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A Comparison of Sperm Health in Declining and Stable Populations of Hellbenders (Cryptobranchus alleganiensis alleganiensis and C.a. bishopi)

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ABSTRACT.—Animals in many freshwater habitats are experiencing decreased recruitment due to declines in reproductive health. Both subspecies of a long-lived aquatic salamander, (*Cryptobranchus alleganiensis alleganiensis* and *C. a. bishopi*) have experienced severe population declines characterized by low recruitment. For many states throughout their geographic range, captive propagation and translocation are the only remaining form of management given the severity of declines. These captive rearing programs should rely on techniques to assess male reproductive health, which are currently lacking. In this study, we compared the sperm health (motility, viability, and concentration) of male hellbenders from declining and stable populations. Sperm motility and viability were similar among populations, but sperm concentrations (sp/ml) were significantly lower in declining Missouri populations than in hellbenders from populations with higher recruitment in the southeast. Sperm from Ozark hellbenders was successfully cryopreserved but with low post thaw motilities. This method for assessing male reproductive health provides the first baseline comparative study among populations of this cryptic species in decline and has broad implications for use in monitoring male health across the distribution of the eastern hellbender.

INTRODUCTION

Anthropogenic encroachment and habitat degradation have placed many populations at risk, with increasingly isolated and fragmented populations threatened (Reed, 2004). Some causal factors, such as overfishing or habitat loss due to deforestation, are relatively easy to diagnose, but determination of other causes can be more difficult. Freshwater species can be especially vulnerable to these more cryptic influences due to a wide array of factors that directly or indirectly influence water quality (Abell *et al.*, 2000). Moreover, freshwater populations often experience minimal gene flow due to low rates of immigration between watersheds (Ward *et al.*, 1994).

When populations experience a decline in recruitment, the decrease in representation of young individuals can be due either to problems with reproduction or early survivorship. In recent years, a number of species of aquatic vertebrates have exhibited adverse effects to reproductive function and fertility. For example, some studies have shown decreased reproductive output, especially by males, in response to the presence of xenobiotic environmental contaminants in streams (Battaglin *et al.*, 1998; Tavera-Mendoza *et al.*, 2002; Solomon *et al.*, 1996, 2008). Adverse physiological effects can include reduced testicular volume and decreased primary spermatagonial cell nests (Tavera-Mendoza *et al.*, 2002), decreased percentages of spermatocytes (Orton *et al.*, 2006), and decreased levels and

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inhibition of testosterone (Hayes *et al.*, 2002; Hecker *et al.*, 2004). Outside of a few declining species and zoos, surprisingly few wild populations of amphibians have been assessed for sperm health (Kouba *et al.*, 2009).

Hellbenders are long-lived, fully aquatic salamanders that have experienced substantial declines in many populations over the past few decades (Burgmeier *et al.*, 2011a). This decline is often characterized by low recruitment (Wheeler *et al.*, 2003) and thus hellbenders are a good model for examining reproductive function in declining freshwater populations. The hellbender currently is subdivided into two subspecies. The distribution of the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) is fairly broad, including central Missouri and extending to the southeastern United States, while the Ozark subspecies (*C. a. bishopi*) is restricted to southern Missouri and the adjoining areas of northern Arkansas (Petranka, 1998). The Ozark hellbender, *Cryptobranchus a. bishopi,* was recently listed as federally endangered (Federal Register 76FR61978).

Breeding of the eastern subspecies in Missouri occurs primarily during a 2 w ''peak period'' between late Aug. and early Oct. (Smith, 1907; Nickerson & Mays, 1973). Ozark hellbenders in Missouri breed slightly later, beginning from late Sep. to mid-Oct. (Nickerson & Mays, 1973). Hellbender testes become enlarged as the breeding season approaches (Ratcliff, 1965; Ingersol *et al.*, 1991). Males excavate breeding areas beneath large rocks or logs, in the stream bedrock, (Alexander, 1927; Bishop, 1941; Nickerson & Mays, 1973; Kern, 1986), or at the base of mud-gravel banks (Nickerson & Tohulka, 1986; Peterson *et al.*, 1988). Unlike most other salamanders, hellbenders exhibit external fertilization in which the sperm must swim through the water to contact the eggs (Petranka, 1998).

