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# Does the Non-lethal Gastric Lavage Method Affect Subsequent Feeding Behavior in Adult and Larval Plethodontid Stream Salamanders?

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Studies on the diet of vertebrates are critical to understanding their importance to food webs and ecosystems. While diverse approaches are regularly used to assess diets that include lethal and non-lethal techniques, it is necessary to evaluate how they can directly influence a study population, particularly when the methods are relatively invasive. Although diet studies can be lethal (i.e., stomach removal from museum or recently euthanized specimens (Rodriguez-Robles et al. 1999), a variety of non-lethal techniques exist, including fecal analysis (Crovetto et al. 2012), stable-isotope analysis (Fenolio et al. 2007), emetics (Jernejcic 1969), fistulas (Krayukhin 1962), forceps (Wales 1962), gastroscopes (Zweiacker 1972), insertion of tubes (Den Avyle and Roussel 1980), intestinal flushing (Baker and Fraser 1976), and gastric lavage stomach flushing (Seaburg 1957). Non-lethal methods to collect diet samples are preferable to lethal sampling, as they minimize the impacts on local populations (Davic and Welsh 2004). Further, the ideal method should remove all stomach contents, be easy, efficient, and inexpensive, avoid long-term internal trauma, should not

impact subsequent feeding behavior, and be applicable to a wide variety of species (Light 1983).

Gastric lavage is one of the oldest and frequently used non-lethal techniques (Fraser 1976; Legler 1977; Romano et al. 2012). The method of gastric lavage (i.e., stomach flushing) is accomplished by pumping distilled or spring water into the stomach via the insertion of a soft plastic tube. Stomach contents are then flushed out and retained for identification. Gastric lavage has been applied to fish (Foster 1977; Light 1983), freshwater turtles (Legler 1977), squamate reptiles (Legler and Sullivan 1979; Waddle et al. 2009; Nifong et al. 2012), birds (Goldsworthy et al. 2016), small mammals (Kronfeld and Dayan 1998), and amphibians (Fraser 1976; Solé et al. 2005; Anthony et al. 2008; Bondi 2015; Crovetto 2012; Costa 2014; Hutton et al. 2017, 2018, 2019). Gastric lavage has been validated against stomach dissection analysis in both salamanders (Salvidio 1992) and frogs (Patto 1998; Wu et al. 2007). Further, Corvetto et al. (2012) reported limitations of fecal analysis compared to gastric lavage in salamanders, as prey digestion rate is taxa-dependent. Despite wide support, it is uncertain how gastric lavage affects individual health and subsequent feeding behavior after returning to the wild.

Previous amphibian studies have documented mortality and internal injury while attempting gastric lavage. Bondi et al. (2015) observed damage to stomach mucosa in three of 124 flushed salamanders; the authors speculated the damage was from the tubing entering the stomach lining. In an anuran study, researchers reported mortality in eight *Bufo ictericus* and one *Scinax granulatus* from tube-punctured gut or lung lining, which then filled with water after attempted gastric lavage on non-anesthetized individuals; however, mortality only occurred in 9 of 583 animals (Solé et al. 2005). Interestingly, the authors performed gastric lavage on 29 frogs, kept them in captivity, and observed the same individuals consuming offered termites two hours after the procedure (Solé et al. 2005).

To our knowledge, no studies have assessed the potential impacts of gastric lavage on subsequent feeding behavior in the wild. As amphibian species continue to decline globally, it is imperative to both consider and understand the possible repercussions of research methods on a population. In this study, we examined the potential effects of non-lethal gastric lavage method on subsequent feeding behavior in adult and larval plethodontid stream salamanders. We hypothesized that there would be no effect of gastric lavage and anesthetization on the subsequent ability of larval and adult stream salamanders to obtain prey items.

## Materials and Methods

*Study Sites.*—Our study sites consisted of ten first-order streams in the Cumberland Plateau in Breathitt, Knott, and Letcher counties in southeastern Kentucky, USA (Hutton et al. 2020). Seven streams were located at the University of Kentucky's experimental research station Robinson Forest (RF) and three streams at Eastern Kentucky University's Lilley Cornett Woods Appalachian Ecological Research Station (LCW). All stream sites were in old-growth and second-growth forests and had low anthropogenic disturbance (i.e., specific conductivity values ranging from 30–418 µS/cm), and the average forest cover at these sites was 99.78% (Hutton et al. 2020). See Martin and Shepherd (1973), Martin (1975), and Phillippi and Boebinger (1986) for description of vegetative communities at our study sites.

