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Prebreeding, Courtship, and Mating Behaviors of Wild Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*)

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Abstract: Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*) are giant, charismatic salamanders of conservation concern. Despite growing interest in their breeding behavior, significant gaps remain in our understanding of hellbender reproduction, particularly the behavior occurring immediately prior to and during breeding because these activities typically occur within the nesting cavity and out of view. In this study, we used custom-built infrared cameras installed underwater in artificial nesting shelters to record prebreeding behaviors, complete mating sequences, and failed mating attempts in 11 shelters. Using these recordings, we describe the basic mating sequence, the presence of potential alternative mating tactics, and two novel behaviors, including possible signal production via wave-based communication and unique egg laying behavior by female hellbenders. These findings add to our understanding of hellbenders' life history as well as informing conservation efforts in both captive and wild environments.

Key words: Alternative reproductive tactics; Cryptobranchidae; Custom-built camera; Nest cleaning; Oviposition; Raspberry pi microcomputer; Reproduction; Satellite males

Understanding a species' reproductive behavior can contribute to advancements in evolutionary ecology as well as more applied conservation efforts. For example, knowledge of breeding behavior can elucidate the evolutionary history of a species by revealing evidence of past or current selective pressures (e.g., Pfennig and Pfennig 2009) and can reveal when reproductive behaviors arose in a clade's evolution (e.g., Ron 2008). From a more applied perspective, improved understanding of reproduction has enabled more effective captive breeding of Giant Pandas (*Ailuropoda melanoleuca;* Martin-Wintle et al. 2015), informed the protection of breeding habitat for Humpback Whales (*Megaptera novaeangliae*; Oviedo and Solís 2008), and highlighted the risk posed by increasing light pollution for fireflies (Lampyridae; Hagen et al. 2015). Additionally, the evolutionary history of a species can inform conservation priorities and approaches. For example, evolutionary distinctness has been suggested as a means for determining conservation priorities in a way that focuses on species evolutionary diversity (Faith 1992; Redding et al. 2014).

Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*) are giant, fully aquatic salamanders that are both evolutionarily distinct (Larson and Dimmick 1993) and of conservation concern (Wheeler et al. 2003; Burgmeier et al. 2011; Graham et al. 2011), yet their reproductive behavior remains poorly understood. Eastern Hellbenders are one of five species in the family Cryptobranchidae that, along with Hynobiidae, shares a basal node with the rest of urodeles, possibly excluding Sirenidae (Larson and Dimmick 1993; Pyron and Wiens 2011). Populations of Eastern Hellbenders, as well as the closely related Ozark Hellbenders (*Cryptobranchus alleganiensis bishopi*), are declining rapidly throughout their range in the United States (Wheeler et al. 2003; Foster et al. 2009; Burgmeier et al. 2011; Graham et al. 2011), and recent work has indicated that failure to produce viable offspring is contributing to population declines (Hopkins et al. 2023). This emphasizes the need to better understand hellbender reproductive biology. Additionally, there are several captive breeding programs currently underway to support hellbender recovery (e.g., Ettling et al. 2013; Civiello et al. 2019; McGinnity et al. 2021), and there remain challenges in encouraging breeding in captive environments and ensuring egg viability (Ettling et al. 2013; Civiello et al. 2019).

Until recently, understanding of hellbender breeding behavior was limited to anecdotal accounts (e.g., Bishop 1941; Peterson 1988; Foster et al. 2009), studies focused on the timing of breeding (e.g., Peterson 1988; Peterson et al. 1989), and one observational study of two captive breeding events (Smith 1907). However, recent research has employed improved technologies to study reproductive behavior in both captive (Settle et al. 2018) and natural (Unger et al. 2020) environments. Research shows that breeding males occupying an active nest (hereafter referred to as occupying males) begin defending nesting cavities several weeks prior to the commencement of breeding. These cavities are visited by one to several females and can receive multiple clutches, which are fertilized externally. The females leave the nest following oviposition, but the occupying male remains with the eggs until the larvae emerge from the nest in early spring. Several mating behaviors have been observed outside the nest cavity prior to breeding, including guarding and patrolling of the nest chamber by the male, nose-to-nose contact between individuals, biting of conspecifics, cleaning of the nest cavity, cloacal sniffing, swimming in tight circles, and tail swishing (Bishop 1941; Settle et al. 2018; Unger et al. 2020). However, critical gaps remain in our understanding of the mating process. Most significantly, all previous studies of hellbender breeding have been restricted to interactions visible from outside of the nesting cavity. The small, enclosed nature of most hellbender breeding cavities means that observing or filming the behavior occurring within them is difficult, so the details of the actual breeding event, and the mating behavior immediately preceding it, have not been recorded. Additionally, previous observations of breeding behavior

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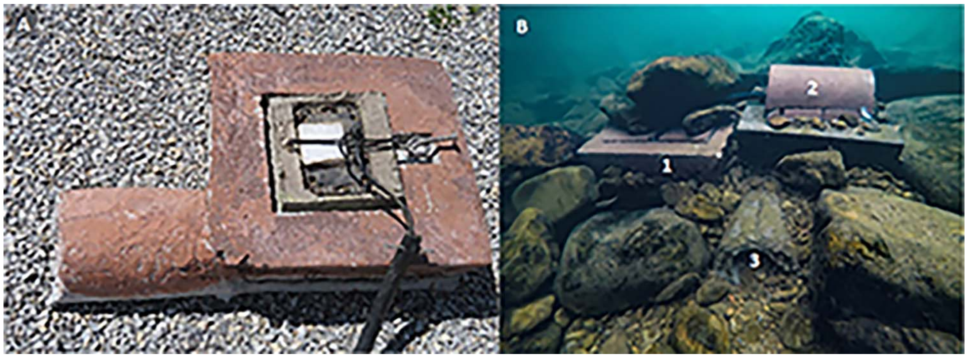

