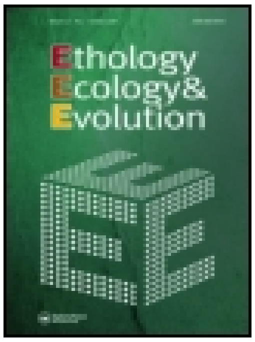

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Genetic confirmation of filial cannibalism in North America's giant salamander, the Eastern hellbender Cryptobranchus alleganiensis alleganiensis

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Introduction

For aquatic species with mating systems characterized by parental care, cannibalism is increasingly recognized as an evolutionary strategy (Manica 2002). Brooding parents may eat some (partial filial cannibalism) or all (total filial cannibalism) of their own offspring to forgo current breeding efforts in order to invest in future reproductive efforts (Rohwer 1978). The incidence of filial cannibalism can be influenced by several factors, such as presence of predators or conspecifics (Chin-Baarstad et al. 2009), reduced food intake (Walls 1998), or poor somatic condition of parental males (Gomagano & Kohda 2008). Experimental data on fish have shown that parents in poor body condition are more likely to cannibalize their brood to replenish energy reserves (Neff 2003; Manica 2004; Takeyama et al. 2013). Among species that cannibalize their offspring, one common theme involves guarding of large clutches of eggs, often with multiple dams (DeWoody et al. 2001). This can be problematic if these evolutionary behaviors remain intact despite changes in recent population demography (e.g., low population densities), which is the case for many amphibians experiencing declines (Wheeler et al. 2003).

The Eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) is a large, fully aquatic salamander found throughout the eastern United States. Due to their cryptic nature, little is known regarding hellbender reproductive behaviors, especially parentage. During the breeding season, males move to breeding sites (large rocks) and excavate a nesting cavity that females visit to deposit eggs; only males actively guard these eggs, for prolonged periods of 5–6 months (Nickerson & Mays 1973). Typically, multiple females may visit den masters (Floyd & Unger 2016), so that males may end up guarding numerous clutches with eggs numbering in the thousands in areas with high population densities. In low-density populations, however, males may only encounter a single dam and guard a few hundred eggs. One well-studied population (Blue River, Indiana) has experienced a dramatic decline over the past few decades (Burgmeier et al. 2011b). Moreover, we have observed eight clutches of eggs within the last decade, yet we have not documented recruitment since 1983 (Kern 1984). This leads us to hypothesize that cannibalism might be a factor in the lack of recruitment in this low-density population.

Cannibalism of eggs and larvae has been documented in hellbenders (Smith 1907; Humphries et al. 2005), but it is not clear whether males are consuming the eggs of conspecifics in nest takeovers or consuming their own genetic offspring. In the closely related *Andrias*, hygienic filial cannibalism occurs whereby guardian males selectively feed on eggs with fungal infections (Okada et al. 2015). However, directly observing these behaviors in the field is challenging, given that breeding takes place under large rocks in low light conditions. Molecular markers provide a powerful tool to characterize the presence of filial cannibalism and genetic parentage through reconstruction of progeny data (Avise et al. 2002; Gopurenko et al. 2007).

Here, we utilize a suite of highly polymorphic microsatellite markers to document filial cannibalism in eastern hellbenders from both a wild-collected, male-guarded clutch and regurgitated eggs from an adult male in the Blue River, Indiana. Specifically, we investigate whether guarding males are consuming their own offspring (filial cannibalism) or those of conspecifics (heterocannibalism), to inform conservation management practices for this endangered species.

