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ORIGINAL ARTICLE

Evaluating levels of genotoxic stress in eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) using the erythrocyte micronucleus assay

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Abstract The micronucleus assay is a count of cells containing fragments of nuclear content (micronuclei, MN) that arise during errors in cell division and when animals are exposed to genotoxic agents such as chemicals or radiation. The assay can be performed (via light microscopy) using any nucleated cell type, such as erythrocytes in amphibians, birds, fish, and reptiles. Most prior studies of MN in amphibians have been performed in laboratory settings. The goal of this project was to determine baseline levels of genotoxic stress (i.e., frequency of MN in erythrocytes) in a free-living population of eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*, a species of giant salamander that lives in rocky streams) in northern Georgia, USA. We obtained and examined blood smears from 51 hellbenders from eight streams during a 3-month survey (June–August) in 2012. Counts of erythrocytes with MN were made from stained thin blood films and expressed as a percentage of total cells counted (1,000 per animal). Micronuclei were detected in 1.16 % of erythrocytes on average in the hellbenders, and there was a negative relationship between snout–vent length and MN frequency, indicating an age-related increase in clearance of cells with nuclear damage. This relationship with size should be factored into future MN assessments of this and other free-living salamander populations.

Keywords Micronucleus · Genotoxicity · Hellbenders · *Cryptobranchus alleganiensis alleganiensis* · Georgia

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Introduction

Micronuclei (MN) are small fragments of nuclear content that appear in the cytoplasm of nucleated cells (such as red blood cells of amphibians, birds, fish, and reptiles), and these form when cells undergo incomplete division or experience damage to their nuclei ("genotoxic" damage). However, so far, the majority of studies utilizing this assay in animal subjects have been performed in controlled laboratory settings to test the genotoxicity of a large variety of chemicals and stressors (e.g., Siboulet et al. 1984; Fernandez et al. 1993; Krauter 1993; Campana et al. 2003; Ergene et al. 2007), with fewer studies using it in field settings (e.g., Al-Sabti and Hardig 1990; Barni et al. 2007; Huang et al. 2007), despite its potential for identifying risks to wildlife populations.

One aquatic species where the MN assay would be ideally suited for use is the giant hellbender salamander (*Cryptobranchus alleganiensis*) of North America (Fig. 1). There are two subspecies of hellbenders, the eastern hellbender (*C. alleganiensis alleganiensis*), with a range encompassing much of the eastern USA, and the Ozark hellbender (*Cryptobranchus alleganiensis bishopi*), which is restricted to a narrow range in Missouri and Arkansas. These animals inhabit large, fast-flowing, and rocky streams, and their populations are thought to be highly sensitive to

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Fig. 1 Eastern hellbender (*C. alleganiensis alleganiensis*) from South Fork Kentucky River at Booneville, Owsley County, KY. Photo credit —John MacGregor, Kentucky Department of Fish and Wildlife Resources

anthropogenic pressures such as water pollution or stream habitat degradation (Graham et al. 2011). Most populations of hellbenders are declining (Wheeler et al. 2003; Foster et al. 2009; Burgmeier et al. 2011a), and thus, there are many current efforts underway to assess aspects of individual and population health (e.g., Burgmeier et al. 2011b; Hopkins and DuRant 2011; Davis and Hopkins 2013).

With average cell lengths of nearly 50 μm (Jerrett and Mays 1973), hellbender erythrocytes are some of the largest in the animal kingdom (Glomski et al. 1997). This means that any cytoplasmic inclusions (i.e., micronuclei) in their cells would be easily seen using conventional microscopy. Another unique feature of hellbenders is their relatively long lifespan compared to most amphibians; hellbenders can live more than 30 years in the wild (Taber et al. 1975). This may result in prolonged exposure to aquatic contaminants over their lifetime and possibly bioaccumulation of toxins in their tissues in older individuals, resulting in higher MN frequencies in older individuals.