A challenge with studies that attempt to assess reproductive health in declining populations is determining appropriate baseline estimates for comparisons. For most species, there is little to no historical data on measures of sperm production, and this lack of historical information is the case for hellbenders. However, qualitatively, there appears to be some geographic variation in the reproductive health of hellbender populations, with some populations exhibiting apparently strong levels of recruitment (Nickerson *et al.*, 2003). In this paper, our approach is to determine baseline sperm parameters and to compare these parameters across populations that are experiencing reduced recruitment (*C. a. alleganiensis* and *C. a. bishopi* from Missouri) with populations that appear to have greater recruitment success *(i.e.*, relatively stable populations of *C. a. alleganiensis* from North Carolina and Georgia). Unfortunately, there are no known relatively stable populations of *C. a. bishopi.* To characterize sperm health we quantified sperm motility, viability, and concentration. To examine the potential for successful large-scale artificial insemination programs, we also developed preliminary techniques for cryopreservation of Ozark hellbender sperm. This methodology can provide state managers with baseline sperm parameters for the species to inform management policies and captive propagation efforts.

METHODS

STUDY AREA

Sperm samples were collected from Missouri eastern hellbenders from the Big Piney and Gasconade Rivers between 18 Sep. and 25 Oct. 2002. Samples from Ozark hellbenders were collected from the Eleven Point and North Fork of the White Rivers between 1 Oct. and 18 Nov. 2002. Samples for the relatively stable comparison populations were collected from eastern hellbenders from Coopers Creek in Georgia on 12–15 Sep. 2002, and the Davidson River in North Carolina on 9–11 Sep. 2002.

SURVEY METHODS AND DESIGN

Hellbenders were detected by wading/snorkeling and lifting large shelter rocks and were captured by hand. A total of 89 (Georgia = 52, North Carolina = 23, Missouri = 14) male eastern hellbenders and 52 male Ozark hellbenders (Missouri = 52) were captured during the 2002 breeding season (Sep. to Nov.). Males were identified by the swollen appearance of the cloaca (Smith, 1912). Males were characterized as being in reproductive condition if they were producing milt. Total length (TL) was measured to the nearest mm using a modified fish board.

We used search effort and percentage of juveniles captured to illustrate the relative health of the NC and GA populations in comparison to the Missouri populations (see also Jensen *et al.*, 2008). Search effort was calculated by multiplying the search hours by the number of individuals searching. The total number of individuals caught per search effort (catch/search effort) was also calculated to obtain information on relative population sizes.

Semen was stripped by hand from all males that were producing milt at the time of capture (Georgia eastern n = 8, North Carolina eastern n = 2, Missouri eastern n = 8, Missouri Ozark n = 30). All samples were collected in the field in 1.5 ml micro-centrifuge tubes, and subsamples were taken for each separate semen analysis (concentration, motility, and viability). The timing of the breeding seasons for populations overlapped, and precise timing of beginning and ending of the seasons was somewhat unpredictable. Unfortunately, we sampled the North Carolina population at the very end of the breeding season and only two individuals were in reproductive condition. Due to this low number of available samples, and because the data for North Carolina and Georgia were similar (see Results below), we combined the data from North Carolina and Georgia for statistical analysis. All semen samples were micropippetted with a Labnet standard 0.01–10 µl micropipette. Disposable tips were used to prevent cross contamination of semen samples.