Materials and methods

Sample collection. The study area where we collected genetic material from individual hellbenders is the Blue River, a tributary within the Ohio River drainage located in southern Indiana, USA. On 25 September 2011, one egg mass containing 120 eggs (stage 12–15; Smith 1912) was regurgitated from a male captured by hand, of which 25 eggs were selected for DNA extraction. On 11 September 2012, we observed a male guarding a fertilized nest of ~ 250 eggs (stage 15–17; Smith 1912) within an artificial nest structure. However, we returned 7 days later and observed that the male had consumed ~ 80% of the egg mass. About 10 of the remaining eggs from the artificial nest structure were set aside for DNA extraction. To identify potential candidate parents, we extracted DNA from tail clips from 101 adults previously collected from the Blue River between 30 June 2007 and 30 September 2011 as part of an ongoing mark recapture study.

Laboratory analysis. We extracted genomic DNA using a standard phenol-chloroform extraction protocol (Sambrook & Russell 2001). We used 12 polymorphic microsatellite markers (Unger et al. 2010) to genotype offspring and adults. PCR conditions followed Unger et al. (2012, 2013a), with the exception that individuals were run on single well reactions to increase ease of scoring and genotyping. We also reamplified ~ 30% of all adults in additional PCR reactions for quality control and to ensure repeatability. As an additional measure of quality control, all offspring (extracted eggs) were re-amplified twice in two separate PCRs and subsequently genotyped in single well reactions. We calculated the frequency of null alleles, test of Hardy Weinberg equilibrium (HWE), and allelic richness in CERVUS.

Genetic documentation of cannibalism. Genotypes from successfully extracted offspring (regurgitated eggs) were compared with the male that regurgitated eggs, to document filial cannibalism. We used CERVUS 3.0 (Kalinowski et al. 2007), a likelihood-based paternity inference program with strict exclusion to evaluate parentage (potential mother and father genotypes), to compare offspring genotypes for all adult individuals within our study system. CERVUS 3.0 assigned LOD (logarithm of odds) values calculated by the natural logarithm of the overall likelihood ratio, with positive LOD scores indicating that the candidate parent is more likely to be the true parent (Kalinowski et al. 2007). Polymorphic information content and probability of exclusion was calculated in CERVUS 3.0 (Kalinowski et al. 2007). We set the error rate in CERVUS at 0.001 following Williams et al. (2009).

Results

We successfully extracted DNA from 18 of the 25 embryos from the regurgitated egg mass. We were unable to obtain high-quality DNA from the subset of eggs (10) collected from the artificial nest structure, probably due to their degraded state. High-quality DNA from all

101 adults was successfully extracted. The sex ratio for 101 adults for adult Eastern hellbender individuals (candidate parents) was 2.2:1 (male:female). Our highly polymorphic genetic markers were well suited to assign parentage. For example, we detected null alleles at very low levels (0.001–0.08; Table 1), mean number of alleles per locus was high (12.58) and ranged from 9 to 18 (Table 1). None of our 12 loci were out of HWE. Lastly, our mean polymorphic information content (PIC) was 0.814 while combined exclusion probabilities were 99.98–99.999 with either one or two parents known, respectively.

We observed two to four alleles for individuals among the regurgitated clutch, consistent with single paternity (Table S1, see Supplemental data). Moreover, CERVUS assigned all offspring from the regurgitated egg mass to a single candidate father (the male that regurgitated eggs) and a single candidate mother (an adult female from the same sample location) without any mismatches (genotype) with > 95% confidence (Table 2). LOD values for paternity ranged from 5.17 to 9.59 (Table 2). While these results reflect observations of a single individual and a single egg mass, they provide the first direct genetic evidence for filial cannibalism in Eastern hellbenders.

Discussion

Filial cannibalism in Eastern hellbenders may have evolved to allow guardian males to defend a nest from conspecifics and ensure that some proportion of the total sired offspring survive. This scenario seems likely, and advantageous, if historical densities were high and allowed males to sire and/or guard multiple clutches. Multiple parentage has been genetically documented in populations where adults have access to a larger pool of potential mates (Unger & Williams 2015), and probably characterizes the genetic mating system in high-density areas. Siring multiple clutches would allow males to consume a much smaller proportion of their total clutch to

Table 1.

