In-vitro Fertilization and Embryology of the Mountain Brook Lamprey

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Abstract: The mountain brook lamprey (*Ichthyomyzon greeleyi*) has no previous embryological description even though the lamprey's primitive developmental patterns are of value to evolutionary biologists. This research describes visible developmental processes of the mountain brook lamprey from the unfertilized ovulated egg to the release of ammocoete larvae into the field. In-vitro fertilization was the process used to produce viable embryos after strip spawning. This process successfully produced about 4000 embryos for descriptive observation. Embryological and larval developmental stages were named using criteria defined for *Lampetra reissneri*. The findings conclude that the timing of early developmental events is similar to that of other lamprey species.

Key words: embryology, in-vitro, lamprey, larvae, strip spawning

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The lamprey is an agnathan (jawless vertebrate) belonging to the order Petromyzontida. Agnathans are basal vertebrates and possess a number of primitive characteristics of interest to evolutionary biologists. The study of these organisms can increase our understanding of primitive vertebrate developmental patterns and the evolution of vertebrates (Kuratani et al. 2001).

The Mountain brook lamprey (*Ichthyomyzon greeleyi*) is a non-parasitic lamprey that inhabits small upland rivers and creeks (Potter and Bailey 1972). Degenerate teeth in the buccal cavity support evidence of their non-parasitic lifestyle. The mountain brook lamprey is characterized by an eel-like body that is devoid of scales and grows to a length of approximately 20 cm. The larva lives a sedentary life submersed in lower gradient stream substrate, composed of sand and organic debris, for a period of 5.2–6.2 years (Potter and Bailey 1972). They filter particulate matter, for nourishment, by exposing the oral hood to water currents (Potter and Bailey 1972). Adults do not feed, and they perish after reproduction.

Metamorphosis to the adult stage is initiated in mid-August and completed by mid-December (Beamish 1982). Upon completion of metamorphosis, the adults swim upstream to higher gradient substrates to build nests. The mountain brook lamprey initiates spawning between late April and early June in streams composed of clean, clear water flowing over cobble and gravel substrate (Hubbs and Trautman 1939). Little more is known about their life history even though this species is listed as threatened (Kentucky State Nature Preserves Commission 2004).

It is hypothesized that the parasitic lamprey (*Ichthyomyzon bdellium*) gave rise to the species mountain brook lamprey (Vladykov and Kott 1979). Additionally, a study recently proposed that "a gene expression in the lamprey mandibular arch was a key event facilitating the evolution of jaws in gnathostomes" (Cohn 2002). Therefore, study of the non-parasitic lamprey species mountain brook lamprey may aid in the evolutionary study of gnathostomes (jawed vertebrates).

The purpose of the mountain brook lamprey in-vitro fertilization and embryology study is twofold. First, to determine if propagation methods used by Bayer et al. (2001) on the western brook lamprey (*Lampetra richardsoni*) can be used to study early life history stages of the mountain brook lamprey. Second, to provide presently unpublished information about the embryology of the mountain brook lamprey and to create a base line for future research and potential recovery efforts.

Study Area

In 2005, a number of adult mountain brook lampreys were discovered in Russell Creek, Adair County, Kentucky. Russell Creek is a small waterway that feeds into a larger stream and is surrounded by a natural wooded area (3704785N, 8518681W). The lampreys were collected near or under flat stones measuring approximately 15–30 cm in diameter, in close proximity to their spawning sites. Spawning sites include flat stones for mating and nests are built downstream of the stones. Spawning occurred in a stretch of shallow choppy water (a riffle), which measured about 100 m^2.

Methods

Propagation of the mountain brook lamprey samples followed the methods used by Bayer et al. (2001), with minor, lab specific,

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adaptations. Deviations included the use of methylene blue to reduce mold and bottled water (Crystal Geyser Natural Alpine Spring Water) to prevent copapod contamination from stream water. The brand Crystal Geyser was used because the Ca^{++} content of 40 mg L^{-1} was similar to the river Ca^{++} range of 28–45 mg L^{-1}. Developmental stages targeted were those listed by Tahara: 1-cell, 8-cell, morula, blastula, gastrula, neural plate, head protrusion, hatching, melanophore, eye spots, gall bladder, and completion of digestive tract (Tahara 1988).