FIG. 1.—Modified hellbender shelters ready to be installed (A) and newly installed (B) in a stream. The camera is located under the white foam on top of the shelter in Image A. In Image B, an external battery enclosure (1) is located next to the installed shelter, and a domed protective concrete cover (2) has been installed to protect the camera housing. The tunnel (3) is pointed downstream. See the Supplemental Materials for more information. A color version of this figure is available online.

have been limited in duration, with observations averaging 10 nocturnal hours in captive environments (Settle et al. 2018) or 3.95 diurnal hours in natural environments (Unger et al. 2020). This means that full breeding interactions were not always captured (Settle et al. 2018).

In this study, we sought to build on previously identified mating season behaviors by capturing uninterrupted footage of the behaviors that occur within the nesting cavity, including the activities of occupying males while alone and his interactions with visiting hellbenders. Using novel infrared camera technology and artificial breeding shelters submerged in a natural stream, we were able to record prebreeding activities in 11 occupying males as well as 6 complete breeding sequences and multiple failed mating attempts.

MATERIALS AND METHODS

Study System

This research took place across four distinct reaches of a stream in southwestern Virginia that support relatively healthy populations of hellbenders. The approximately 100–200-m-long reaches are dispersed across 27.3 channel km, and for the past 10 yr they have been part of an ongoing research effort utilizing fully submerged concrete shelters. These shelters consist of a rectangular chamber with a single tunnel entrance and are installed in arrays of ≥30 shelters/reach. Hellbenders readily utilize the shelters for both cover and nesting (Bodinof Jachowski et al. 2019; Hopkins et al. 2023), and males that nest in the shelters are comparable to those utilizing wild nests in mass and snout–vent length (SVL; R. O'Brien, personal observation). For this project, we added 19 shelters over the span of 2 breeding seasons, which were modified to accommodate a camera to provide an aerial view of the interior of the shelter (Fig. 1). Eleven of these shelters were occupied by males during the period of study. Occupying males that successfully procured eggs were captured, weighed, and measured for total length within 4 d of oviposition.

Video Recording

We recorded footage in our modified shelters using a microcomputer (Rasberry pi Foundation, Cambridge, UK) attached to an infrared camera (FOV 175° 5-Megapixel Camera Module; Sainsmart, Lenexa, KS, USA). We programmed the cameras to operate for 48-h cycles and then shut down so that we maximized the battery life but avoided forced shutdowns that could damage the computer over time (see Supplemental Material Text files S1 and S2, available online). The recording was continuous except for pauses to exchange batteries and data storage devices or when circumstances, such as very high flows, prevented access to the stream or otherwise prevented data collection. The cameras saved video footage in 15-min segments at a frame rate of 15 frames/s and a resolution of 1640 × 1232 pixels.

Our study took place in the fall breeding seasons of 2020 and 2021. We recorded some footage in late July that was not analyzed but used to test the cameras, and then formally installed the cameras at the beginning of nesting, between 29 August and 10 September (Table 1). Based on a decade of monitoring in these streams, we know that hellbenders within the same stream reach breed relatively synchronously, so after a week of checking shelters in a reach without finding any new nests, we determined that breeding in that reach was complete and stopped filming. All of the shelter arrays with camera-equipped shelters had high occupancy (54.6–68.9%), so we had multiple nests in each reach to indicate the cessation of breeding. Additionally, we continued to check for eggs in the shelters that had not produced nests for 2 wk following the end of breeding, so we were able to confirm that all shelters were accurately categorized as either having or not having received eggs. The total duration of our recordings per shelter ranged from 18.5 to 521.9 h (i.e., <1 to 21.7 d; Table 1) depending on how soon after installation, if at all, the shelter received eggs.

Behavioral Coding and Analysis

We used ELAN v6.4 software (Brugman and Russel 2004) to code focal samples (Altmann 1974) of the behaviors of occupying males and other visiting hellbenders within each shelter. When occupying males were alone in the shelter, we analyzed 15 min of behavior every 4 h to gain an understanding of their basic behaviors in the shelter. When a visiting hellbender (an animal other than the occupying male) entered the shelter, we analyzed the footage continuously from the start of the segment when the visiting animal entered the shelter through the end of the segment in which the visiting animal left the shelter. We coded the behavior of all animals, including the occupying male and any other

TABLE 1.—Date, duration, and location of camera installation and removal from underwater shelters as well as occupying Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*) male mass and total length. Only successfully breeding male hellbenders were captured and measured. Shelter G–K did not receive eggs and so the oviposition date, time, mass, and total length are not applicable, or N/A.

Shelter	Stream reach	Date installed	Date removed	Total filmed hours	Oviposition initiation date	Oviposition initiation time EST (military notation)	Male mass (g) at oviposition	Male total length (cm) at oviposition
A	1	4 Sep 2020	5 Sep 2020	21	5 Sep 2020	1037	590	45.4
B	2	1 Sep 2020	10 Sep 2020	207	10 Sep 2020	0554	440	42.2
C	2	9 Sep 2020	10 Sep 2020	19	10 Sep 2020	1220	475	44.9
D	3	31 Aug 2020	11 Sep 2020	267	11 Sep 2020	1420	480	44.4
E	4	4 Sep 2021	13 Sep 2021	224	13 Sep 2021	1614	720	47.8
F	2	29 Aug 2021	8 Sep 2021	237	6 Sep 2021	0251	870	50.9
					6 Sep 2021	1042[a]		
					8 Sep 2021	0827[a]		
G	4	1 Sep 2020	15 Sep 2020	336	N/A	N/A	N/A	N/A
H	3	29 Aug 2021	20 Sep 2021	522	N/A	N/A	N/A	N/A
I	3	4 Sep 2021	20 Sep 2021	383	N/A	N/A	N/A	N/A
J	4	10 Sep 2021	20 Sep 2021	231	N/A	N/A	N/A	N/A
K	1	31 Aug 2020	16 Sep 2020	391	N/A	N/A	N/A	N/A

