damage to hemocytes) into a 20 mL Nalgene® test tube. An equal amount of a modified Alsever's Solution (Sigma-Aldrich, St. Louis, MO, USA), an isotonic, balanced salt solution containing ethylenediaminetetraacetic acid (EDTA), was used as a rinse solution to prevent the spontaneous aggregation of hemocytes (Chen & Bayne, 1995) upon extraction from the mussel. All successive hemolymph samples were immediately pooled in a 20 mL Nalgene® test tube to minimize inter-individual variability, until the necessary volume for the given experiment was obtained. Typically, 7 to 9 mL of hemolymph was collected from 7 to 11 individual mussels, with an equal amount of Alsever's solution, thus the final working volume of the hemolymph-Alsever mixture was between 14 to 18 mL, which will be referred to as the "hemolymph mixture". The hemolymph mixture was used in experiments immediately after extraction.

In Vitro*: Exposure Procedures*

The following procedures were conducted in a laboratory without direct sunlight and the florescent overhead

lights were shielded with an ultraviolet (UV) protective coating to minimize background levels of DNA damage in hemocytes from the UV radiation. All *in vitro* exposures were conducted in triplicate using flat bottom, 18 well, non-tissue culture treated plates (Corning® Costar®, Sigma-Aldrich, St. Louis, MO, USA) with lids. In addition, all exposures included controls and hydrogen peroxide (H_2O_2) as a reference genotoxicant and positive control (VWR International, West Chester, PA, USA), as well as a solvent control of acetone for the PAH exposures. For the in vitro exposures, 650 µL of the hemolymph mixture was aliquoted into the experimental wells of the plate and the H_2O_2 or PAH mixture (Alaskan North Slope crude oil (Battelle, Duxbury, MA, USA) and creosote (CAS # 8001-58-9; AccuStandard Inc., New Haven, CT, USA) containing 48 different PAHs; similar to Thorsen et al., 2004) was added to the appropriate wells. In this study, the Alaskan North Slope crude oil and creosote (3:1, volume:volume) was diluted in acetone. The target concentrations for the sum total 48 PAHs in the two working stock solutions used for the exposures were 0.1 µg/µL and 1.0 µg/µL. The plate was then covered and agitated gently for 1 min, placed in a dark incubator at 4 °C for a 4-h exposure period (Tice et al., 2000). Agitation of the plate was conducted for 1 min at 30-min intervals during the exposure period. Upon exposure completion, the hemolymph mixture was transferred via micropipette from the wells into individually labeled 2 mL microcentrifuge tubes. A Ca2+ and Mg2+ free 1 X phosphate buffered saline (1XPBS) (Cambrex Bio Science, Walkersville, MD, USA) solution mixed 1:1 with Alsever's solution was prepared and used to rinse the wells. The exposed hemolymph mixture was centrifuged at 1100 g for 4 min, supernatant decanted and the hemocyte pellet resuspended in 600 µL of the 1XPBS-Alsever solution, and repeated 2 times. After the final rinse, the exposed hemocytes were brought to a final working volume of 325 µL with 1XPBS-Alsever solution.

In Vitro*: Cell Viability*

A prudent approach for selecting definitive exposure concentration ranges for *in vitro* comet assays is to perform cell viability testing with the compound of interest so that testing concentrations which decrease cell viability by more than 30% (Tice et al., 2000), compared to the control cells can be avoided, as low cell viability negatively influences comet assay results. Cell viability tests were conducted with the CellTiter-Glo® Luminescent assay (Promega, Madison, WI, USA), a fluorometric method for estimating the number of viable cells present based on the quantification of adenosine 5' –triphosphate (ATP), an indicator of metabolically active cells (Crouch et al., 1993). To convert relative luminescence units (RLUs), a measurement of the intensity of the emitted light detected by the luminometer (Fusion™, Packard Instrument Company, Meriden, CT, USA) into ATP concentrations, a linear calibration curve was prepared using 0.025-2.0 µmol/L of 100 mM rATP (Promega, Madison, WI, USA). The calibration curve was used to extrapolate the levels of ATP from the measured RLUs recorded from the unexposed and exposed hemocytes isolated and resuspended in 1XPBS-Alsever buffer solution in 96-well plates (Corning® Costar®, Sigma-Aldrich, St. Louis, MO, USA). Based on the need for data normalization to protein content in the cell viability assay, the Bradford Protein assay (IBI-Shelton Scientific, Peosta, IL, USA), a kit containing 0.5 mg/mL bovine serum albumin (BSA), 0.15 M NaCl and a Bradford Reagent consisting of Coomassie blue, a dye that binds protein, was used to determine the protein concentration in unexposed hemolymph by generating a BSA linear standard curve plotting absorbance at 595 nm (Spectronic® Genesys™, Milton Roy Company, Rochester, NY, USA) versus protein concentration.

Range-finding cell viability tests were performed with the reference genotoxicant H_2O_2 (positive control) to determine the appropriate concentrations for the 4-h *in vitro* exposures. Once the optimum H_2O_2 concentrations (0-640 µM H_2O_2) were determined, the PAH mixture underwent the same range-finding cell viability tests to confirm an acceptable exposure concentration range (0-200 µg/L total PAH). The concentration of acetone (solvent control) required for complete PAH solubility was determined by using the greatest concentration of acetone required in the preparation of the PAH treatments and ensuring cell viability was within the acceptable level. Cell viability was expressed as the changes in intracellular ATP levels, or µmol ATP/µg of protein converted to a percentage and compared to the unexposed hemolymph or baseline levels measured immediately after extraction.