Sperm concentrations and total volume of semen.—Sperm concentration and total concentration estimates for all individuals were performed in the field within 10 min directly following sample collection. Since hellbender sperm maintains motility in water (Baker, 1963), raw semen was diluted using a 1:10 ratio, in which 1 µl of raw semen was mixed thoroughly with 9 µl of 10% distilled vinegar (which kills sperm) to accurately count the nonmoving sperm. 10 µl of total solution was placed on a hemocytometer and the number of sperm was counted on five diagonal squares (Fig. 1). We then multiplied the number of sperm counted on these five squares using a standard formula for sperm concentration (# of sperm cells/ml) = DR × HF × NS × C, where DR is the dilution ratio, HF is the hemocytometer factor (volume factor), NS is the number of sperm cells counted, and C is concentration (conversion factor) to convert cubic millimeters to cubic centimeters to number of sperm cells/ml (Bearden & Fuquay, 1999). The total volume of milt collected per individual was also noted.

Motility.—Motility is defined as the number of free-moving sperm cells observed (Bearden & Fuquay, 1999). A new subsample of the 1.5 ml semen sample was made containing 10 µl of undiluted raw semen mixed with 50 µl of river water. Of this, a total of 200 sperm cells were enumerated per individual animal as moving or nonmoving using 2–4 viewing fields per slide under 10× magnification. The overall motility estimate was then expressed as the total number moving per 100 cells (Schmitt & Webb, 2002). All motility estimates were made on site to minimize the effect of post ejaculate decrease in motility due to sperm death following sample processing (Billard *et al.*, 1995).

Viability.—We utilized three different methods to assess viability: staining of live vs. dead sperm, presence of sperm with intact acrosomes, and rate of forward progression (RFP).