[a] The exact initiation of oviposition could not be determined because of the presence of previously laid eggs, so the reported times are when the female entered the shelter on her ovipositing visit.

individuals in the shelter. Once the visiting animal left, we returned to the reduced sampling scheme until another visiting hellbender entered. We measured the duration of each visiting animal's stay in the shelter and coded behavioral data using the ethogram in Table 2. Our ethogram was based on previously identified breeding behaviors (Guimond and Hutchison 1973; Settle et al. 2018; Terry et al. 2018; Unger et al. 2020) as well as novel behaviors we identified that occurred across more than one individual and appeared significant to the breeding process. To enable a more easily interpretable description of breeding behaviors, we allowed for overlapping behavioral codes. We also recorded mutually exclusive data on the position of the occupying male in the shelter. The lead author did all coding.

We confirmed the sex of the occupying male through passive integrated transponder tags or, for untagged animals, through the observation of distinct morphological and behavioral characteristics during successful breeding events. Gravid females that did not breed were sexed based on their appearance and behavior: They were swollen with eggs, moved sluggishly while in the shelter, and elicited attentive behavior from the resident male. Individuals that entered and were not obviously gravid females were classified as intruders and not assigned a sex. When possible, we used distinguishing characteristics such as scars, spotting patterns, or physical deformities to identify when the same animal entered the shelter more than once (Fig. 2).

Analysis

We calculated time budgets for the occupying male's position in the shelter when alone and when visited by a female, and described the basic sequence of events for breeding interactions. We also used *t*-tests to compare behaviors between males that were successful and unsuccessful at procuring eggs as well as between females on ovipositing and nonovipositing visits. All statistical analyses were done using Program R v3.6.2 (R Core Team 2022).

RESULTS

We captured the breeding season behavior of 11 shelter-guarding males as well as 38 gravid females and 29

TABLE 2.—Ethogram of hellbender behaviors coded during video analysis. Cleaning, rocking, and guarding behaviors were defined based on Guimond and Hutchison (1973), Terry et al. (2018), and Unger et al. (2020), respectively. Nose-to-nose and tail swishing were based on Settle et al. (2018). Entering, threading, and quivering were all newly described from our observations.

Behavior	Description
Nest cleaning	Movement of lose substrate using the hind legs in a kicking motion or shoveling with the nose. Sometimes very fast undulation of hind legs and tail to achieve the same effect.
Conspecific biting	Biting of the head, tail, side, or leg of another animal.
Tail swish	Male sways hips to move tail in a lateral waving motion.
Nose-to-nose	Occupying male and visiting female touch or nearly touch noses.
Rocking	Lateral back and forth movement of the body while all four feet remain grounded.
Entering	The time between when a female's nose first became visible in the shelter chamber until her hind legs entered the chamber.
Threading	Weaving in and out of the egg mass.
Quivering	A rapid contraction of the animal's side.
Other	Eating shed skin, resting in chamber, striking at or running toward targets outside of shelter tunnel, struggling with unseen subject in the tunnel, flicking tail, biting at material floating in water, rolling, yawning, scratching face, shaking leg, opening and closing mouth, resting.

Position	Description
Nest guarding	Male's face or entire body is in the tunnel.
Absent	Male is absent from the camera's field of view after exiting the tunnel. Subsequent re-entry is face-first.
Chamber	Male is in the chamber without his face in the tunnel.