In Vitro*: Genotoxicity*

Once cell viability was determined to be no less than 75% below baseline levels for all exposure concentrations and the 4-h *in vitro* exposure period was complete, the comet assay was performed using rinsed, isolated, and resuspended hemocytes. The procedures differed slightly from the traditional comet assay methods developed by Singh et al. (1988) in that CometSlides™ (Trevigen, Gaithersburg, MD, USA), specially treated two-well microscope slides were used in accordance to the manufacturer's protocol (Trevigen, 2007).

During processing, the humidity of the laboratory was monitored and maintained below 60%. The isolated hemocytes, suspended in 1X PBS-Alsever solution at approximately 1 X 105 mL-1 were combined with 500 µL molten 37 °C low melting agarose (LMA) (Trevigen, 2007). Then, 50 µL of the hemocyte-LMA mixture was

pipetted onto each of the two sample wells of the coded CometSlide™ and repeated in duplicate. Each exposure, including the controls and solvent controls, was represented by a total of six slides. The prepared CometSlides™ were placed on slide trays and incubated at 4 °C for 30 min before immersion in pre-chilled cell lysis solution (Trevigen, 2007) in coplin jars and incubated at 4 °C for 60 min. After cell lysis, the slides were drained and transferred to coplin jars containing freshly prepared pH > 13 alkaline solution containing NaOH (Mallinckrodt Baker Inc., Paris, KY, USA) and 200 mM EDTA (Trevigen, 2007) for 20 min to unwind and denature the DNA. Next, the slides were placed on a recirculating, horizontal electrophoresis apparatus (Fisher Scientific, Pittsburgh, PA, USA), voltage set to 1 volt/cm, and freshly prepared alkaline electrophoresis solution (pH > 13, 300 mM NaOH, 1 mM EDTA) was added until the current reached 300 mA. Electrophoresis was performed for 40 min and once complete, the slides were rinsed 3X in distilled water, fixed in 70% ethanol (EMD Chemicals, Inc., Gibbstown, NJ, USA), and stored flat in desiccators to dry.

When the CometSlides™ were ready for analysis, each sample well was stained with 50 µL SYBR® Gold Nucleic Acid gel stain (Molecular Probes, Eugene, OR, USA), a fluorescent DNA intercalating dye, and visualized by epifluorescence microscopy. A computer imaging analysis system, Komet™ 5.5 (Andor Technology, South Windsor, CT, USA) was used to capture and analyze, or score, the levels of DNA damage. The parameters measured and reported include % tail DNA, the percentage of DNA in the comet tail, and olive tail moment (OTM), the product of the fraction of DNA in the tail and tail length. The hemocytes were scored 25 cells per well (50 cells per slide) in duplicate per treatment with three replicates per treatment for a total of 300 cells per exposure concentration. Each slide was labeled with a code unrelated to treatment and processed randomly to reduce potential bias during image analysis.

In Vivo *PAH: Exposure Concentrations and Experimental Design*

The same 3:1 mixture of Alaskan North Slope crude oil and creosote dissolved in acetone with the 48 different PAHs that was screened *in vitro* was also tested *in vivo* through aqueous exposures. The concentration range for the sum total 48 PAHs in this test was determined based on measured concentrations commonly reported within freshwater, aquatic environments (USGS, 1999) and was similar to that used in the *in vitro* tests (0-200 µg/L total PAH). All PAH preparations were dissolved in acetone and all test exposure concentrations of total PAHs in the *in vitro* and *in vivo* tests were validated with empirical measurements using standard analytical methods, as previously described (Thorsen et al., 2004). All measured total PAH concentrations in the test treatments from this study averaged 98% (range 96-101%) of the target concentrations at test initiation.

For this experiment, mussels were collected from the Eno River as previously stated. Immediately upon collection from the river, two mussels were randomly selected to represent baseline or background levels of DNA damage. Hemolymph was sampled from these mussels as previously described and they were not used for further experimentation. The remaining mussels were returned to the lab and were acclimated in aerated coolers containing river water to the test temperature of 20 °C. The mussels were not fed during the 3-d acclimation or during the 3-d experiment. Once acclimated, the shells of the mussels were gently cleaned of debris with a soft-bristled brush and returned to clean, aerated coolers containing reconstituted soft water (ASTM, 2006). Upon start of the experiment, mussels were selected at random, weight and length measurements recorded, and distributed to labeled, aerated, glass aquaria, containing 2-L of soft water (ASTM, 2006). The exposure consisted of adding the Alaskan North Slope crude oil and creosote mixture in concentrations of 1, 10, 50, 100, and 200 µg/L total PAHs into the appropriate 2-L glass aquarium with a glass syringe, for a total exposure duration of 3 d (similar to Thorsen et al., 2004). A positive control consisting of 30% H_2O_2 was also used in this experiment, but the optimum peroxide concentration from the *in vitro* exposures was increased to 1500 µM H_2O_2 for the *in vivo* exposure. The increased peroxide concentration was chosen based on an *in vivo* study performed with *Mytilus edulis* exposed for 1 h at concentrations up to 1000 µM H_2O_2 (Wilson et al., 1998). All exposure concentrations, including the controls, H_2O_2, and acetone solvent controls were conducted in triplicate. A 48-h renewal of test concentrations and exposure water was conducted for all treatments with measurements of water chemistry, following standard methods, to analyze dissolved oxygen, conductivity and temperature using a YSI Model 556 MPS (Yellow Springs Instruments, Yellow Springs, OH, USA) calibrated multiprobe meter. Analysis of pH was performed with a Beckman Model Φ 240 (Beckman Instruments, Fullerton, CA, USA) calibrated meter. Alkalinity was determined by titration with 0.02 N H2SO4 to pH 4.5, and hardness by titration with 0.01 M EDTA.