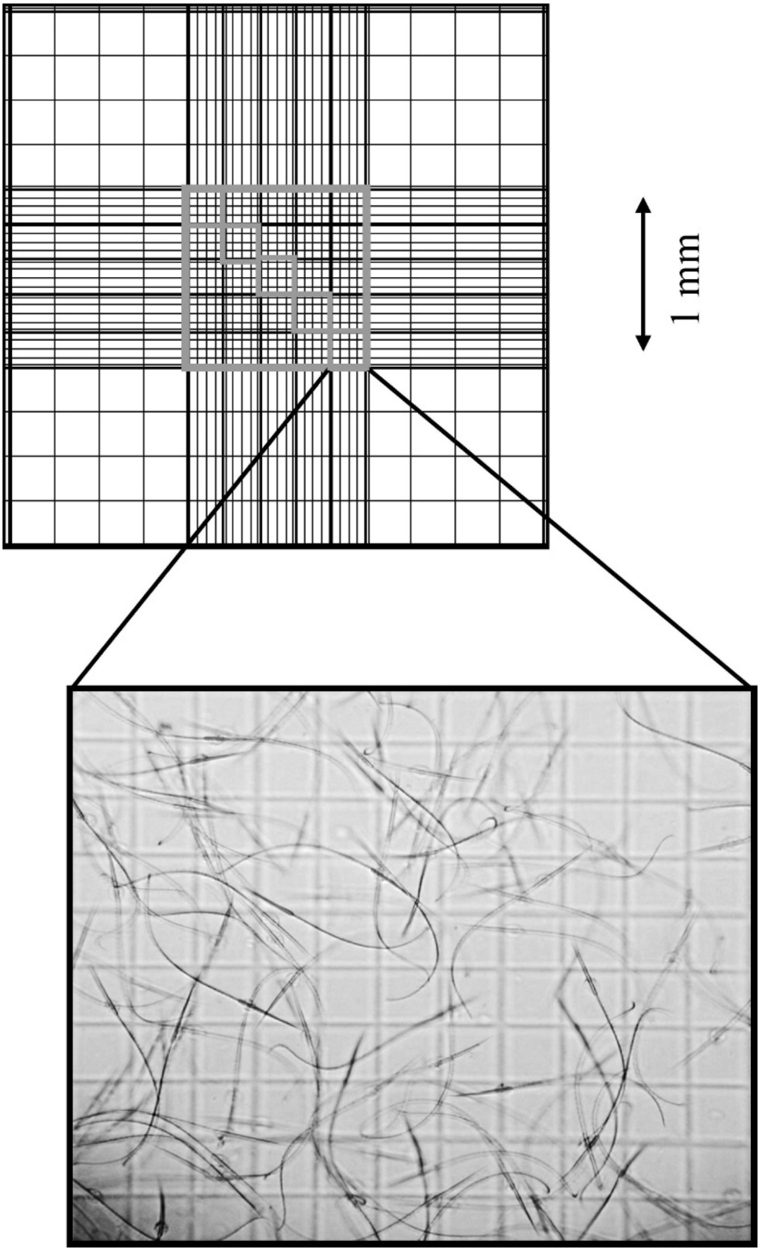

FIG. 1.—Counting Chamber diagram (adapted from Hausser Scientific Horsham, Pennsylvania) showing 5 central diagonal squares used for sperm concentration estimates according to Bearden & Fuquay (1999)

Viability was initially assessed by the ratio of live to dead sperm cells (Aalseth & Saacke, 1986; Schmitt & Webb, 2002), determined by whether parts of the sperm cell take up the stain and react (dead) or do not take up the stain (live) (Bearden & Fuquay, 1999). A new subsample of the 1.5 ml semen sample (10 μl) was combined with 5 μl of EosinB/fast green dye on a slide and immediately air-dried using a standard hair dryer to heat fix the slide. All slides were made within minutes of obtaining the sample and estimates were performed within an hour following staining. Eosin is a differential stain that can penetrate nonliving cell membranes but cannot pass through living cell membranes, while Fast green helps to make the unstained sperm heads visible (Bearden & Fuquay, 1999; Schmitt & Webb, 2002).

To be viable, sperm must have intact acrosomes (Bearden & Fuquay, 1999; Schmitt & Webb, 2002). As a second estimate of viability in addition to viability staining, 200 sperm cells from an additional subsample (10 μl) were enumerated and assigned to four categories, (1) Live sperm with intact acrosomes, (2) Live sperm with acrosome reacted, (3) Dead sperm with intact acrosomes, and (4) Dead sperm with acrosome reacted. Category 1 was the healthiest and indicates the highest level of viability while Category 4 was the lowest level of viability. The total ratio of live to dead sperm was estimated as was the percent of intact sperm.

RFP is the third measure of viability that we quantified (Schmitt & Webb, 2002). We used RFP is an additional measure of viability because it reflects the degree of motile, live sperm. Motility slides were also used to estimate RFP in six categorizes; (0) no movement, (1) head movement only (no forward progression), (2) slow forward progression (with labored head movements), (3) faster forward progression, (4) even faster forward progression, and (5) fastest linear forward progression. These ratings are subjective so the same observer evaluated all samples. Sperm (N = 200) were counted and the average RFP was determined. RFP is an important assessment of viability since a rating of less than three may not be adequate for fertilization (Schmitt & Webb, 2002).

Cryopreservation.—A subset of samples collected for this study were used in cryopreservation experiments to assess freezing techniques for spermatozoa. Sperm samples from a subset of Ozark hellbenders (2 males from 1 Oct., 1 male from 11 Oct., 2 males from 12 Oct.) were kept refrigerated (5 C) in the laboratory at MSU for a cryopreservation experiment. Samples from 1 Oct. were kept in the refrigerator for 24 h, and samples from 11 and 12 Oct. were kept refrigerated for 3–4 d prior to experimental cryopreservation on 2 and 15 Oct. 2002.

