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Preliminary Evidence that American Bullfrogs (*Rana catesbeiana*) Are Suitable Hosts for *Escherichia coli* O157:H7[▽]

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We orally inoculated *Rana catesbeiana* tadpoles ($n = 23$) and metamorphs ($n = 24$) to test their suitability as hosts for *Escherichia coli* O157:H7. Tadpoles were housed in flowthrough aquaria and did not become infected. Metamorphs were housed in stagnant aquaria, and 54% tested positive through 14 days postinoculation, suggesting that they are suitable hosts for *E. coli* O157:H7.

Escherichia coli O157:H7 is a major food safety concern, because it produces Shiga toxin, which causes hemolytic uremic syndrome in humans (12). Cattle are known reservoirs of *E. coli* O157:H7, and there is considerable effort by cattle farmers and the food safety industry to minimize its prevalence in herds (3, 14, 15, 23, 25). If infected cattle defecate in water sources (e.g., farm ponds), transmission to herd members (23) and exposure to aquatic vertebrates, such as amphibians, are possible. Moreover, considering that amphibians become infected by other strains of *E. coli* (2, 13), those that inhabit farm ponds and wetlands where cattle defecate may serve as a "spillover" reservoir (sensu Daszak et al. [4]) for the pathogenic *E. coli* serotype O157:H7. If *E. coli* O157:H7 is capable of infecting tadpoles, then the pathogen could be shed in their feces and infect cattle that drink contaminated water. Further, tadpoles metamorphose and may be capable of dispersing up to 1 km from natal ponds (22); thus, tadpoles could serve as overland vectors of *E. coli* O157:H7. Water contaminated with *E. coli* O157:H7 by tadpoles or metamorphs also could be a source of infection for vegetables if the water is used for irrigation. Lastly, humans consume bullfrogs (5); thus, direct transmission of *E. coli* O157:H7 to humans is possible through consumption of undercooked frog legs if this frog species is a suitable host. Ultimately, this pathogen could be transmitted to humans through consumption of meat or vegetables, especially if these food products are uncooked or undercooked.

Therefore, the objective of this pilot investigation was to determine if American bullfrog (*Rana catesbeiana*) tadpoles and metamorphs are suitable hosts for *E. coli* O157:H7. We chose American bullfrogs as our model species because they are native to the eastern United States (6), have been introduced into wetlands in the western United States (7, 17), and are shipped globally as a food product (5). In addition, we investigated bullfrog tadpoles because they usually develop for >1 year (6), resulting in overlapping cohorts, and thus could hypothetically maintain *E. coli* O157:H7 continuously in an aquatic system through pathogen shedding and infection of new individuals. Metamorphs were investigated because they are typically the primary dispersing unit in a population (21) and hence are the age class most likely to transport a pathogen overland to a naïve aquatic system.

American bullfrog tadpoles (at Gosner [10] stages 31 to 41) were collected from two wetlands in eastern Tennessee and housed at the Joe Johnson Animal Research and Teaching Unit on the University of Tennessee—Knoxville campus. All tadpoles were screened for *E. coli* O157:H7 by fecal testing, acclimated for 14 days prior to inoculation, and randomly assigned to two treatment groups: tadpoles and metamorphs. Each tadpole in the tadpole group ($n = 23$) was randomly assigned to a 38-liter aquarium that was independently connected to a closed, dechlorinated flowthrough system. Water turnover in aquaria was approximately once per hour. Each tadpole in the metamorph group ($n = 24$) was randomly assigned to an individual 10-liter aquarium with floating platforms that allowed individuals to emerge from the water following metamorphosis. Metamorph tanks were not connected to the flowthrough system, because this system maintains high water quality and could have delayed metamorphosis. Addition of 3,5,3′-triiodothyronine (T3; Sigma-Aldrich, St. Louis, MO) to aquarium water (6 ppb) was necessary to induce metamorphosis (9) in three tadpoles.

The water temperatures in all aquaria were maintained between 26 and 28°C, which is similar to water temperatures in Tennessee ponds during summer (M. J. Gray and A. C. Schmutzer, unpublished data). All animals were exposed to a 12/12-h light/dark cycle (18) and fed commercial feed once per day. Metamorphs were switched to one cricket and mealworm after their mandibles developed. Air was supplied to water in all tanks at a constant rate and diffused with air stones.