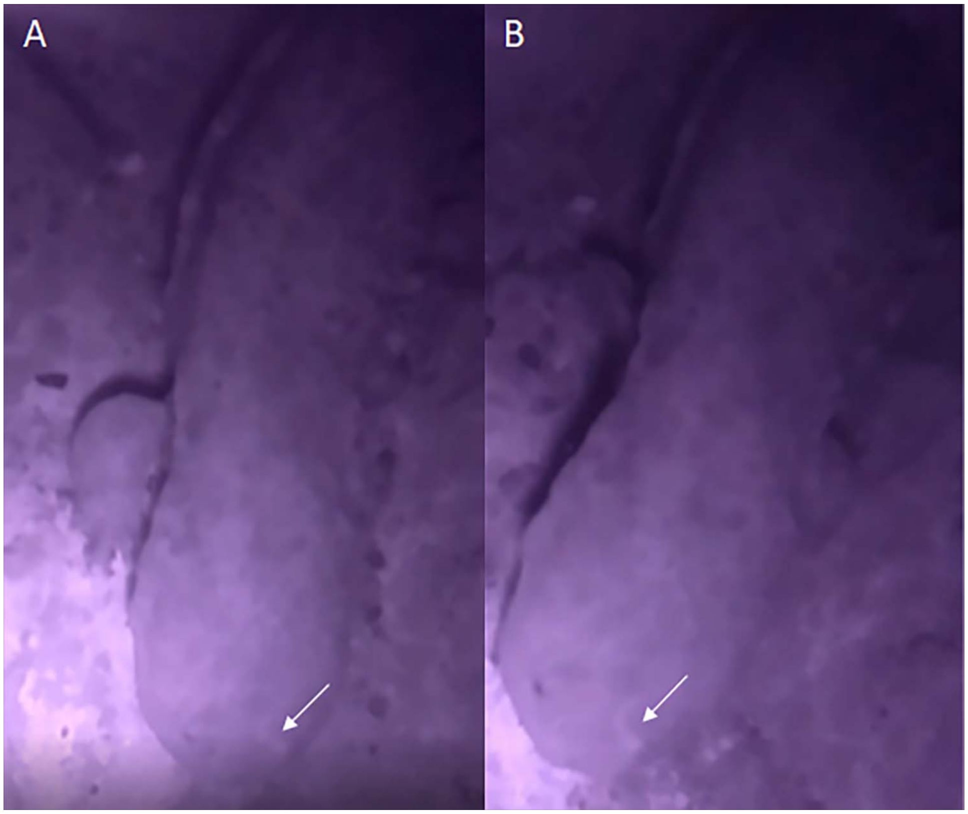

Fig. 2.—To determine instances of repeated entry by the same individual Eastern Hellbender, we took note of features such as spotting patterns, scars, or other physical deformities. This image shows a breeding female at first (A) and second (B) entry into a shelter. The carved scar on her face highlighted by the arrow, as well as her spotting pattern, helped identify her as the same female. A color version of this figure is available online.

individuals whose sex could not be definitively identified. From our recordings, we determined how males spent their time alone in the shelter prior to receiving eggs as well as how they and visiting hellbenders behaved when together. Six of the males that we recorded were successful in procuring eggs, and we were able to record and describe the breeding event in its entirety from the female's entry into the shelter through her exit after depositing eggs.

Our positional time budget revealed that males spent the majority of their time guarding the tunnel of the shelter when alone, but once a female entered the shelter, they switched to spending the majority of their time in the chamber (Table 3). After oviposition began, they resumed some guarding of the tunnel, but still returned to the chamber to a greater extent than they did when alone. Males only left the shelter when no female was present.

While alone in the shelter and prior to receiving their first clutch, occupying males primarily spent their time rocking (mean percent time = 7.0 ± 1.2 SE) and cleaning (mean percent time = 2.4 ± 0.7 SE). Cleaning behaviors included pushing sediment using the nose or feet, as well as rapid movement of the tail and hind legs, and they occurred both in the main chamber and at the entrance to the tunnel (see Supplemental Video SV1, available online). Based on footage captured before the study began (not formally analyzed), males began cleaning as many as 7 wk prior to breeding. Other behaviors that males engaged in while alone (each for <1% of the total recorded time) included striking at targets outside the shelter, twitching their legs, eating shed skin, scratching their faces, rolling, biting at material in the water, opening and closing their mouths, clenching their jaws, flicking their tails, and fighting with unseen animals in the tunnel. Hellbenders also spent time resting in the chamber.

Occupying males (both successful and unsuccessful at procuring eggs) had one to six gravid females enter their shelters throughout the course of the filming, with these interactions occurring during both day and night. Ovipositing females spent between 2.02 and 4.21 h in the shelter during a visit, with an average time of 3.01 ± 0.26 h. For females that did not oviposit, the time spent in the shelter was far less than ovipositing females, ranging from 1.6 min to 50.8 min with an average stay of 14.6 ± 2.0 min. Regardless of whether they oviposited or not, females always left of their own volition. However, they were often nipped on the tail by the occupying male as they left.

Individuals whose sex could not be definitively identified (hereafter, "intruders") also frequently entered the shelters, and six of the eight breeding events had at least one intruder present during breeding and/or oviposition. Intruders were always smaller than the occupying male and did not appear to have abdomens distended with eggs. They rarely (9.7% of the time in the shelter) were present in the shelter without a gravid female. Intruders were present more often when ovipositing females were in the shelter (94.4% ± 2.8% of the time they were in the shelter) as compared with when nonovipositing females were present (13.1% ± 8.9% of the time they were in the shelter). Intruders typically remained motionless in the shelter after entry except for short bursts of rapid movement. The occupying male usually ignored the intruders, particularly when a female was present, but did periodically attempt to

Table 3.—Positional time budgets for occupying male Eastern Hellbenders. Some behaviors are only applicable to successful or unsuccessful males, and the alternative is therefore marked as not applicable, or N/A.

		Percent time spent in position			
Circumstance	Position	All	Successful males	Unsuccessful males	
Alone	Guarding	77.6 ± 3.7	79.2 ± 5.0	74.8 ± 5.7	
	Absent	1.8 ± 0.5	2.2 ± 0.6	1.1 ± 0.5	
	Chamber	20.6 ± 3.8	18.6 ± 5.0	24.1 ± 6.0	
Female that oviposited present	Guarding	9.9 ± 7.0	2.0 ± 2.0	17.9 ± 20.2	
	Absent	0 ± 0	0.0 ± 0.0	0.0 ± 0.0	
	Chamber	90.1 ± 7.0	98.0 ± 2.0	82.1 ± 13.5	
Female that oviposited present (prior to oviposition)	Guarding		0.5 ± 0.3		
	Absent	N/A	0.0 ± 0.0	N/A	
	Chamber		99.5 ± 0.3		
Female that oviposited present (after oviposition begins)	Guarding		32.8 ± 2.9		
	Absent	N/A	0.0 ± 0.0	N/A	N/A
	Chamber		67.2 ± 2.9		

drive them out by biting them. Intruders never behaved aggressively toward the occupying male, suggesting they were not seeking to displace him, but at least one intruder did appear to release sperm over a clutch of eggs while in the shelter (indicated by sudden cloudiness of the water). Several times, multiple intruders were present in the shelter simultaneously, with one shelter containing four intruders at once in addition to the occupying male and ovipositing female. The same intruder often came and went from a shelter several times.