To conduct a baseline experiment on cryopreservation of Ozark hellbender sperm, we used several concentrations of two different cryoprotectants: 2.5%, 5%, 7.5%, 10%, and 15% Dimethyl Sulfoxide (DMSO), and 2.5%, 5%, 10% Glycerol. Three different extenders, sucrose (10%), suspension buffer (SB), and amphibian ringer solution (ARS), were used to dilute the sperm to a 1:3 semen:extender ratio (Costanzo *et al.*, 1998; Mugnano *et al.*, 1998; Browne *et al.*, 2002). Individual samples were randomly assigned different concentrations and type of cryoprotectant. Samples were mixed with extenders and cryroprotectants either slowly (1 drop at a time over an hour) or quickly (over 3 min) and placed into 0.5-ml plastic freezing straws. Samples were then frozen either rapidly (immediately), slowly (over 10–15 min), or rapidly following a 10 min equilibrium period. The purpose of the ''slow'' treatment was to reduce the potential for osmotic shock (Mazur *et al.*, 1972; Arav *et al.*, 2002). Samples were frozen at −80 C (Mugnano *et al.*, 1998) and were thawed several days later by placing them in a 35 C water bath for 10 s.

STASTICAL ANALYSIS

Comparisons of sperm parameters among populations were performed using a Kruskal-Wallis test in Minitab13. Nonparametric multiple comparison tests were used to compare individual populations to each other (Zar, 1984). Nonparametric statistical analyses were used because one of the study populations (Missouri eastern) violated the assumption of normalilty for parametric tests. To assess the possibility of senescence influencing sperm health in the declining population, we used a Spearman Rank Correlation analysis to compare sperm parameters with total lengths of individuals; in hellbenders, length is correlated with age (Peterson *et al.*, 1983). The North Carolina population was excluded from motility and viability estimates because individuals had little or no motility and were likely spent.

RESULTS

Search effort for the 2002 breeding season varied among populations, with the greatest concentration of effort for declining populations (Georgia eastern = 22.3, North Carolina eastern = 15.2, Missouri eastern = 45.1, Missouri Ozark = 124.4 h searching X number of individuals searching). The total catch per unit effort (catch/search effort) during 2002 also varied among populations (Georgia eastern = 2.33, North Carolina eastern = 1.15, Missouri eastern = 0.31, Missouri Ozark = 0.418), with the total number of individuals caught per unit effort higher for presumed stable populations (Georgia and North Carolina eastern) than for both Missouri populations (eastern and Ozark). Size distributions were noticeably different between populations in Missouri and the southeast. Distributions of males from the Missouri populations were highly skewed, with most individuals in larger size classes, above 200 mm total length (Missouri eastern = 100%, Missouri Ozark = 97%). The population from North Carolina appeared less skewed (100% above 200 mm TL; less proportion of individuals in the 450–499 size class) and the population from Georgia contained a higher proportion of small (young) individuals (45% below 200 mm TL).

Sperm concentrations and total volume of semen.—Qualitatively, the sperm counts of the two males from North Carolina (47,500,000 and 45,500,000) were similar to those of the males from Georgia (range = 37,000,000–67,000,000). Therefore, the data for the ''stable'' populations consisted of sperm measurements from North Carolina and Georgia combined.

Sperm concentrations differed significantly among populations of eastern hellbenders collected from ''stable'' populations (North Carolina and Georgia) and eastern and Ozark hellbenders in Missouri (Kruskal-Wallis $H = 12.54$, df = 2, P = 0.002, Fig. 2). Sperm concentrations were significantly lower for males from both Missouri populations than for males from populations in North Carolina/Georgia (Nonparametric multiple comparison tests: North Carolina/Georgia versus Missouri eastern, $Q = 4.88$, $P < 0.01$; North Carolina/Georgia eastern versus Missouri Ozark, $Q = 4.34$, $P < 0.01$). Missouri Ozark and Missouri eastern populations did not differ significantly ($Q = 1.33$, $P > 0.20$).

Motility.—Motility was relatively high for all populations, with averages for the three populations (Georgia eastern, Missouri eastern, & Missouri Ozark) ranging from 84% to 89% and no significant difference in motility among populations (Kruskal-Wallis, $H = 0.25$, df = 2, P = 0.881; Table 1). Three males (two from North Carolina, one from Georgia) were excluded from the analysis because they had extremely low motilities (<65%) and were likely ''spent'' following the peak of the breeding season (Smith, 1907; Bishop, 1941; Peterson *et al.*, 1989).