Nine adult lampreys were collected from Russell Creek by seine in mid-April 2005. The lampreys were transported in a cooler equipped with airflow, to the Campbellsville University Stream Lab where they were placed in one communal 70-L aquarium. Water for the aquarium was retrieved from the lamprey collection site and maintained at ambient room temperature (17–17.9 C). Timer controlled incandescent lights were used to simulate natural photoperiod. Lighting exposure at sunrise with dim lights from 0700–1000 hours, middle of the day with full lighting from 1000–1400 hours, dim lights through sun set from 1400–2000 hours, and all lighting terminated from 2000–0700 hours.

Two days after collection, external morphological changes were observed in the males with the urogenital papillae protruding from the urogenital pore. Lampreys were then segregated by gender and held in two separate 70-L aquaria until they exhibited nest-building behaviors by both males and females. Nesting behavior includes moving large rocks with the oral disk, and flagellating their bodies to rake gravel and sand substratum while attached to solid objects by their oral disk. At maturation, the adults were anesthetized for strip spawning of gametes into a single collection container and when fertilization was verified, the combined egg and milt solution were separated into smaller containers (Bayer et al. 2001).

Two males and two females were used in this study. Mature lampreys were moved to a dissection tray and anesthetized in 1000 ml solution of tricaine methanesulfonate (MS–222), concentration of 65 mg L^{-1}, until they were unresponsive to stimulations. Lampreys were rinsed with fresh water for one minute to remove anesthetic. Eggs were stripped first by squeezing the abdomen down toward the urogenital pore, and into a 5-cm petri dish pre-filled with spring water (Figure 1). The process was repeated until no further gametes were expelled and a trace amount of blood was present. Milt from the males was obtained in the same manner. Gametes from a single female and male were stripped into an individual petri dish. A combined total of 3899 eggs were produced from the two females. Adult lampreys were returned to one communal aquarium where they engaged in polygamous mating until they died from natural causes.

Eggs and milt were mixed by placing the petri dishes on an orbital shaker for a minimum of 30 min. After fertilization was verified by presence of the polar spot in the animal hemisphere, zygotes were separated into 40 5-cm petri dishes (~100 specimens per dish).

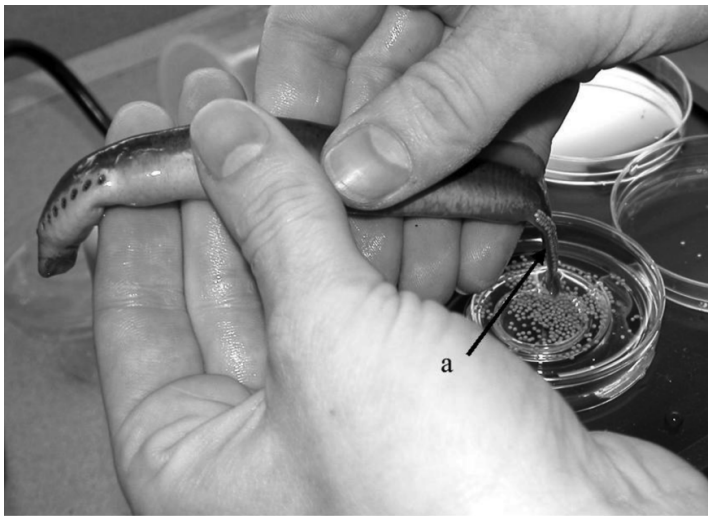

Figure 1. Strip spawning of gametes from the female into a 5-cm petri dish. A. ribbon effect of the eggs due to a sticky residue that coats the eggs.

Water was replenished daily in each petri dish by adding fresh spring water and three drops of methylene blue 0.3 mg L^{-1} for mold control. The concentration of methylene blue was increased to 0.4 mg L^{-1} eight days after fertilization and maintained through the duration of the study. The temperature of the water was kept ambient with room temperature, which fluctuated day and night (16.1–18.9 C). Embryos were exposed to indirect photoperiod lighting and aeration was not provided. Inspections were performed twice daily to check for live, dead, and abnormal individuals. When dead individuals were identified, they were removed with a wide mouthed pipette and abnormal individuals were isolated for further observation.