In Vivo *PAH Exposure: Hemolymph Collection and The Comet Assay*

On day 3 of the *in vivo* PAH exposure, 300 µL of hemolymph was extracted per mussel and expelled into individually labeled microcentrifuge tubes containing 300 µL Alsever's solution. The hemocytes were isolated by

centrifugation at 1100 g for 4 min, supernatant decanted, and the hemocyte pellet rinsed 2X with 1XPBS-Alsever solution and brought to a final working volume of 300 µL with 1XPBS-Alsever solution. As described previously, the comet assay procedures were performed immediately following hemocyte isolation. Quantification of DNA damage was performed as described for the *in vitro* experiments.

Quality Control

CometAssay Control Cells™ (Trevigen) were used to assess the comet assay procedure in the laboratory and to ensure the validity of results. The Control Cells™ consisted of a negative control (CC0) and three DNA damaged cell treatments (CC1, CC2, and CC3) that had been pretreated with increasing concentrations of etoposide (a model genotoxicant) and cryopreserved. When electrophoresed, the Control Cells™ exhibit a dose-response of DNA damage. The results obtained with the Control Cells™ were compared to the results published by Trevigen (2007) within their protocol. The Control Cells™ were run in conjunction with the mussel hemocytes during all *in vitro* comet assay procedures, and produced the following levels of DNA damage, reported as % tail DNA (SD in parenthesis): CC0 11.4 % (3.4), CC1 29.0 % (2.6), CC2 39.4 % (2.6), and CC3 49.6 % (3.5). The levels published by Trevigen were the following in % tail DNA: CC0 5.8 % (7.7), CC1 28.4 % (14.0), CC2 39.7 % (21.8), and CC3 56.8 % (23.6). All results obtained within this study using the Control Cells™ were reflective of the mean % tail DNA values established by Trevigen.

Statistical Analysis

Differences in the amount of DNA damage detected and quantified by image analysis, using the parameters of % tail DNA and OTM, were performed with JMP Statistical Analysis software (version 5.1, SAS Institute, Cary, NC, USA) by use of analysis of variance (ANOVA) followed by a Dunnett's test for means comparison ($\alpha = 0.05$) between the control and each treatment.

RESULTS

In Vitro*: Cell Viability and Genotoxicity*

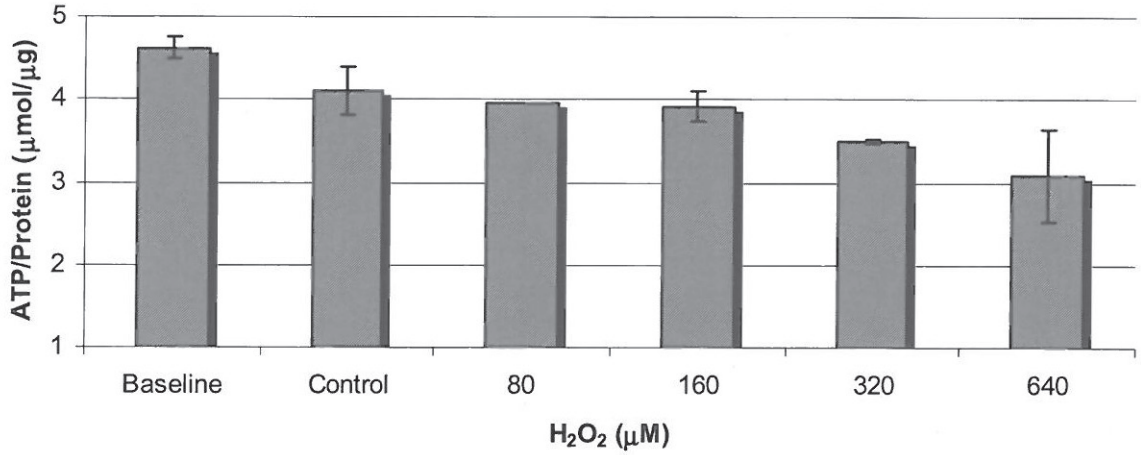
Hemolymph from a total of 139 *Elliptio complanata* was used during the *in vitro* experiments. The average weight of test mussels was 81.2 g (range 44.8 - 149.6 g) and the average length was 300.8 mm (range 188.3 - 322.7 mm). The concentration range tested to establish a suitable positive control using 30% H_2O_2 was 80 to 640 µM. These concentrations yielded a concentration response decrease in cell viability from 86% to 67% relative to baseline (n = 3) levels (Figure 1). Thus, 160 µM H_2O_2 was chosen as the positive control concentration because it provided an acceptable level of cell viability, approximately 80%, and yielded a statistically significant ($p < 0.05$) amount of genotoxicity in comparison to the controls for all *in vitro* exposures. Overall genotoxicity results are reported as % tail DNA and OTM in Figure 2, for all controls and 160 µM H_2O_2, processed in triplicate per *in vitro* experiment. The controls yielded a mean % tail DNA of 17.9 % (2.6) and OTM of 4.2 (0.8) (n = 18). The 160 µM H_2O_2 yielded a mean % tail DNA of 39.7 % (4.3) and OTM of 13.3 (2.1) (n = 17).