Inoculation of tadpoles and metamorphs occurred on the same day. The animals were netted from their tanks, orally inoculated with 1.18×10^6 CFU of *E. coli* O157:H7 (ATCC 35150) using a pipette with disposable tips, and returned to their original tanks. During inoculations, we performed a 100% water change in the metamorph tanks.

Tadpole fecal samples were collected on days 1, 3, 5, 7, 14, 21, and 28 postinoculation (p.i.) to document *E. coli* O157:H7

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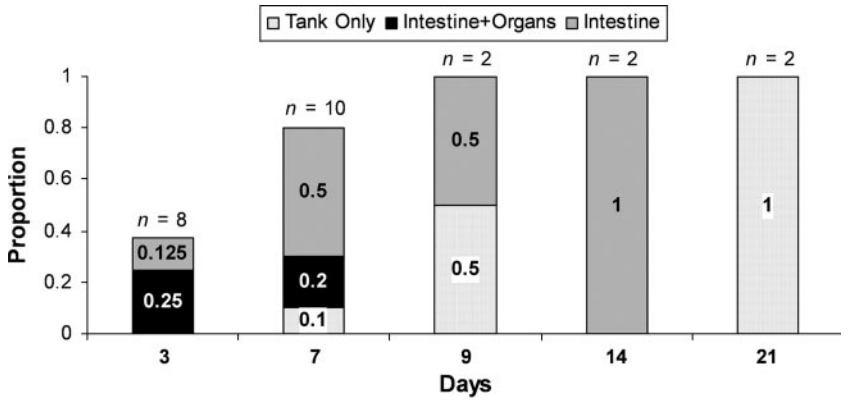

FIG. 1. Proportions of *E. coli* O157:H7-positive tank water, intestine, and intestine and organ homogenate samples for American bullfrog (*Rana catesbeiana*) metamorphs euthanized at 3, 7, 9, 14 and 21 days p.i. The organ homogenates were prepared using spleen, liver, heart, and kidney.

shedding. Each tadpole was rinsed with sterile water and placed in a jar containing 250 ml sterile water for 3 h, after which 20 ml of a feces-water solution was collected. The tadpoles were euthanized when they metamorphosed or after 28 days p.i. to test whether organs were infected. Three, nine, four, three, and three tadpoles metamorphosed and were euthanized on days 3, 7, 9, 14, and 21 p.i., respectively. Only one tadpole did not metamorphose within 28 days p.i. Metamorph feces were not collected, because defecation was minimal due to the restructuring of the digestive system during metamorphosis (24). Metamorphs were euthanized following complete tail resorption, which was considered the point at which they became juvenile froglets and were capable of dispersal (8). On days 3, 7, 9, 14, and 21 p.i., we euthanized 8, 10, 2, 2, and 2 metamorphs, respectively. Also, on the day of euthanasia, a 20-ml water sample was collected from the respective metamorph tank to document *E. coli* O157:H7 shedding. At 28 days p.i., four uninoculated control tadpoles and metamorphs were euthanized. Each individual was euthanized in a separate sterile container by immersion in a benzocaine (250-mg/liter) water bath (1) and then shipped on ice overnight to the University of Georgia Veterinary Diagnostic and Investigational Laboratory for *E. coli* O157:H7 testing. All captive husbandry and euthanasia procedures followed an approved University of Tennessee IACUC protocol (no. 1571-0806).

Intestines, spleen, liver, heart, and kidneys were collected from each individual. An organ homogenate was manually prepared from the last four organs, and it was processed separately from the intestines to determine the extent of *E. coli* O157:H7 colonization. Samples were homogenized in 500 μl of sterile distilled water. Fifty-microliter aliquots of each sample were inoculated into a MacConkey agar plate and incubated overnight at 37°C in an aerobic incubator. All lactose-positive colonies were subcultured into MacConkey agar-sorbitol plates and incubated overnight. All sorbitol-negative isolates from the MacConkey agar-sorbitol plates were screened using a commercially available latex agglutination kit (Remel, Lenexa, KS) for confirming the O157:H7 serotype, by first using O157 antiserum and then using H7 antiserum on positive isolates. All O157- and H7-positive colonies were confirmed as *E. coli* O157:H7. Similarly, fecal and tank water samples were centrifuged at 3,000 rpm for 20 min, supernatants were removed, and 50-μl aliquots were tested as described above.