This paper describes the results of an initial attempt to quantify baseline MN levels in wild hellbender salamanders focusing on a population in northern Georgia, USA. Georgia is the southern limit to the eastern hellbender range, with local populations only in the northernmost portion of the state (Albanese et al. 2011). This work differs from most other micronucleus studies in the fact that our study subjects varied in age. In the majority of micronucleus studies, captive-reared larvae are used and then only at specific developmental stages (e.g., Jaylet et al. 1986; Mouchet et al. 2006; Li et al. 2010). Field-based studies have also restricted their collections to larvae of uniform stages (Lajmanovich et al. 2005; Marquis et al. 2009). In contrast, our collection of wild-caught hellbenders included individuals of all sizes (i.e., ages). This allowed us to examine if the frequency of MN varied with age, a question that has rarely been addressed in nonhuman vertebrates. Based on studies of humans, there is reason to expect a positive relationship between age (body size) and MN frequency in hellbenders. In humans who are chronically exposed to low levels of radiation, MN frequency tends to increase with age (da Cruz et al. 1994; Thierens et al. 1996). Also, since many bodily functions (including cellular replication) begin to break down or become less efficient with age, we might expect greater MN in older animals regardless of pollutant exposure.

Materials and methods

Study sites Hellbenders were collected from streams that are either within the main stem or are tributaries to the Hiawassee, Nottley, and Toccoa rivers within Fannin, Union, and Towns counties, thus spanning much of the extant distribution of the hellbender within the Tennessee River drainage of Georgia (Fig. 2).

Capturing hellbenders From June through August 2012, a team of personnel from the Georgia Department of Natural Resources surveyed the streams above for hellbenders. When a hellbender was captured, it was immediately brought to the stream bank, where it was measured (snout–vent length), assigned to gender where possible, and given a uniquely numbered passive integrated transponder tag for later identification. This involved intramuscular injection of the tag with a 12-gauge needle into the side of the tail. In most (but not all) cases, this caused a minor amount of blood to well from the insertion point, and wherever possible, this

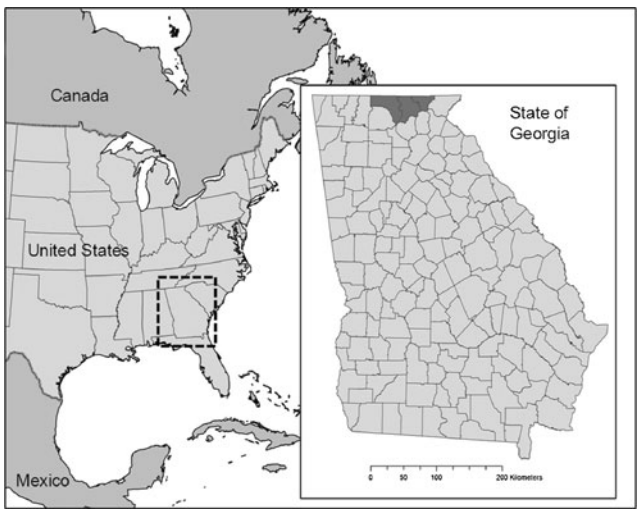

Fig. 2 Map of the State of Georgia, USA, showing the three counties (Fannin, Union, and Towns counties) where streams were surveyed for hellbenders for this project in 2012

blood was collected using heparinized microcapillary tubes. The blood was then used to make thin blood films. All animals were released at the site of capture.

Determining MN frequency In the lab, all hellbender blood films were stained with a buffered Wright–Giemsa stain (CAMCO Quik Stain II), then examined with a light microscope at ×1,000 by one of us (AKD) for micronuclei. At least 1,000 erythrocytes were examined per film (Krauter 1993; Campana et al. 2003; Lajmanovich et al. 2005), and all cells containing micronuclei were counted (Figs. 3a,b). To be considered micronuclei, the inclusions had to have no connection with the larger nucleus and be the same color and intensity as the main nucleus. Cells with cleaved nuclei (Fig. 3c) were also counted. A small number of individuals (n=3) appeared to be infected with an intraerythrocytic bacterial parasite that forms cytoplasmic vacuoles which vaguely resemble micronuclei (Fig. 3d), but these can be differentiated on the basis of the staining color and internal morphology (Davis and Cecala 2010). For each individual, the frequency of cells with micronuclei (including cleaved nuclei) was expressed as a percentage of the number of erythrocytes counted. These data were log-transformed (+1) to approximate normality. We then compared (transformed) MN frequency to snout–vent length using Pearson correlation, and we compared frequencies in male versus female hellbenders using Student's t tests.