In accordance with the comet assay protocol (Tice et al., 2000), each comet assay experiment included 5 to 8 test concentrations of total PAHs (minimum of 3 recommended). All of the concentration ranges chosen yielded cell viability levels greater than 85%, and were thus used for the 4-h *in vitro* comet assay exposures, these included total PAH mixture; 0.05 to 200 µg/L total PAHs. Of all the concentrations tested during the 4-h *in vitro* exposure, excluding 160 µM H_2O_2, only the 50 and 100 µg/L total PAH mixture yielded statistically significant ($P < 0.05$) levels of DNA damage compared to the controls for both DNA damage parameters, with % tail DNA of 40.7 % (2.1) and 38.6 % (0.08) and an OTM of 12.4 (2.1) and 11.0 (0.3), respectively, shown in Figure 3. No other PAH concentrations elicited statistically significant levels of DNA damage in comparison to the controls.

In Vivo*: PAH Genotoxicity*

A total of 26 *Elliptio complanata* were used during the *in vivo* 3 d PAH experiment, and had an average weight of 103.1 grams (range 75.6 - 128.7 g) and average length of 309.4 mm (range 302.5 - 317.6 mm). The baseline, controls, and solvent controls all yielded similar levels of DNA damage (% tail DNA and OTM), 10.4 % (1.3) and 1.8 (0.4), 11.6 % (3.4) and 2.3 (0.9), and 11.8 % (4.3) and 2.0 (1.2), respectively (n = 3), shown in Figure 4. In contrast, the 1500 µM H_2O_2 yielded statistically significant ($P < 0.05$) levels of DNA damage with % tail DNA of 21.9 % (1.2) and OTM of 5.2 (0.4). However, none of the PAH exposure concentrations elicited statistically significant levels of DNA damage in comparison to the controls under the tested conditions. The level of DNA damage for the total PAH exposures, reported as % tail DNA ranged from 10.9 to 15.4 % and OTM of 1.6 to 2.9.

(a)



(b)

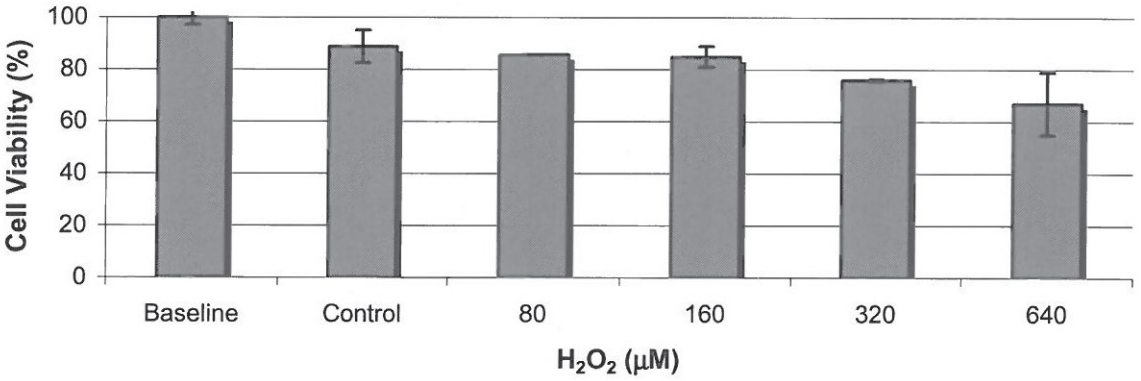


FIGURE 1

The concentration range for the positive control (n = 3), hydrogen peroxide (H_2O_2), during the *in vitro* exposure of freshwater mussel hemolymph with cell viability expressed as (a) µmol ATP/µg of protein and (b) converted from µmol ATP/µg of protein to cell viability (%) in comparison to baseline levels.