After hatching, larvae were placed in two 3-L plastic storage containers lined with clean sand and filled with fresh spring water to mimic natural conditions. The containers were placed on the counter for indirect exposure to photoperiod lighting and tubes were placed at either end to provided aeration. Water level was maintained by adding fresh spring water every four to five days. Two full droppers of methylene blue (0.4 mg L^{-1}) were added when water was replenished to prevent mold. Water temperature remained ambient with room temperature (16.1–18.9 C). Larvae were fed a yeast solution (~4 g L^{-1} yeast to warm water) every three days.

Larval development was observed and recorded using a micro video camera and video cassette recorder (VCR). Observation oc-

curred every 30 min after fertilization until the first cell cleavage at four hours, then samples were taken at the following hours: on the hour from hours 5–12, every two hours at hour 12–32, every four hours from hour 32–60, and then at 72 hours. After 72 h, samples were taken once a day. Observations continued until ammocoetes were released downstream from adult collection site. All equipment surfaces in direct contact with sample specimen were sterilized in a solution of chlorine bleach and bottled spring water (Crystal Geyser), concentration rate of 62.5 ml L^{-1}.

Results

Ovulated but unfertilized eggs were creamy white in color, spherical in shape, and the outer edge (chorion) measured 1 mm in diameter. Fertilization took place within 15 min observed by the presence of a dimple-like polar spot at the animal hemisphere and fluid perivitelline membrane surrounding the zygote (Figure 2A). The first cell division (cleavage) took place four hours after fertilization, increasing the chorion diameter to 2 mm (Figure 2B). The second cleavage evenly divided the two cells to create four equally sized cells (Figure 2C). Cell division was holoblastic, where the entire egg separated into individual blastomeres, until the morula stage which occurred in the sixth cleavage at hour 16. Developing yoke in the vegetal pole marked the beginning of the meroblastic cell division stage.

During blastulation, at hour 23, the animal hemisphere smoothed out, a blastocoel appeared as a fluid-filled translucent membrane, and the fertilization germ ring migrated across the equator (Figure 2D). At hour 56, gastrulation began, the blastopore enlarged, and the anterior lip flattened (Figure 2E).

Neurulation began at day 4 with the formation of a groove in the dorsal mid-line of the neural plate (Figure 2F). At day 5, the neural folds fused and the anterior end began to protrude. Mid-neurulation, at day 7, presented head protrusion (Figure 2G). During the same period, development caused the gut to expand. At the conclusion of neurulation on day 8, the head demonstrated a hook shape, the pharyngeal arches fused at the ventral mid-line, and muscle myomeres were visible.

Larval head movement, visceral pouches, and the pericardial cavity were visible on the ninth day. Sporadic heartbeats and development of the upper coelum, optic placodes, and liver were noted on day 11.

The appearance of hemoglobin occurred on day 12, allowing observation of active inflow to the heart ventricle and a regular heartbeat. The tail bud and esophagus were also visible at this time.

Hatching began at day 22 with all visceral pouches, gill slits, eyespots, and melanophores present. During the hatching stage, the oral hood and velum were present and gill movement was observed (Figure 2H). Hatching was complete on day 27. Following the hatching phase, the gallbladder appeared as a small, round, blue spot near the pericardium at day 28. Until burrowing began six days after hatching, larvae were seen actively swimming and resting on the substratum. On day 38, the final stage was observed and marked by complete resorption of yolk. Total average length, at this time, was ~8.91 mm ($n = 10$).