*Salamander Surveys and Diet.*—We surveyed for adult and larval salamanders in a single 10-meter stream reach at each site. Stream reaches were selected to contain similar widths, depths, and current velocities. All stream reaches contained a pool, run, and riffle section to provide likely habitat to increase detections of all possible species and life stages (Petranka 1998; Hutton et al. 2020). Each stream reach was sampled four times (ca. every 29 days) from April to July 2017. Searches were conducted during daylight hours (0800–1700 h) and in baseflow conditions. Salamanders were captured using systematic dipnetting and bank searches (Price et al. 2011). Dipnetting consisted of one person, moving from downstream to upstream, searching for salamanders around and under submerged rocks, logs, and other cover within the 10-m reach. One person then conducted bank searches, which included searching under rocks, logs, leaf litter, and other material within 1-m of the wetted width of the stream. Stream searches were limited to 0.5 hours and bank searches to 0.25 hours (Price et al. 2011). After sampling, we recorded the species and life stage (larval or post-metamorphotic individuals).

Salamanders were anesthetized in the field, using a solution of 1-g Maximum Strength Orajel® to 1-L distilled water (Cecala et al. 2007). Once the salamanders failed to right themselves after being flipped over, their stomach contents were obtained using the current non-lethal gastric lavage method for amphibians (Solé et al. 2005; Cecala et a. 2007; Bondi et al. 2015; Hutton et al. 2017, 2018, 2019). Salamanders were placed on their dorsum on a folded paper towel, and an ca. 6 cm long piece of water-lubricated tubing was slowly inserted into the esophagus until there was resistance. Distilled water was then pumped into the tubing. Specifically, Nipro® 3-mL syringes with 22-gauge needles and 0.8 mm and 1.3 mm OD PTFE tubing were used (Zeus Inc., #AWG24). As in previous studies, salamander stomachs were pumped at least two additional times after the last prey item was extracted to verify removal of all contents (Solé et al. 2005; Cecala et a. 2007; Bondi et al. 2015; Hutton et al. 2017, 2018, 2019). The total amount of water pumped into each salamander was dependent on salamander size, the amount of prey items, and the size of prey and their resistance to removal. However, in general, significantly less water was required for larval and juvenile salamanders than adults.

After lavage, each salamander was measured for snout–vent length (SVL: from the tip of the snout to the posterior angle of the vent) and total length (TL: from tip of the snout to the tail's terminus) to the nearest 0.01 mm with a digital caliper, and mass (except larvae ≤ 30 mm TL) to the nearest 0.1 g with a digital scale. We calculated body condition (mass/TL) on all salamanders ≥ 30 mm TL; salamanders missing tails or parts of their tails were excluded (Karraker and Welsh 2006). Salamanders ≥ 30 mm TL were then marked with unique fluorescent visible implant elastomers (VIE) to allow for capture-mark-recapture (CMR) identification. Each salamander was then placed in a recovery container of stream water until they could right themselves and responded to tapping, which took ca. 15 min. Salamanders were returned to their exact location of capture within 1.5 h. After the first survey, each site was surveyed three additional times and recaptured animals were stomach flushed again to examine potential changes in diet.

Animals in the stomach contents were then identified to lowest taxonomic level possible using a dissecting microscope along with appropriate keys and guides (Peckarsky 1990; Merritt and Cummins 1996; Wagner 2005; Fisher and Cover 2007; Bradley 2012; Evans 2014). Additionally, invertebrate life

stage (larval or adult) was reported, if applicable. For Shannon diversity calculations, largely different sized prey or prey with unique characteristics in a single order, family, or genera were considered to be separate morphospecies. The individual prey items were then grouped into larger sections based on order/class, life stage, and presumed origin (Hutton et al. 2018). Samples were then placed into individually labeled vials containing 70% ethanol. Samples are stored in the Branson Museum collection at Eastern Kentucky University, Richmond, Kentucky.

*Diet Analysis.*—To calculate prey volume, we measured the length and width of each prey item to the nearest 0.01 mm using a digital caliper and estimated volume as a prolate spheroid using the equation (Dunham 1983):

$$\text{Prey Volume } (v_x) = \left(\frac{4\pi}{3}\right)\left(\frac{\text{length}}{2}\right)\left(\frac{\text{width}}{2}\right)^2$$

The number of prey items, number of prey types (i.e., morphospecies), average prey volume, total prey volume, Shannon diversity, and body condition of individuals were calculated for each capture event for adult, larvae, and combined groups. We used t-tests to compare the above diet parameters between first and second captures and between adults and larvae. All parameters were log-transformed prior to analysis to fit assumptions, averages and their standard deviations (SD) were back log-transformed to the scale of the data. Number of days since the previous capture was also examined for correlation to the diet parameters. We found 17 out of 245 prey items as potential outliers (volume ≥ 50 $mm^3$), only 6 of which were in the first capture event, and 2 salamanders had prey items ≥ 50 $mm^3$ in both capture events. However, due to the euryphagous behavior of stream salamanders (Jaeger 1981) and the random sampling, we feel it was unfitting to remove the larger prey items from the dataset. Further, the average volumes of the large prey items from each capture event did not differ ($P$ = 0.93). All analyses were performed in the statistical program R (Version 3.4.3).