During interactions between hellbenders, we observed a previously undescribed "quivering" behavior, characterized by rapid contractions of the hellbender's sides (Supplemental Video SV2, available online). Although this behavior was much more common among females, we also observed quivering in occupying males and intruders. Occupying males showed evidence of responding to female quivering by changing their position in the shelter. We witnessed males responding to quivering in several shelters, but it was particularly striking in one shelter (Shelter A), where the occupying male spent 76% of his time guarding the tunnel while the ovipositing female was present. This male periodically returned to the interior chamber and approached the female. After a short period, the female would begin to quiver, the male would return to the tunnel, and the female would continue her ovipositing activities in the shelter. Nonovipositing females spent a greater percentage of their time in the shelter quivering prior to oviposition than did ovipositing females throughout their visit (Table 4).

Interactions between occupying males and visiting females followed a similar pattern prior to oviposition across all recorded breeding pairs (Fig. 3) as well as pairs that did not ultimately breed. Females entered the shelter slowly when males did not interfere, but the males sometimes bit the females' heads before they completely entered and pulled them into the shelter (Supplemental Video SV3, available online). While the female was entering, males performed a tail swish in 59.5% of all interactions (Supplemental Video SV4, available online), and they touched noses in 25% of all interactions (Supplemental Video SV5, available online). Females began quivering in some cases the moment they became visible on the camera, and continued to quiver off-and-on throughout their interactions with the occupying male.

Occupying males typically bit visiting females one to several times after entry, most commonly on the head (56% of bites) but also on the tail, leg, and side. Tail bites typically occurred as the female was leaving the shelter after the breeding event was completed. Bites on the leg, side, and tail were typically fairly brief (an average hold time of 9.5 ± 5.8, 1.9 ± 0.5, and 1.6 ± 0.2 s respectively), but head bites were often held for prolonged periods with an average of 6.3 ± 1.3 min). One occupying male held a continuous head bite of a female for 38 min. This bite was, in practical terms, even longer, because it immediately followed a 15.5-min head bite with only 32 s of rest in between. Of the eight successful breeding events we captured on film, three did not include the male biting the female prior to laying. However, two of these three breeding events took place after the male had already received one clutch of eggs. When not holding a bite, the male climbed over and around the visiting female, often nudging her side and cloaca and crawling under her belly and resting there. Occupying males also frequently rested under the female's chin. Females remained relatively passive during these interactions, moving lethargically and submitting to male bites.

Table 4.—Statistical *t*-test comparisons of behaviors of female Eastern Hellbenders that oviposited on a given visit and those that did not. Ovipositing females spent significantly longer in the shelter and quivered significantly less prior to oviposition than nonovipositing females did during the duration of their visit.

	Percent or total time spent on behavior					
Behavioral variable	All	Female that oviposited	Female that did not oviposit	t	df	P
Time to enter (s)	75.9 ± 16.67	154.1 ± 69.6	60.3 ± 13.6	−1.32	5.39	0.24
Total time present (min)	47.8 ± 11.2	180.7 ± 15.4	14.6 ± 2.0	−10.66	7.25	<0.001
Time present prior to oviposition (for ovipositing females) or exit (for nonovipositing females) (min)	18.6 ± 3.6	46.1 ± 16.4	13 ± 1.8	−2.00	5.12	0.10
Percent time quivering prior to oviposition (for ovipositing females) or exit (for nonovipositing females)	29.0 ± 3.7	15.3 ± 3.8	31.8 ± 4.2	2.91	20.05	0.01

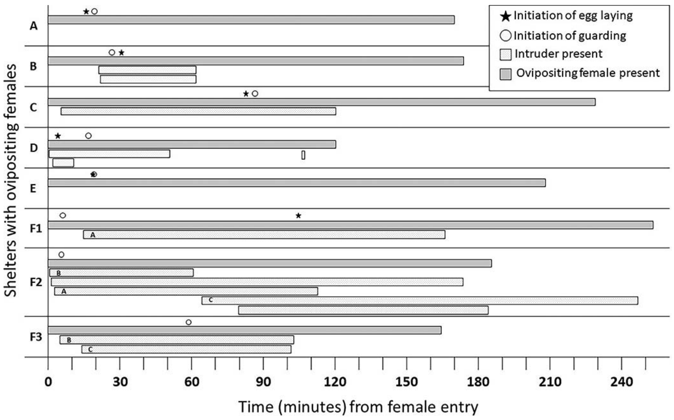

FIG. 3.—Sequence of events taking place in nests of male Eastern Hellbenders that successfully received eggs. The initiation of egg laying for the second and third females in Shelter F could not be determined because of the large number of eggs already in the shelter. Letters in intruder bars denote the same individual returning multiple times with different females. One intruder in Shelter D entered twice while the same female was present.

Females began to lay eggs an average of 41.8 ± 17.1 min after entering the shelter. Throughout the period of oviposition, all the recorded females undertook a previously undescribed behavior (hereafter referred to as "threading") in which they wove in and out of the egg mass while ovipositing, effectively tying the eggs in a large knot (Supplemental Video SV6, available online). This threading behavior appeared to continue even after the female had finished laying eggs. After the ovipositing female had left, we observed a few occupying males also performing this threading behavior, although in their case it did not affect clutch morphology. Ovipositing females increased rocking behavior throughout the course of breeding, rocking 1.8% ± 1.1% of the time before and 11.3% ± 2.7% of the time after oviposition began.