All control animals and all preinoculation fecal samples tested negative for *E. coli* O157:H7. We cultured *E. coli* O157:H7 from fecal samples of four tadpoles. Three of these positive samples were tested at 1 day p.i. and one was tested at 14 days p.i. No positive samples were detected in the intestines or organ homogenates of euthanized tadpoles. The 1-day-p.i. positive results likely represent passage of viable bacteria through the tadpole digestive tract without infection. The 14-day-p.i. positive sample may reflect infectivity, although subsequent fecal, intestine, and organ homogenate samples from this individual were negative. Therefore, it is possible that this individual experienced either a localized or a short-term infection or that this result represented delayed transport of *E. coli* O157:H7 through the digestive system. Similar transport (i.e., 13 days p.i.) of viable organisms without infection has been reported to occur in tadpoles for *Cryptosporidium* (11).

We cultured *E. coli* O157:H7 from tank water, intestine, or organ homogenate samples for 17 euthanized metamorphs. Intestines and organ homogenates were positive for *E. coli* O157:H7 for 37.5, 70, 50, and 100% of metamorphs euthanized on days 3, 7, 9, and 14 p.i., respectively (Fig. 1). The intestines and organ homogenates of the two metamorphs euthanized at 21 days p.i. were not positive (Fig. 1). Tank water samples were positive for five metamorphs (21%), of which only one had positive intestines. All control metamorphs and fecal samples prior to inoculation tested negative for *E. coli* O157:H7. Positive tank water samples provide some evidence that infected American bullfrog metamorphs may be capable of shedding this pathogen. The positive tank water and negative intestine samples may represent pathogen clearing after shedding or false negatives in the intestines. Indeed, our method for recovering *E. coli* O157:H7 (i.e., culturing via a MacConkey plate) was not the most sensitive among available techniques. Future studies using immunomagnetic separation followed by PCR or culture for increasing the likelihood of *E. coli* O157:H7 detection are planned.

We documented infection of intestines or organ homogenates by *E. coli* O157:H7 in 54% of American bullfrog metamorphs and interpret these results to be cases of systemic

infection. In contrast, there were no positive organs in tadpoles. We hypothesize that this difference between age classes may be related to reduced immunocompetence in metamorphs. Amphibian immunocompetence declines during metamorphosis as endogenous cortisol is produced during the restructuring of the immune system (19, 20). Thus, reduced immunocompetence may have facilitated higher infection rates in metamorphs.

Another possibility for the differential infection rates between age classes may be related to the aquatic systems. Tadpoles were housed in a flowthrough system, whereas metamorphs were in stagnant water. Although we did not compare the water quality levels of the systems, it is reasonable to assume that the water quality was lower in metamorph tanks. Lower water quality may induce stress in metamorphs, resulting in immunosuppression. Further experimentation is necessary to determine whether development, water quality, or both were mechanisms driving *E. coli* O157:H7 responses in metamorphs and tadpoles.

Our findings suggest that American bullfrog metamorphs are suitable hosts for *E. coli* O157:H7, though this may be influenced by the type of water system. Inasmuch as habitats used by American bullfrog metamorphs often are stagnant (e.g., farm ponds), a cattle herd member infected with *E. coli* O157:H7 that defecates in an aquatic system may cause infection of metamorphs. Infected metamorphs may continue to release this pathogen, infecting other metamorphs or herd members, and possibly contaminate water sources used for crop irrigation. Additionally, infected metamorphs may transport the pathogen overland to adjacent aquatic systems and herds. It is possible that American bullfrog tadpoles and adults also are suitable hosts for *E. coli* O157:H7 in stagnant aquatic systems, but this remains to be tested.

Cattle, sheep and hogs are usually considered the primary reservoirs of *E. coli* O157:H7 and may release this pathogen into aquatic environments through defecation. The environmental persistence of *E. coli* O157:H7 in pond water outside a host is relatively unexplored (16, 26). However, it has been reported that this organism may survive in river water up to 27 days (16). Thus, persistence of *E. coli* O157:H7 in aquatic environments must be maintained through periodic inputs by primary reservoirs or shedding by suitable wildlife hosts. Our results suggest that American bullfrog metamorphs could function as a "spill-over" reservoir for *E. coli* O157:H7 and thus contribute to its persistence in aquatic environments. Further, given that metamorphs are capable of dispersal, they may have a role in the epidemiology of this pathogen.

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