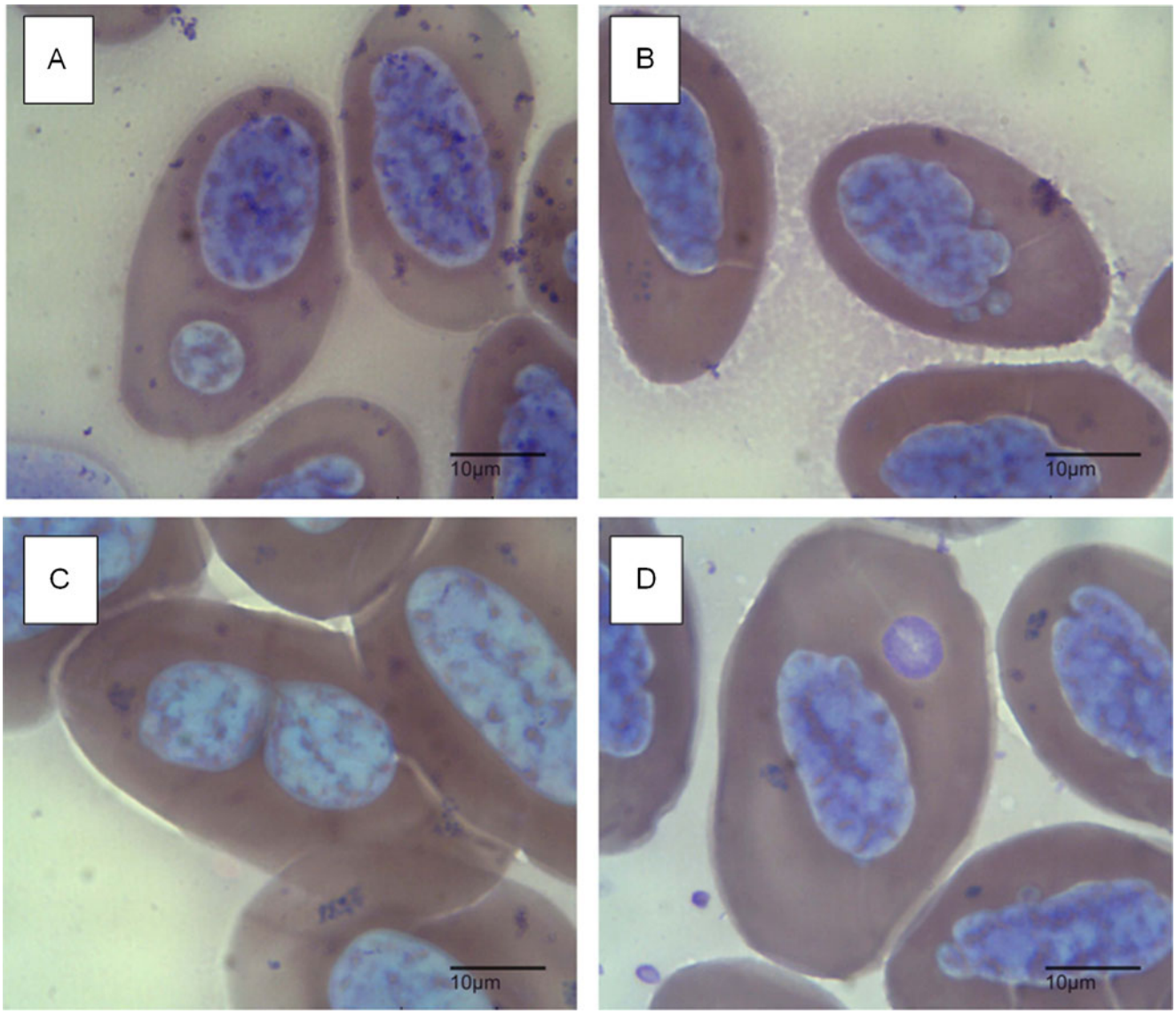

Fig. 3 Micrographs of hellbender erythrocytes showing cells with micronuclei (**a**, **b**), cleaved nucleus (**c**), and infection with a rickettsia bacteria (**d**)

Results and discussion

There were a total of 78 hellbenders captured during the 2012 surveys, and of these, there were 51 usable blood films. Of the individuals of known sex (n=62), there were 38 (61.3 %) males and 24 (38.7 %) females. The average length (snout–vent length, SVL) of the hellbenders from this region was 22.4 cm (3.9 SD). No external parasites (i.e., leeches) were found on any individual, and none showed obvious signs of illness or deformity. There were also no hemoparasites (Davis and Hopkins 2013) found in the blood smears of these hellbenders. Three individuals had infections with intraerythrocytic rickettsial bacteria (Davis and Cecala 2010, Fig. 3d), which are not known to cause disease.