Once females began to oviposit, occupying males across all eight shelters remained in the chamber for a few minutes (average 3.1 ± 0.7 min) before moving to the tunnel to guard the chamber (although two males began guarding before oviposition began; Fig. 3). After guarding commenced, the male would periodically return to the chamber to crawl over and around the female and the eggs, which the female often responded to with quivering. These returns to the chamber happened 5 to 16 times throughout the course of egg laying, or an average of once every 8.9 min. It was unclear whether males were depositing sperm during this time because sperm release was not always clearly visible on the recordings because of disturbed sediment, skin secretions, and/or the presence of pre-existing sperm clouding the water.

We found no significant difference between successful and unsuccessful occupying males in terms of percent time biting visiting females or percent time spent cleaning the nest prior to breeding, which were the most prominent breeding season behaviors of probable interest to females (Table 5). However, successful males did spend longer holding head bites than unsuccessful males, suggesting that further investigation into biting as a possible courtship behavior is warranted (Table 5). The behavior of females that laid their eggs vs. those that did not differed more notably than did successful and unsuccessful male behaviors. Prior to oviposition, females that laid their eggs spent a significantly smaller percentage of their time in the shelter quivering than did nonbreeders during the duration of their visit (Table 6). Ovipositing females also entered the shelter 48% more slowly than did nonovipositing females (Table 6).

In three shelters, the ovipositing female entered the shelter, interacted with the occupying male and left, only to later return and lay eggs. The time difference between the first and second entry varied between these three shelters with times of 3.42, 10.00, and 49.65 h between the initial nonbreeding and the later ovipositing visits. Although in one shelter the occupying male did not bite the female during either visit, in the other two the occupying male bit more and held the bites for longer on the female's initial visit than he did on the second visit where oviposition occurred (Table 6). Females also behaved differently between visits depending on whether or not they laid eggs during the visit. They spent more time quivering and did so more continuously on their initial visit where they did not oviposit than on the later visit where they did. Females also took less time to enter the shelter on their initial visit than they did at oviposition, although this may be due in part to males biting females and pulling them into the shelter on their initial visit rather than allowing them to enter at their own pace at oviposition. In two of the three shelters, the same intruder individuals entered with the female during both of her visits.

Most of the shelters we recorded received only one clutch of eggs, but we recorded one shelter that received three clutches of eggs from three distinct females over a period of three d. Ovipositing females entered this shelter at 2017 h on 5 September, 1032 h on 6 September, and 0826 h on 8 September with no overlap between visits. This shelter also had two females enter but not lay eggs after the occupying male had received the first clutch (a circumstance we also observed in other shelters). The occupying male bit and held the head of the first visiting female for 3.52 min, but he did not bite the second female at all, and bit the third only on the tail as she left the shelter. The females that visited when eggs were already present in the nest cavity cannibalized a small number of the previously laid eggs both before and during their own oviposition without any apparent response from the male. Each subsequent female spent less time in the shelter (252.22, 185.51, and 164.52 min for the

TABLE 5.—Statistical *t*-test comparisons of the percent time male Eastern Hellbenders spent cleaning and average time biting each visiting female hellbender's head (the coded behaviors most likely to influence mating success). There were no significant differences between males that were successful and unsuccessful at procuring eggs.

		Percent time or total minutes spent on behavior					
Behavior	Circumstance	All	Successful shelter	Unsuccessful shelter	*t*	df	*P*
Percent time cleaning	Alone	2.4 ± 0.7	2.1 ± 1.0	2.9 ± 0.8	0.57	8.96	0.58
Total time holding head bite of female (min)	Female present prior to oviposition	6.3 ± 1.3	7.2 ± 2.1	5.4 ± 1.5	−0.71	33.49	0.49

Table 6.—Average duration of behaviors (bouts of quivering or hold times for bites—not applicable, or N/A, to entering) and average total time spent on behaviors for shelters where ovipositing female Eastern Hellbenders entered multiple times. We only measured these behaviors prior to oviposition.

Behavior	Average duration of behavioral bout (min)		Average total time spent on behavior (min)	
	Initial visit	Oviposition	Initial visit	Oviposition
Quivering	0.65 ± 0.18	0.18 ± 0.03	5.45 ± 3.16	4.59 ± 2.90
Entering	N/A	N/A	0.39 ± 0.15	3.62 ± 2.30
Biting	3.79 ± 2.39	1.00 ± 0.47	7.58 ± 2.46	4.01 ± 1.73

first, second, and third ovipositing female, respectively) and spent less time quivering (32.5, 40.4, 5.9 s quivering for the first, second, and third ovipositing female, respectively) than had the previous ovipositing female. The occupying male in this shelter was larger than the other males in our study in both mass and total length (Table 1).

Discussion

This research represents one of only a few studies of hellbender breeding behavior, and it is the first to make observations within nesting cavities rather than outside of them. Our results confirmed some previous observations about hellbender breeding behavior and identified novel behaviors and mating strategies that had not been previously described. These findings shed light on the evolution of hellbender life history and offer considerations for future conservation management of the species.

One of the new behaviors that we identified in our filming was the behavior we called threading, where during and in some cases continuing after oviposition, females wove in and out of the clutch, resulting in a knotted mass of eggs. Although clutch morphology in other species of Cryptobranchids (*Andrias*) has not been explicitly described, accounts of the females rotating (Luo et al. 2018) or spinning (Kuwabara et al. 1989) during oviposition suggest that threading may be a characteristic shared across Cryptobranchidae. We were unable to find any reports of similar clutch morphology in other species of urodeles, suggesting that it may be unique to Cryptobranchids.