Viability.—Animals among populations did not differ in Rate of Forward Progression (Kruskal-Wallis, $H = 0.79$, df = 2, P = 0.673; Table 1). The majority of semen samples (>90%) of all populations were categorized as live with intact acrosomes (Category 1) and there was no significant difference among populations (Kruskal-Wallis test, $H = 3.70$, df = 2, P = 0.157).

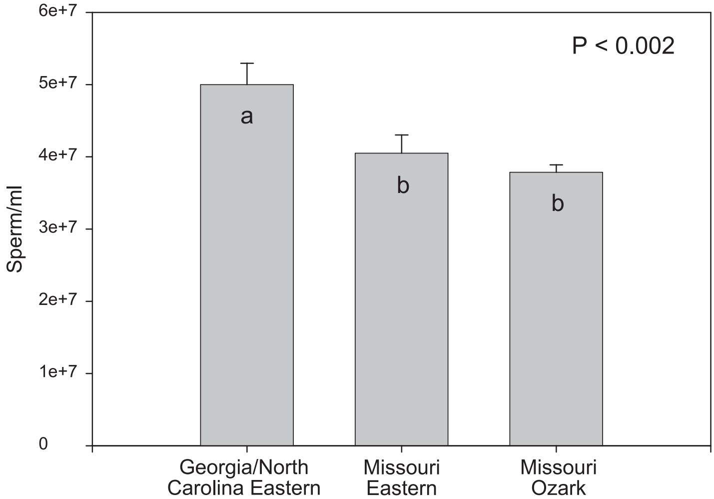

FIG. 2.—Sperm counts ($\bar{X} \pm 1$ SE) for eastern hellbenders from relatively stable populations (Georgia/ North Carolina) and declining eastern and Ozark hellbenders from Missouri. The P-value is for a Kruskal-Wallis ANOVA. Different letters indicate significant differences for nonparametric multiple comparison test

Body Size.—Sperm concentration and motility were not significantly correlated with salamander total length [Sperm Concentrations: (Spearman rank correlation, Georgia/ North Carolina, $r_s = -0.067$, $P = 0.855$; Missouri eastern, $r_s = -0.429$, $P = 0.289$; Missouri Ozark, $r_s = -0.197$, $P = 0.296$), Motility: (Spearman rank correlation, Georgia/North Carolina, $r_s = 0.218$, $P = 0.638$; Missouri eastern, $r_s = 0.220$, $P = 0.601$; Missouri Ozark, $r_s = 0.193$, $P = 0.308$)]. RFP was significantly positively correlated with total length for Ozark hellbenders but not for the other two populations (Spearman rank correlation, Georgia/ North Carolina, $r_s = 0.214$, $P = 0.645$; Missouri eastern, $r_s = -0.143$, $P = 0.736$; Missouri Ozark, $r_s = 0.409$, $P = 0.025$).

Cryopreservation.—Quick freezing, thawing in 35 C bath water for 10 s, and 2.5% DMSO resulted in the highest post-thaw motility (20%). However all samples post-thaw had low rates of forward progression (~Category 2). Samples with high concentrations of DMSO (15%, 10%) resulted in less than 5% motility. Samples mixed slowly resulted in zero post thaw motility. We observed that for up to three days after collection, samples kept at 5 C

TABLE 1.—Comparison of Mean Sperm Motility, Viability (live vs. dead), and Rate of Forward Progression estimates (mean ± SE), for Eastern hellbenders "healthy populations," declining Eastern hellbenders (*C.a. alleganiensis*) from Missouri, and declining Ozark hellbenders (*C.a. bishopi*) from Missouri

	GA, NC Eastern	MO Eastern	MO Ozark
Sperm Motility	89% ± 3.21	83.5% ± 6.51	86.3% ± 2.15
Sperm Viability	100% ± 0.01	95.6% ± 0.87	96.5% ± 0.80
Rate of Forward Progression (Index)	3.6 ± 0.12	3.3 ± 0.25	3.6 ± 0.07

remained motile (50–70%). However after four days there was a noticeable drop in motility (40–50%) from original estimates.