The average frequency of MN across all hellbenders in this study was 1.16 % of erythrocytes (±0.98 SD) and ranged from 0.1 to 5.0 % (Fig. 4). There was no statistical difference in MN frequency (log-transformed) between male and female hellbenders (t test, df=42, t=−1.59, p=0.118). Comparison of SVL and MN frequency of all individuals indicated a weak negative relationship (r=−0.25, p=0.0809), although when we examined data from within one stream that had a large number of samples, we found a much tighter and significant negative correlation between SVL and MN frequency (r=−0.84, p=0.0046; Fig. 5).

To our knowledge, this project represented the first attempt to assess micronucleus frequencies of hellbenders, which is somewhat surprising given (1) the high level of concern for the viability of hellbender populations throughout their range and (2) the extremely large size of hellbender blood cells (Fig. 3), which greatly facilitates micronucleus counting. The relationship we discovered between hellbender body size and MN frequency appears to be a novel pattern within the micronucleus literature, and it was in the opposite direction we expected. In contrast to that found in humans (da Cruz et al. 1994; Thierens et al. 1996), MN do not appear to increase with age in hellbenders. In fact, MN frequencies appear to be highest in the smallest (youngest) individuals (Fig. 5). Given that MN are primarily produced during errors of cell replication and production, it may make sense that they would be more prevalent in younger individuals, which have higher growth rates (Taber et al. 1975) and, by extension, higher metabolism and rates of cell production or division. Moreover, there was one prior study that found higher background rates of MN in larval frogs compared to adults (Barni et al. 2007), which is consistent with this idea. For the investigator, this means that for assessments of MN frequency as part of routine health screenings or monitoring programs, age must be accounted for in the target animals, either statistically (by capturing a range of ages) or by limiting the samples to animals of known age across the sites to be assessed.

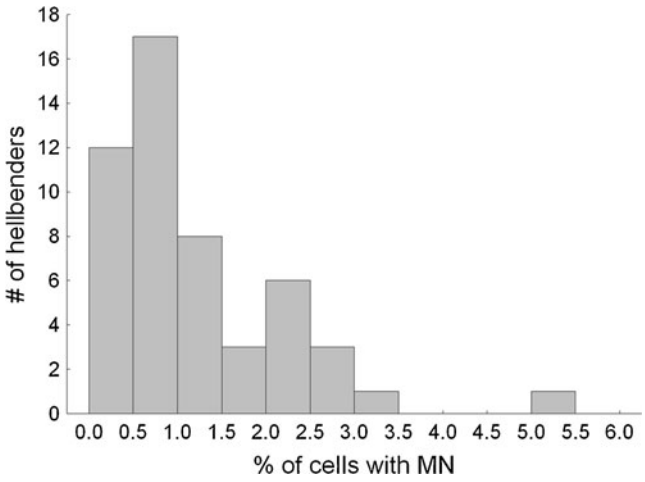

Fig. 4 Histogram showing range of micronucleus frequencies in the hellbenders sampled in this project

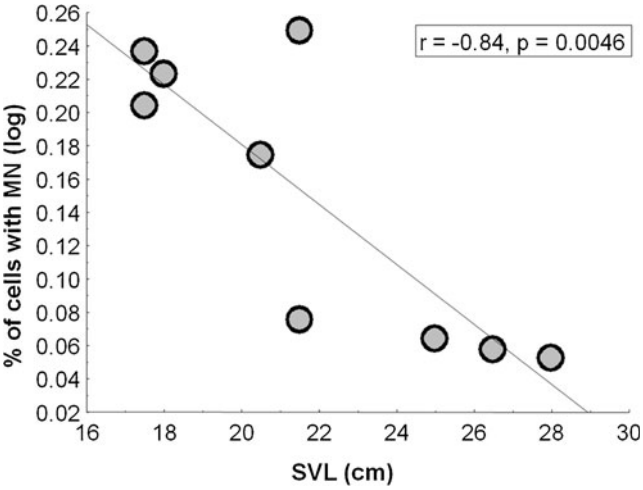

Fig. 5 Relationship between body size (snout–vent length, a proxy for age) and micronucleus frequency in hellbenders from one stream in Georgia

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