There are several possible explanations for threading. One possible explanation is that the knotted clutch enables more effective management of the eggs during parental care and/or reduces the likelihood that loose strands caught by the stream's current pull the clutch out of the nest cavity, which is known to occur in some instances (O'Brien 2023). This would represent a novel solution to clutch management and an alternative to attaching the eggs to substrate as is seen in many other aquatic amphibians (Altig and McDiarmid 2007). It is also possible that threading improves fertilization success. Hellbenders fertilize externally, so weaving in and out of the clutch may help disperse milt and ensure that the entire clutch is fertilized.

Another possibility is that threading behavior spreads antimicrobial skin bacteria or skin secretions on the eggs. Skin peptides from hellbenders have been found to inhibit bacterial growth (Pereira et al. 2023) and other amphibian species have also been found to produce antifungal compounds and/or host cutaneous bacteria that could reduce egg infection (Austin 2000; Lauer et al. 2007; Banning et al. 2008). Weaving in and out of the eggs may spread these beneficial compounds and/or bacteria. If this explanation for threading is true, it has implications for captive rearing because in-vitro fertilization (IVF) would deprive eggs of these compounds and could adversely affect offspring survival and health. This may be one reason why eggs collected for IVF have a lower survival rate than eggs that have been raised under the father's care (D. McGinnity, personal communication; McGinnity et al. 2021). However, there are additional parental care behaviors that are absent with IVF that likely also play a role in reduced survival compared with eggs reared by occupying males (Settle et al. 2018; Unger et al. 2020). Additionally, if threading spreads beneficial bacteria, this suggests that ongoing efforts to maintain the skin microbiome in captive hellbender breeding stock are highly valuable (Hernández-Gómez et al. 2019; Kenison et al. 2020; D. McGinnity, personal communication).

Another novel behavior that we observed was the quivering motion, which is likely some form of signal given the response it elicited from occupying males in some shelters. The sensory mode that quivering appeals to is difficult to determine without further study. It may be that quivering produces a wave-based signal (we use wave-based to encompass both vibrational and acoustic signal possibilities because of the lack of clarity in distinguishing the two [Hill 2008]). Hellbenders have been found to sense vibrations via their jaws and lateral lines, but little is known about their signal production (Nickerson and Mays 1973). Wave-based communication and the sensing of vibrations via the lateral line has been identified in Hynobiidae (Tanaka 1986, 1989; Usuda 1995; Park et al. 1996, 2008; Kim et al. 2009). However, these waves are produced through body undulations or tail waving rather than the quivering that we observed. Acoustic communication has also been identified in the closely related *Andrias davidianus* (Evans and Ding 2005), but how the sound is produced is unclear. Evans and Ding (2005) noted that it is sometimes accompanied by bubble release, suggesting that the lungs may be involved. We never observed bubble production during quivering in our recordings, but the potential involvement of the lungs in this signaling is intriguing because hellbenders primarily respire cutaneously, and our understanding of the function of their lungs remains poor (Guimond and Hutchison 1973; Coe et al. 2016). An interesting possibility is that hellbenders are drumming against their lungs in a manner similar to the way fish drum against the swim bladder to produce sound (Jones and Marshall 1953). However, this possibility remains highly speculative and would need further investigation.

An alternative possibility to sound production is that quivering is related to pheromone production. Previous research has suggested that olfactory signals may be utilized by hellbenders during breeding season (Peterson 1988), and in the aquatic newt, *Cynops pyrrhogaster*, males release a pheromone and then waft it toward the female using tail vibration (Kikuyama and Toyoda 1999). Something similar could be occurring in hellbenders with the quivering serving to release or propel the chemical signal away from the skin. It is unlikely that the quivering is purely a tactile signal because it was often produced without physical contact between animals.

Although multiple males have previously been observed entering nests during breeding (Smith 1907; Unger et al.

2020), this is the first study to provide evidence of possible alternative reproductive tactics in hellbenders. In most breeding events, we observed the presence of one to several intruding individuals in the shelter during mating. Although we did not capture any of the intruders to confirm their sex, we have, in previous field seasons, captured up to four males with swollen cloacae (indicating sexual readiness) in a single shelter with an ovipositing female. Additionally, our observations of intruder behavior suggest the intruding individuals are likely males employing an alternative mating tactic. These individuals likely represent a satellite mating strategy that has been documented in Japanese Giant Salamanders (*Andrias japonicus*) (Kuwabara et al. 1989; Kawamichi and Ueda 1998; Okada et al. 2015 and untranslated citations therein) as well as in some territory-guarding species of Hynobiidae (Hasumi 2015).

Satellite males are submissive individuals that, rather than defending a territory and/or attracting females on their own, exploit the mating events of larger males by sneaking fertilizations (Dunbar 1982; Taborsky et al. 1987). Consistent with a satellite mating strategy, the intruders we observed were typically much smaller than the occupying male. Given the strong relationship between age and size (Taber et al. 1975), it is likely that satellites are young individuals that may not yet be large enough to defend their own nesting cavity. However, we also observed some successful males that were quite small, so what determines whether a male becomes a satellite or an occupying male may be a matter of nest cavity availability and competition among males. The tolerance that occupying males displayed toward intruders suggests that the risk of lost fertilization opportunities as a result of intruders does not justify the cost of defending the nest. Given the likely young age of the intruders and our limited observations of obvious sperm release by them, fertilization by intruders may not always occur. Additionally, there may be a high cost to the occupying male of driving the intruders out such as physical injury, energy loss, disruption of mating, and/or the risk of inadvertently pushing the clutch out of the nest cavity into the stream current. Taborsky (2001) identifies three characteristics that support the evolution of satellite mating in fish, including indeterminate growth, the prevalence of external fertilization, and paternal investment, all three of which are present in Cryptobranchidae.

