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First record of polyembryony in Hellbenders, *Cryptobranchus alleganiensis alleganiensis* (Caudata: Cryptobranchidae)

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Many caudates breed in microhabitats that are difficult to access for researchers, such as under large boulders and logs. Due to the secretive life histories of some salamanders, their egg clutches are not as frequently studied as more conspicuous breeders (e.g., pond breeding amphibians). Therefore, knowledge about the frequency of polyembryony (twinning) from caudates in the wild are scarce. Twinning in caudates has been documented three times. Once in *Ambystoma tigrinum* Green, 1825, where three out of 7846 eggs were polyembryonic (Lindberg, 1995). Another observation was recorded by Hamed et al. (2015) of one clutch with one polyembryonic egg in *Hemidactylium scutatum* Temminck & Schlegel, 1838. The last observation was recorded in two clutches of *A. maculatum* Shaw, 1802, one of which had more than eight and one of which had more than five polyembryonic eggs (McDaniels et al., 2020).

Polyembryony has not been documented in *Cryptobranchidae*. Herein we report, to the best of our knowledge, the first observations of polyembryony in *Cryptobranchidae* and specifically in the Eastern hellbender, *Cryptobranchus alleganiensis alleganiensis* (Daudin, 1803). We took photographs of 87 clutches of eggs laid in artificial shelters in 2019 and 2020. Underwater artificial shelters used in our study system are made from concrete and consist of a rectangular-shaped chamber that hellbenders can access through a single tunnel entrance (Button et al., 2020 a, b). The chamber can be accessed via a lid for monitoring occupancy and nesting by hellbenders. Each clutch was temporarily removed from the artificial shelter, placed in a sterile container with a small volume of water, and gently compressed with a transparent sheet of plexiglass. This resulted in a more homogeneous distribution of eggs which we photographed. We then visually checked each egg in each photograph for polyembryony. Seven out of 87 nests (8.05%) contained ≥1 polyembryonic egg (Fig. 1A). In total, this represented 46 out of the 26,369 eggs (0.17%) that we visually examined. Of the seven clutches that had polyembryonic eggs, the average number of twins per clutch was 6.6 ± 3.79 SE (range 1 – 27 twins per clutch). Based on visual inspection, the polyembryonic eggs were developing at the same rate as monoembryonic eggs at approximately day 29 after oviposition (see Fig. 1B). Visual inspection indicated that all polyembryonic eggs had their own section within a single shared external egg capsule divided by a vitelline membrane. This is in line with observations of *A. maculatum* (McDaniels et al., 2020). On one occasion the vitelline membrane separation was absent and the *C. a. alleganiensis* twins were conjoined; both twins were still showing signs of proper development 32 days after oviposition. Whether these twins ultimately hatch and produce viable offspring in the wild remains unknown.

While this is only the fourth report of polyembryony in

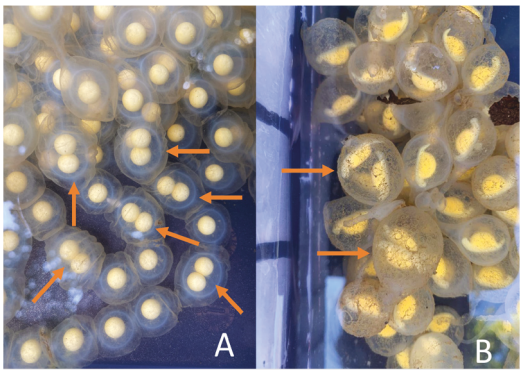

Figure 1. (A) Arrows indicating polyembryonic eggs at approximately 5 days post oviposition, and (B) eggs at approximately 29 days post oviposition. Photos by J. Groffen (A) and E. Fralin (B).

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caudates, it is possibly more common than publications indicate. Further work is needed to determine whether polyembryonic eggs are viable or if this represents a deleterious developmental abnormality.

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