Genetic diversity estimates including locus specific allelic richness (K), observed heterozygosity (H_o), expected heterozygosity (H_e), and frequency of null alleles (Null) for 119 (including 18 offspring) Indiana Blue River Eastern hellbenders.

Locus	K	H_o	H_e	Null
Call 171	9	0.714	0.714	0.04
Call 127	12	0.756	0.856	0.06
Call 351	13	0.756	0.857	0.05
Call 204	10	0.832	0.862	0.01
Call 205	10	0.655	0.751	0.06
Call 232	10	0.824	0.817	0.006
Call 347	18	0.912	0.892	0.001
Call 282	18	0.763	0.803	0.025
Call 341	13	0.816	0.793	0.001
Call 261	13	0.746	0.886	0.083
Call 26	16	0.906	0.906	0.003
Call 266	9	0.798	0.823	0.001

Table 2.

Parentage results for regurgitated egg mass offspring across 12 polymorphic markers. Pair LOD score refers to the log-likelihood ratio for a parent–offspring relationship according to CERVUS 3.0.

Egg mass offspring	Pair loci mismatching for candidate parent (Father/Mother)	Pair LOD score
Offspring 1	0	9.41E + OO*
Offspring 2	0	6.55E + OO*
Offspring 3	0	7.88E + OO*
Offspring 4	0	7.20E + OO*
Offspring 5	0	6.70E + OO*
Offspring 6	0	9.0lE + OO*
Offspring 7	0	9.59E + OO*
Offspring 8	0	8.83E + OO*
Offspring 9	0	7.33E + OO*
Offspring 10	0	7.79E + OO*
Offspring 11	0	7.94E + OO*
Offspring 12	0	5.57E + OO*
Offspring 13	0	5.17E + OO*
Offspring 14	0	5.48E + OO*
Offspring 15	0	7.02E + OO*
Offspring 16	0	5.86E + OO*
Offspring 17	0	7.79E + OO*
Offspring 18	0	6.19E + OO*

*For strict pair confidence. All 12 markers used for all offspring/candidate parent comparisons, with all alleles from candidate mother and candidate father.

sustain themselves. On the other hand, filial cannibalism may not be advantageous in low-density populations. Population densities within our study site are the lowest reported in the literature (Burgmeier et al. 2011a), and thus probably result in single parentage (one mother, one father), far fewer eggs for the male to guard, and a disproportionate effect on recruitment if the male cannibalizes his eggs.

Filial cannibalism as observed in our study may be a result of increasingly senescent, guarding males with reduced overall body condition (Burgmeier et al. 2011a; Unger et al. 2013b). Though Eastern hellbenders can withstand starvation in captivity for several months and recover once released (Nickerson & Mays 1973), males in our study may be below a critical threshold for prolonged guarding and subsequently consume their eggs to restore energy reserves. Alternatively, our observation of cannibalism in the artificial nest structure may be the result of our physical disturbance during nesting 7 days before we returned to collect eggs. This behavior has been documented in female long-tailed skinks, *Mabuya longicaudata*, in which the frequency of intruders increased whole-clutch filial cannibalism (Huang 2008). In either of these scenarios, cannibalized young appear to be a major food source for guarding males.

Filial cannibalism is currently not considered within the conservation management programs of Eastern hellbenders. Unfortunately, many of the Midwestern states are now left with dangerously low population densities where filial cannibalism can become a limiting factor to population growth. To this end, we recommend that in areas with low population densities researchers practice continued collection of available clutches (immediately following deposition or during the early breeding season, to avoid guardian male consumption of eggs) for captive rearing. We also recommend applying alternative DNA extraction methods to increase yields from potentially degraded eggs and number of offspring individuals for parentage analyses. Further research efforts should focus on assessing body condition, behavioral observations during reproduction, and the implementation of artificial nest structures to address population declines across the range of the species.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplemental data

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