The criteria that females used for mate choice were not clear from our observations, and we did not find evidence of highly stereotyped courtship rituals. However, there were some behaviors that individuals exhibited across nearly all breeding events. For example, in all breeding events, the occupying male crawled under and around the visiting female and nudged her belly in a way similar to that described in *Andrias* (Kuwabara et al. 1989). Periodically during this crawling, the occupying male rested under the female's chin and torso while she quivered, which may have been an opportunity for him to more strongly sense her signaling (Nickerson and Mays 1973). We also observed bite-and-hold behaviors in nearly all breeding interactions. Biting of gravid females by males has previously been seen in *Andrias* (Kuwabara et al. 1989; Luo et al. 2018). However, previous hellbender research has identified aggressive interactions primarily between males (Unger et al. 2020) or between females (Settle et al. 2018) rather than between sexes, likely because these recordings were taken outside of the nest. Successful males held the bites for longer on average than did unsuccessful males, so it is possible that biting plays some role in sexual selection, but further research is still needed to determine whether hold time plays a role in female choice. We observed touching noses in about a quarter of interactions between occupying males and females, but neither this behavior, nor tail swishing (which occurred in over half of interactions) seemed related to mating success. Still, it cannot be ruled out given our small sample size.

Males cleaned their nests in all recorded shelters, but the exact purpose of this behavior and whether it was subject to sexual selection remained unclear. Terry et al. (2018) suggested that this behavior in Japanese Giant Salamanders might serve primarily to enlarge and shape the nesting cavity or to reduce the threat of water mold to eggs by minimizing the amount of other substrate for growth. Both of these possibilities would be expected to make clean nests more attractive to potential mates, but we did not detect any relationship between time spent cleaning and mating success with our small sample sizes. However, if cavity enlargement due to cleaning is a factor in mate choice, the artificial nature of our nesting cavities could remove or minimize the importance of this variable from female choice, meaning we might only be able to detect this effect in natural cavities. It could also suggest a possible unintended effect of artificial shelter design and installation, particularly if nest maintenance is an indicator of other aspects of male quality. An alternative explanation is that the piles of sediment that accumulate outside of the tunnel as a result of cleaning might help deter intruders by narrowing the nest entrance and making it easier to defend (Svensson and Kvarnemo 2003; Unger et al. 2020). Given how far in advance of breeding we recorded nest cleaning in anecdotal observations, it is also possible that this behavior does not have a purpose related to mating and is simply normal shelter cavity maintenance. Future video recordings at other times of year and of nonnesting individuals could help eliminate this possibility.

Our observations suggest that hellbenders potentially engage in mate choice copying and comparative mate evaluation (Pruett-Jones 1992; Bateson and Healy 2005). In the shelter where we recorded multiple clutches being deposited, each subsequent ovipositing female spent less time in the nest and spent a smaller percentage of the time present quivering. The occupying male also only substantially bit the first female to enter. These differences in behavior suggest that the females may have been relying in part on the presence of eggs to enable a faster assessment of the male's quality. Another finding was that in several nests we recorded females entering the same nest multiple times before ovipositing. Although re-entry into a nesting cavity by the same female has been observed in *Andrias* (Kawamichi and Ueda 1998), this study was, to our knowledge, the first confirmation that re-entry by females also occurs in hellbenders. In some instances we began recording only shortly before oviposition began; therefore, it is possible that this behavior also occurs more frequently than we recorded. The existence of re-entry suggests that the females were evaluating potential mates and possibly comparing them with alternative choices. When deciding between males that vary in more than one characteristic, the available choices can influence which characteristics the female emphasizes in making her selection (Sedikides et al. 1999). If comparative evaluation is taking place in hellbenders, the choices that females make may vary depending on the quality and quantity of alternative males (Bateson and Healy 2005). This may highlight a new implication for declining hellbender populations and health. Females may not be able to make many comparisons if populations are too small, or they may be comparing males that differ in ways that are not typical of a healthy population. This could cause changes in how females evaluate potential mates or which characteristics they emphasize in making a choice (Bateson and Healy 2005), and suggests there may be a minimum population size at which optimal mate choice decisions can be made. In captive populations, comparative mate choice suggests that providing females with several potential mates (as done by Settle et al. 2018) is beneficial and that care should be taken in selecting these potential mates.

Future Directions

Although this study has deepened our understanding of hellbender behavior, there is much that remains to be discovered. Further investigations into the significance of threading and quivering, as well as the specific characteristics used in selection of mates would be exciting next steps that would further our understanding of hellbender natural history and promote conservation efforts such as captive rearing. Confirmation of our observations in a natural nest to remove any self-selection bias (Webster and Rutz 2020) would also be an exciting, although challenging, future study. By continuing to advance our knowledge of hellbender breeding ecology, we can improve our understanding both of their evolutionary history and the future conservation of this remarkable species.

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SUPPLEMENTAL MATERIAL

Supplemental material associated with this article can be found online at https://doi.org/10.1655/HERPETOLOGICA-D-23-00002.S1; https://doi.org/10.1655/HERPETOLOGICA-D-23-00002.S2; https://doi.org/10.1655/HERPETOLOGICA-D-23-00002.SV1; https://doi.org/10.1655/HERPETOLOGICA-D-23-00002.SV2; https://doi.org/10.1655/HERPETOLOGICA-D-23-00002.SV3; https://doi.org/10.1655/HERPETOLOGICA-D-23-00002.SV4; https://doi.org/10.1655/HERPETOLOGICA-D-23-00002.SV5; and https://doi.org/10.1655/HERPETOLOGICA-D-23-00002.SV6.

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