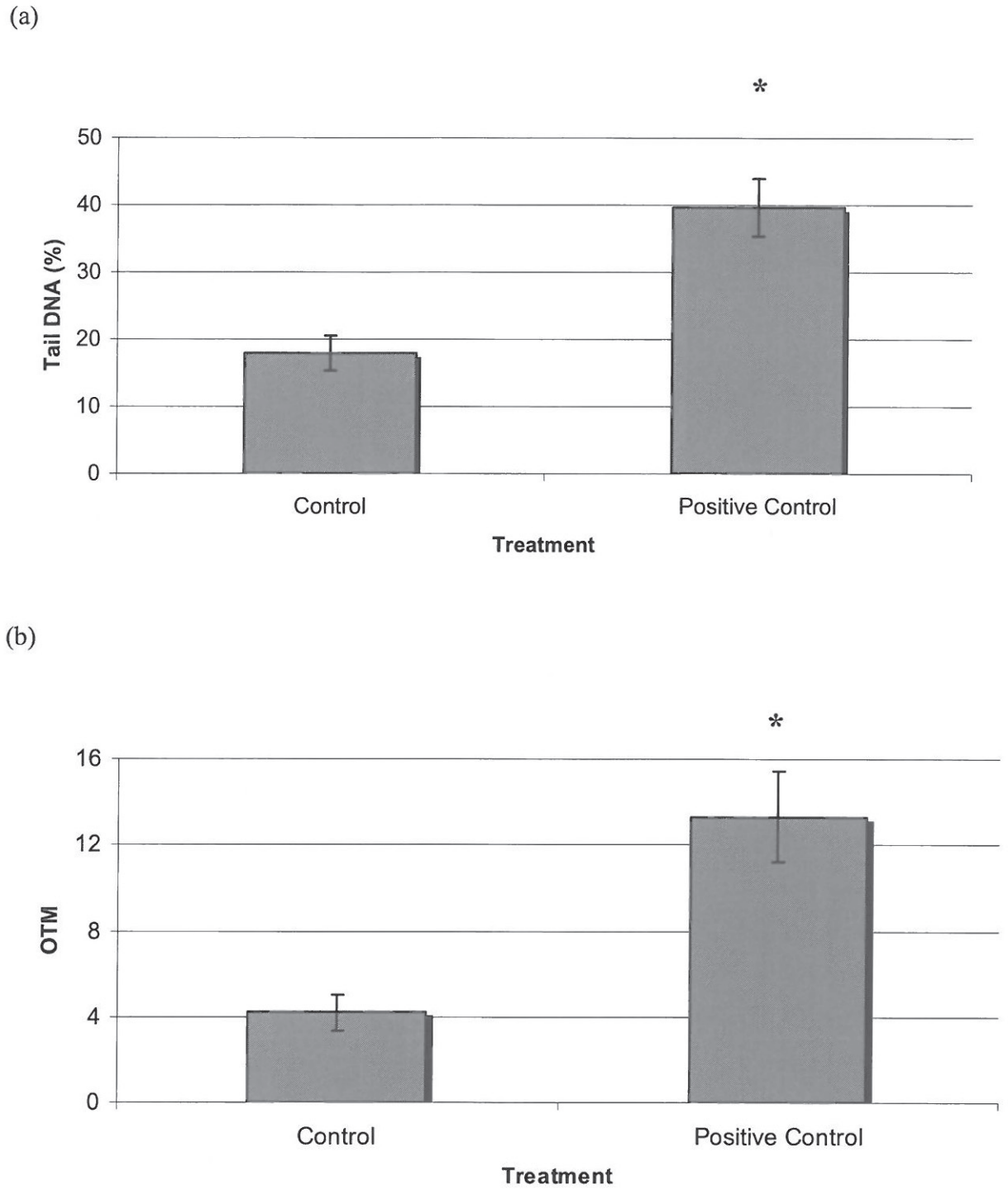


FIGURE 2

Overall mean genotoxicity of the *in vitro* controls (n = 18) and positive control, hydrogen peroxide (H_2O_2) (n = 17), in freshwater mussel hemolymph, (a) % tail DNA, (b) olive tail moment; OTM. *Indicates significantly different from the control ($P < 0.01$).