DISCUSSION

Sperm concentrations for male hellbenders from declining populations (Missouri) appear to be significantly lower than that of males from stable populations (Georgia, North Carolina). Sperm concentrations were statistically lower for both eastern and Ozark subspecies from Missouri than from the two southeastern populations. Because hellbenders have external fertilization, lower sperm concentrations could result in low fertilization success and may be an indicator of reduced reproductive health (Poole & Dillane, 1998). The sperm health for Missouri is within the range of other populations in decline (Burgmeier *et al.*, 2011b). Previous surveys in 2001 in Missouri (Unger, unpubl.) yielded spent males with clumped or no semen, indicating high yearly variance in reproduction in these populations. There is also likely some seasonal difference in timing of the breeding and spermatogenesis among populations sampled which could lead to some of the differences we detected in overall sperm health given the large geographic range and time period in which we sampled.

The potential influence of endocrine-disrupting chemicals on reproductive health has recently become a cause for concern, although their possible role in amphibian declines is unknown (Crisp *et al.*, 1998). Interestingly, Swan, *et al.* (2003) conducted a similar comparison of geographic differences in sperm concentrations in humans and found that males from Missouri had relatively low sperm counts, which they suggested were due to the close proximity of this population to pesticides associated with industrial agriculture. Hellbenders in Missouri at our sample locations are exposed to a variety of potentially estrogenic chemicals, but exposure levels appear to be relatively low (Solis *et al.*, 2007). However, due to the limited scope of our study we were unable to quantify this relationship.

Sperm motility and viability were similar among populations for both declining (Missouri) and stable (Georgia) populations. Overall sperm viability, rate of forward progression, and motility among populations were high compared to expected patterns associated with reductions in male reproductive function. The high percentage of sperm cells classified as live, with intact acrosomes (Category 1), are consistent with the high rate of forward progression observed for all populations. The high motility estimates we observed are similar to those observed for other species of externally fertilizing amphibians; *Xenopus laevis*, *Bufo marinus*, and *Rana pipiens* (Browne *et al.*, 1998; Costanzo *et al.*, 1998).

Because populations in Missouri are dominated by older, larger individuals, we investigated the possibility of senescence having a negative effect on fertility (Nickerson & Mays, 1973; Peterson *et al.*, 1983). For example, in brown trout, *Salmo trutta*, sperm concentrations were negatively correlated with length (age), and this decline resulted in decreased fertilization ability (Poole & Dillane, 1998). However we did not find a significant correlation between sperm counts with body size (total length).

The cryopreservation of hellbender semen has never been attempted and this information may be helpful for ongoing conservation management efforts. Experiments were conducted using differing concentrations of cryoprotectants, buffer solutions, and freezing and thawing regimes. There are no taxa-wide protocols for freezing amphibian sperm, partly because it has been poorly studied, and also because tolerances to freezing techniques vary among species (Costanzo *et al.*, 1998). In this study we were successful in freezing and thawing sperm that were viable, albeit at a greatly reduced motility (quick-freezing and low percent DMSO resulted in the greatest post-thaw motility). Cryopreservation of hellbender sperm and embryos may one day help to increase the fertilization success in artificial insemination procedures.

Although our estimates for sperm counts were relatively low, other aspects of our data are encouraging. Male hellbenders in Missouri are still producing viable sperm. Moreover, although sperm counts are significantly lower than in some healthy populations, other characteristics, including motility and viability, are overall high and similar to that of healthy populations. In addition, we have observed some successful reproduction in these populations in 2002 (Unger, 2003) and in 2007 (Gall and Mathis, 2010), indicating high motility may be enough to offset lower concentrations of sperm under nest rocks, where males can successfully fertilize eggs. Future work should thus focus on increasing post thaw motility of cryopreserved sperm using different crypoprotectants under different thawing regimes. When taken together, the results of this study illustrate the high efficacy of monitoring male health to better inform management strategies for this cryptic species.

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