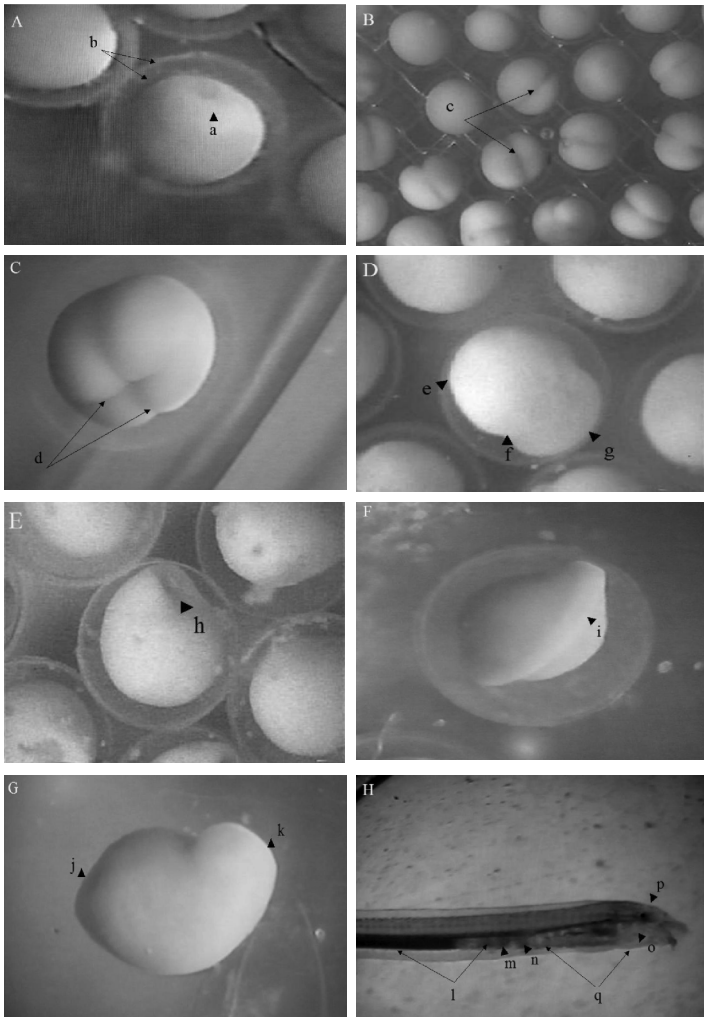

Figure 2. *I. greeleyi* embryonic development noted, in parentheses, by time in minutes, hours, and days after fertilization. A: (15 min) fertilized egg, B: (hour 4) two-cell stage, C: (hour 6) four-cell stage, D: (hour 23) blastula stage with germ ring at the equator, E: (hour 56) gastrula stage, F: (day 5) neurula stage, G: (day 7) mid-neurula stage, H: (day 27) post-hatching stage. a: polar spot, b: perivitalline membrane, c: first cleavage furrow, d: two cleavage furrows, e: animal hemisphere, f: germ ring, g: blastocoel, h: blastopore, i: neural groove at the dorsal mid-line, j: blastopore, k: head protrusion, l: yolk sac, m: liver, n: pericardial cavity, o: first gill pore, p: pigmented retina, q: pharynx.

Discussion

The primary developmental stages of the mountain brook lamprey were named according to stages listed for *Lampetra reissneri* (Tahara 1988). However, to gain a more clear insight to the 12 de-

Table 1. Developmental comparison among *P. marinus, L. reissneri,* and *I. greeleyi*

Developmental phases	*P marinus*[a]	*L. reissneri*[b]	*I. greeleyi*	Standard deviation
2-cell	2 h	6.5 h	4 h	2.3 h
8-cell	10 h	15.5 h	9 h	3.5 h
Morula	19 h	28 h	16 h	6.2 h
Blastula	24 h	48 h	23 h	14.2 h
Gastrula	64 h	78 h	56 h	11.1 h
Neural plate	4 d	5 d	4.5 d	.5 d
Head protrusion	6 d	7.5 d	6 d	0.9 d
Hatching	10 d	11 d	22 d	6.7 d
Melanophore	13 d	16 d	14 d	1.5 d
Eye spots	15 d	18 d	18 d	1.7 d
Gall bladder	17 d	24 d	28 d	5.6 d
Completion of digestion tract	33 d	31 d	38 d	3.6 d
Temperature	18.4 C	15 C	16.1 C–18.9 C	

a. Paivis 1971
b. Tahara 1988

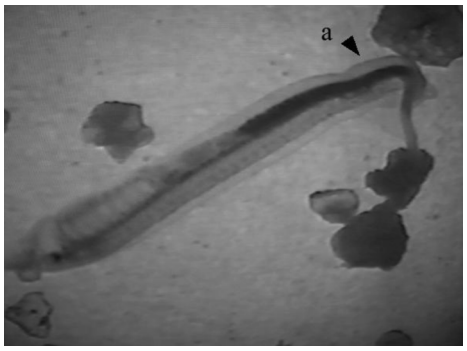

Figure 3. Day 23. This animal was manually removed from its chorion. Though this individual had a structural tail deformity it was capable of swimming a: deformity appearing as a hook shape.