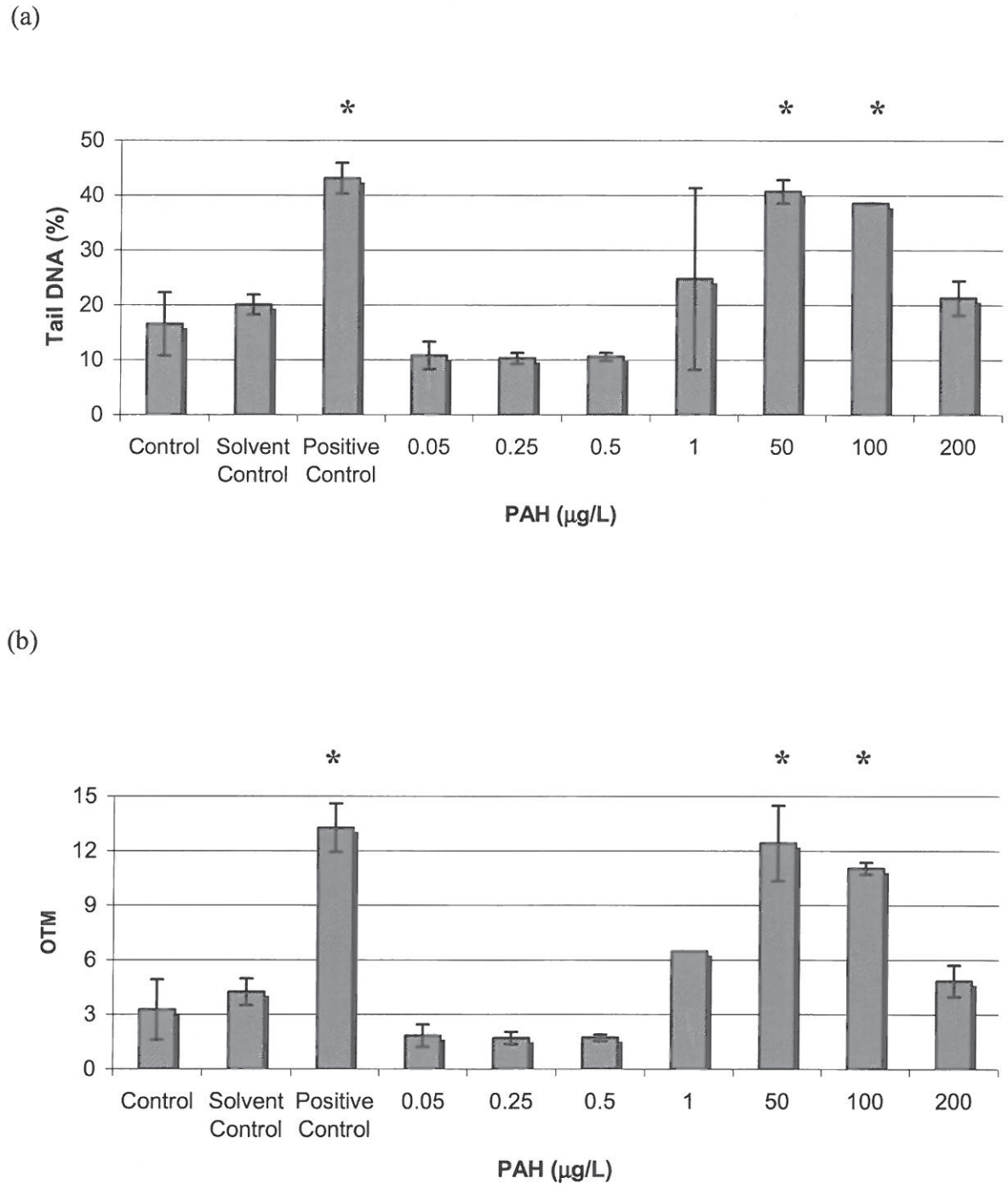


FIGURE 3

In vitro genotoxicity of total PAHs in freshwater mussel hemolymph (n = 3) (a) % tail DNA, (b) olive tail moment; OTM. *Indicates significantly different from the control ($P < 0.01$).

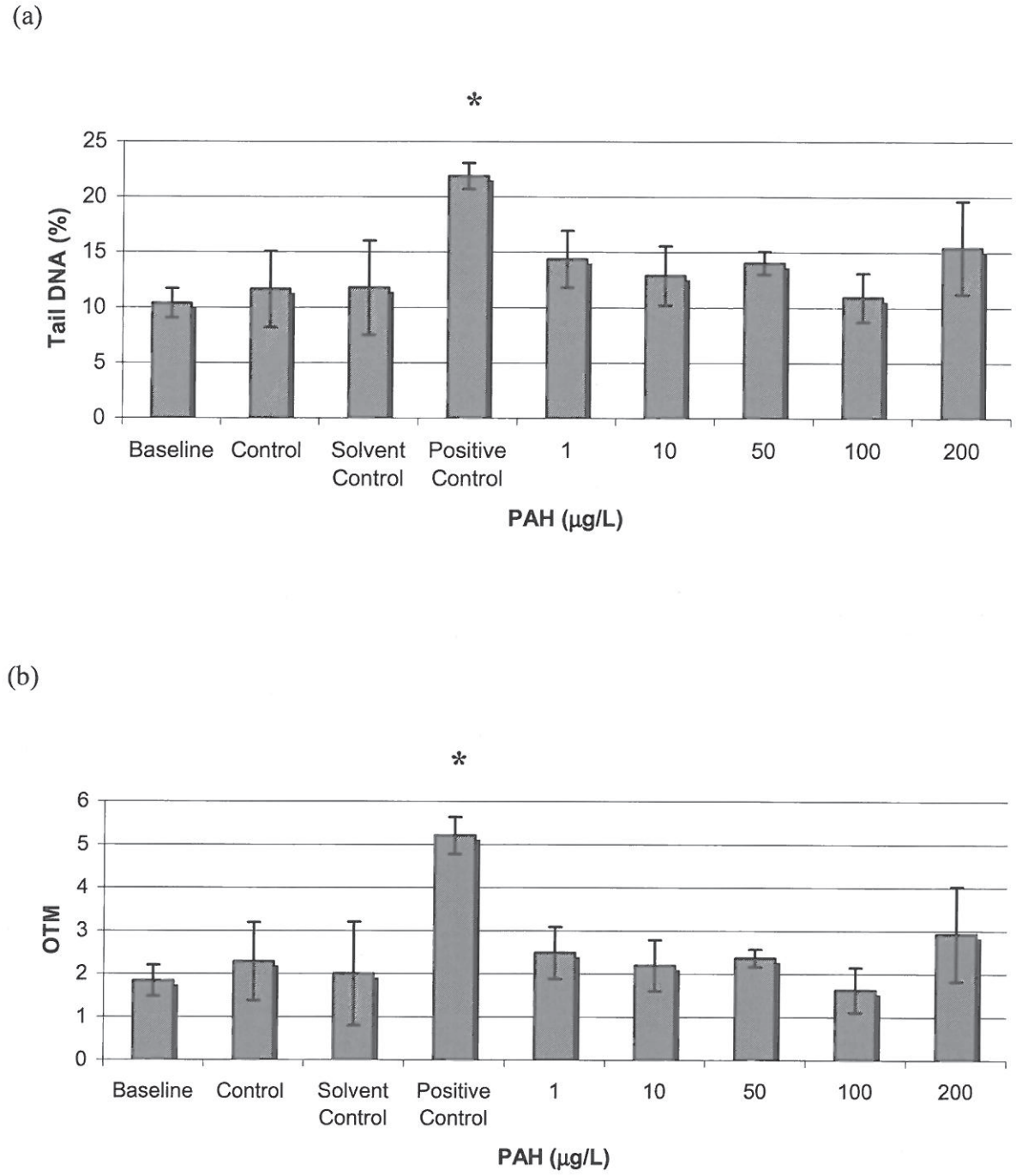


FIGURE 4

In vivo genotoxicity of total PAHs in freshwater mussel hemolymph (n = 3) (a) % tail DNA. *Indicates significantly different from the control (P = 0.0027), (b) olive tail moment; OTM *Indicates significantly different from the control (P = 0.0011).