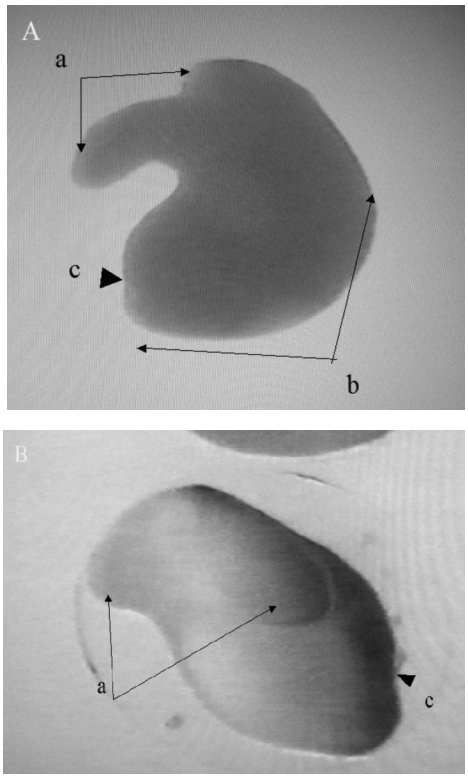

Figure (4A, B). This 39-day-old animal developed with two heads. Neural fold fusion extended from posterior end of the animal to the distal end of the pericardial cavity. Organogenesis differed from healthy specimens with only minimal organ development of the pericardial cavity, esophagus, and visceral pouches. a: two heads, b: area of connection, c: blastopore.

velopmental stages in the mountain brook lamprey, comparisons were made both with *L. reissneri* and the sea lamprey (*Petromyzon marinus)* (Piavis 1971) (Table 1).

These comparisons revealed some developmental variations between the species. At the first cell division, the mountain brook lamprey developed slower than the sea lamprey by two hours, and then developed faster until protrusion of the head, which occur at the same time. The mountain brook lamprey developed faster than *L. reissneri* until development of eye spots, which happened at the same time, then developed slower in the final two stages. The sea lamprey developed at a faster rate than *L. reissneri* until the final stage. For the 12 documented developmental phases, progression was most similar between the mountain brook lamprey and the sea lamprey.

A critical factor to consider when comparing developmental variation among species

is water temperature (Tahara 1988). There were notable differences between rearing temperatures in the species; sea lamprey (18.4 C) (Piavis 1971), *L. reissneri* (15 C) (Tahara 1988), and mountain brook lamprey (not controlled but constant between 16.1–18.9 C). In a separate study, five species of lampreys were reared at a constant temperature and developed with time variations both between species and within each species (Smith et al. 1968). This is evidence that variations are not dependent solely on temperature. Regardless to the temperature variances, early development among the species appeared to progress along a similar rate with minimal deviations and one exception in hatching. The cause of the one extremely slower hatching time in the mountain brook lamprey is unknown.

In addition to later hatching times for mountain brook lamprey, defects in larval body structure were observed. About 100 individuals developed deformities in their tails that did not prevent mobility; more than 50% corrected themselves within a few days (Figure 3). One individual developed two heads, and lived with full movement of both heads until day 45 when it died and was preserved in 4% paraformalin solution (Figure 4 A, B). It is hypothesized that the deviations of bottled water and addition of methylene blue may be the cause of these defects.

The methodology used for in-vitro fertilization and rearing described by Bayer (2001) allowed for a broad observation of embryo and early larval development in the mountain brook lamprey, previously unavailable for this species. Successful application of in-vitro fertilization illustrates potential use of this technique for

further study in early developmental stages and recovery efforts within both this and similar species.

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