DISCUSSION

This study demonstrated the *in vitro* and *in vivo* use of hemolymph, sampled non-lethally from a unionid mussel (*Elliptio complanata*) to detect DNA damage, or genotoxicity using the comet assay upon exposure to a reference genotoxicant (H_2O_2) and to environmentally relevant concentrations of a known aquatic ecosystem contaminant. This study also extends the previous research on genotoxicity assessment with unionid mussels (Conners & Black, 2004; Stambuk et al., 2008; 2009) by incorporating CometAssay Quality Control Cells™ (Trevigen, 2007) into the protocol. Most commonly, genotoxicity experimentation results in the destruction of the organism due to the invasive nature of cell or tissue collection, especially when gill, liver or digestive gland cells are used. Therefore, the successful use of hemolymph sampled non-lethally from a unionid mussel is an important finding from this study because of the global imperilment of this fauna and the fact that they have been rarely utilized in genotoxicity assessments. To date, research within bivalve genotoxicity has focused mainly on marine species. Most often, *Mytilus sp*. is used as a sentinel species in biomonitoring studies (Rocher et al., 2006; Lee & Steinert, 2003; Wilson et al., 1998), but Stambuk et al., (2009) have recently used a unionid species (Unio pictorum) placed in cages in two Croatian rivers to assess genotoxicity in polluted freshwaters. There are many advantages to the use of mussel hemolymph with the comet assay, for example, few cells are required and mussel hemolymph contains numerous hemocytes, with a median level of 1018 cells/µL (Gustafson et al., 2005b). Moreover, the results provided by mussel hemocytes, as demonstrated by the overall mean of the control and positive control data (Figure 2), were extremely reproducible throughout all tests. In addition, minimal manipulation of the hemolymph and hemocytes was required for the comet assay, thus creating less opportunity for error.

The PAHs and hydrogen peroxide were genotoxic in *in vitro* exposures of whole hemolymph under our testing conditions and two total PAH concentrations (50 and 100 µg/L) produced statistically significant levels of DNA damage, or genotoxicity in comparison to the controls. Because genotoxicity was detected during the *in vitro* exposure with PAHs, an *in vivo* exposure with PAHs was performed to assess the predictive capabilities of the *in vitro* test. The *in vitro* PAH exposure produced a much greater genotoxic response with both parameters (% tail DNA and OTM) than was detected *in vivo*, in which only the hydrogen peroxide yielded statistically significant levels of DNA damage. Thus, under the conditions tested in this study, *in vitro* exposure was unable to predict a similar *in vivo* response. This may be due in part to the inability to definitively determine or measure the exact exposure concentration of PAHs reaching the hemolymph through the waterborne exposure route. A 3-d *in vivo* PAH exposure allowed for *Elliptio complanata* to reach steady state with the PAHs (Thorsen et al., 2004), however, the actual exposure concentration of the hemocytes to PAHs *in vivo* remains unknown. Nonetheless, our results demonstrated a high degree of method accuracy, evident in the consistent levels of DNA damage measured in the CometAssay Control Cells™ and hydrogen peroxide treatments. For those reasons, we are confident that if the concentrations of PAHs were genotoxic under the tested conditions, the effects would have been detected. A variable that may have influenced the outcome of the *in vivo* tests with the PAHs is the physiological role of mode of action and metabolism. Of particular concern is when the mode of action causing genotoxicity is dependent on the formation of reactive metabolites or metabolic activation. For example, PAHs are well known genotoxic agents, demonstrated to cause DNA damage in marine mussels, *Mytilus sp*., either by direct DNA strand breakage via the generation of reactive oxygen species or indirectly by the formation of reactive intermediates that form unstable DNA adducts (Mitchelmore et al., 1998; Hartl et al., 2004). The extent to which the PAHs were inhibited in their mode of action and/or metabolism or caused toxicity other than DNA damage in whole hemolymph exposed in this study is unclear and requires further research.

There are also multiple procedural steps in the comet assay that involve the factor of time, all of which have varied considerably from study to study (Fairbairn et al., 1995). As a consequence, the influence of time could potentially impede the detection of genotoxicity. For this study, the length of time for cell lysis, alkaline unwinding, and electrophoresis were partially dictated by the manufacturer's protocol (Trevigen, 2007). The protocol suggests conducting cell lysis for 30 min to 1-h and alkaline unwinding for 20 min to 1-h, whereas Tice et al., (2000) state that a minimum cell lysing of 1-h and unwinding of 20 min is preferred. Therefore, cell lysing was conducted for 1-h and alkaline unwinding was 20 min. Longer lysing times, up to 24-h, were evaluated, but the specially coated slides could not withstand the high salts and detergents of the solution, reflected in the degradation of the agarose gel. Thus, our confidence is maintained that the times used for cell lysing and alkaline unwinding in this study provided a sufficient amount of time to liberate and unwind the DNA. Electrophoresis is another influential and variable step, where the conditions of which have varied from laboratory to laboratory based on time, temperature, size of electrophoresis unit, power supply, and set voltage

(Tice et al., 2000). The manufacturer's protocol recommended 20 to 40 min (Trevigen, 2007), whereas Singh et al., (1988) performed electrophoresis for 20 min, and the guidelines (Tice et al., 2000) report a range of 5 min to 40 min, stating that 20 min is sufficient. Through preliminary research using the CometAssay Control Cells™ that were run as a measure of quality control with every *in vitro* experiment in this study, we determined that 40 min of electrophoresis was optimal to reach the reported means for % tail DNA, a time well within the recommendations. Moreover, the same electrophoresis unit and power supply, set to a constant voltage of 1.0 V/cm2 and brought to 300 mA was used throughout the study. Therefore, minimal variation was expected to have arisen from the electrophoresis procedure because all variables were kept constant, partially evident in the negligible standard deviations of the CometAssay Control Cells™.

The intent of this study was to evaluate the genotoxicity of a class of environmentally relevant compounds (i.e., PAHs) at ecologically relevant concentrations; given that mussels are facing peril within their own habitats (Cope et al., 2008). Although testing realistic exposure concentrations of PAHs was of importance, future genotoxicity studies with unionids and PAHs might benefit from an expanded and higher concentration range, as well as evaluating mixtures of these and other compounds, which would represent an even greater realistic exposure scenario. The genotoxic potential of PAHs has been extensively studied, as mentioned previously, in mixture form or singly, most notably benzo[a]pyrene (B[a]P]). The concentration range of total PAHs used in our study was similar to that used in an in vivo experiment with the Pacific oyster, *Crassostrea gigas*, which demonstrated adverse effects on fertilization capability and larval development (Jeong & Cho, 2005). This could be explained by the results obtained from another study with *C. gigas*, in which embryos were used to investigate the relationship between the embryotoxic and genotoxic effects of B[a]P (Wessel et al., 2007). A positive and significant correlation was demonstrated in the oyster embryos between genotoxicity and embryotoxicity; such a connection between embryotoxicity and genotoxicity caused by the PAH B[a]P, a widespread aquatic contaminant, is of great concern at the individual and community level. It is probable that the near persistent exposure to B[a]P can lead to sub-lethal effects in bivalves and over time decrease their population, yet the exact cause may be unidentifiable at the time of the observed decline.

Although variables associated with the exposure conditions, method, test concentrations or mode of action may have influenced the detection of genotoxicity in this study, the significant hydrogen peroxide and CometAssay Control Cell™ data demonstrate the accuracy and reliability of the results obtained. We, therefore, remain confident that if the concentrations of PAHs tested during both the *in vitro* and *in vivo* experiments had been genotoxic, the effects would have been detected with the assay. This research investigated the use of a non-lethal genotoxicity screening tool using unionid mussel hemolymph. Based on our results, additional testing and evaluation is needed before this tool could be widely implemented in biomonitoring programs to detect all potential classes of genotoxicants. Moreover, there is need for a better understanding of unionid mussel hemolymph and the functions and capabilities of hemocytes in their defense and repair of genotoxic compounds.

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OUR PURPOSE

The Freshwater Mollusk Conservation Society (FMCS) is dedicated to the conservation of and advocacy of freshwater mollusks, North America's most imperiled animals. Membership in the society is open to anyone interested in freshwater mollusks who supports the stated purposes of the Society which are as follows:

1) Advocate conservation of freshwater molluscan resources;

2) Serve as a conduit for information about freshwater mollusks;

3) Promote science-based management of freshwater mollusks;

4) Promote and facilitate education and awareness about freshwater mollusks and their function in freshwater ecosystems;

5) Assist with the facilitation of the National Strategy for the Conservation of Native Freshwater Mussels (Journal of Shellfish Research, 1999, Volume 17, Number 5), and a similar strategy under development for freshwater gastropods.

OUR HISTORY

The FMCS traces it's origins to 1992 when a symposium sponsored by the Upper Mississippi River Conservation Committee, USFWS, Mussel Mitigation Trust, and Tennessee Shell Company brought concerned people to St. Louis, Missouri to discuss the status, conservation, and management of freshwater mussels. This meeting resulted in the formation of a working group to develop the National Strategy for the Conservation of Native Freshwater Mussels and set the ground work for another freshwater mussel symposium. In 1995, the next symposium was also held in St. Louis, and both the 1992 and 1995 symposia had published proceedings. Then in March 1996, the Mississippi Interstate Cooperative Research Association (MICRA) formed a mussel committee. It was this committee (National Native Mussel Conservation Committee) whose function it was to implement the National Strategy for the Conservation of Native Freshwater Mussels by organizing a group of state, federal, and academic biologists, along with individuals from the commercial mussel industry. In March 1998, the NNMCC and attendees of the Conservation, Captive Care and Propagation of Freshwater Mussels Symposium held in Columbus, OH, voted to form the Freshwater Mollusk Conservation Society. In November 1998, the executive board drafted a society constitution and voted to incorporate the FMCS as a not-for-profit society. In March 1999, the FMCS held it's first symposium "Musseling in on Biodiversity" in Chattanooga, Tennessee. The symposium attracted 280 attendees; proceedings from that meeting are available for purchase. The second symposium was held in March 2001 in Pittsburgh, Pennsylvania, the third in March 2003 in Raleigh, North Carolina, the fourth in St. Paul, Minnesota in May 2005, the fifth in Little Rock, Arkansas in March 2007, and the sixth in Baltimore, Maryland in April 2009. The society also holds workshops on alternating years, and produces a newsletter three times a year.

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