## Results

We captured six stream salamander species during our active searches (*Desmognathus fuscus* [DF], *D. monticola* [DM], *D. welteri* [DW], *Gyrinophilus porphyriticus* [GP], *Pseudotriton ruber* [PR], and *Eurycea cirrigera* [EC]). Larvae ≤ 30 mm TL were excluded from this study because they were too small to mark safely and uniquely with VIE. Overall, 260 salamanders, 134 adult and 126 larvae (39 DF, 78 DM, 4 DW, 12 EC, 105 GP, and 22 PR; Table 1) were stomach flushed and VIE marked across our 10 stream sites. We recaptured 36 individuals (28 adult and 8 larvae; Table 1), 4 of which were recaptured at least twice (3 DM, 1 GP), for a total of 41 separate recapture observations. Overall, all salamanders contained at least one prey item in their stomachs, for a total of 245 identified and volume-estimated prey items (Table 1). No anesthetization or lavage-based mortality or signs of internal trauma occurred before release.

When all adults and larvae were combined, we found no differences between the first and second capture events in the number of prey ($P$ = 0.273), number of prey types ($P$ = 0.241), Shannon diversity ($P$ = 0.513), or body condition ($P$ = 0.562). However, there were differences in the average prey volumes (mean = 4.15 ± SD 4.79 $mm^3$ and 8.01 ± SD 3.83 $mm^3$; first and second capture, respectively; Table 2) and total prey volumes (mean = 9.33 ± SD 5.16 $mm^3$ and 20.42 ± SD 3.89 $mm^3$; Table 2), with larger volumes in the second capture events ($P$ = 0.045 and 0.021, respectively). In combined groups, we found no correlation between the days since the last capture and the following: number of prey (r = -0.086), number of prey types (r = -0.058), average prey volume (r = -0.016), total prey volume (r = -0.069), Shannon diversity (r = -0.181), or body condition (r = 0.149).

Table 1. Salamander species and life stage capture and recapture for *Desmognathus fuscus, D. monticola, D. welteri, Eurycea cirrigera, Gyrinophilus porphyriticus,* and *Pseudotriton ruber* over four sampling periods at 10 stream reaches in southeastern Kentucky, USA. Numbers in parentheses represent the number of recaptured individuals.

| Species | Adult | Larvae |
|---|---|---|
| *Desmognathus fuscus* | 39 (4) | – |
| *Desmognathus monticola* | 78 (19) | – |
| *Desmognathus welteri* | 4 (4) | – |
| *Eurycea cirrigera* | 12 | – |
| *Gyrinophilus porphyriticus* | 1 (1) | 104 (8) |
| *Pseudotriton ruber* | – | 22 |
| TOTAL | 134 | 126 |

When we examined just the adult salamanders, the results were similar to all salamanders combined group. Among the adults, we found no differences between the first and second capture events in the number of prey ($P$ = 0.155), number of prey types ($P$ = 0.234), Shannon diversity ($P$ = 0.589), or body condition ($P$ = 0.616). However, there were differences in average prey volumes (mean = 3.67 ± SD 4.85 $mm^3$ and 8.58 ± SD 3.42 $mm^3$; Table 2) and total prey volumes (mean = 8.49 ± SD 5.50 $mm^3$ and 23.19 ± SD 3.59 $mm^3$; Table 2), with larger volumes in the second capture events ($P$ = 0.019 and 0.010, respectively). We found no correlation between the days since the last capture and the number of prey (r = 0.029), number of prey types (r = 0.051), average prey volume (r = -0.221), total prey volume (r = -0.205), Shannon diversity (r = -0.143), or body condition (r = 0.079).

Lastly, when we examined the larval salamanders, we found no differences between the first and second capture events in the number of prey ($P$ = 0.891), number of prey types ($P$ = 0.494), average prey volume ($P$ = 0.838), total prey volume ($P$ = 0.411), Shannon diversity ($P$ = 0.633), or body condition ($P$ = 0.557). For the larvae, we were unable to analyze correlation of the days since first capture to the diet parameters due to sample size restraints. Among all salamanders, the least amount of time between capture events was 18 days and the greatest was 65.

A disproportionate volume of large (i.e., ≥ 50 $mm^3$) individual prey items were found in the second capture event compared to the first for the combined (all) and adult groups. Six large individual prey items from the first capture event had a total volume of 540.13 $mm^3$, whereas the 11 large items in the second capture event totaled 934.02 $mm^3$, despite the average volumes of the large prey items from each event (83.92 ± 1.49 and 85.53 ± 1.52 $mm^3$, respectively) not being statistically different ($P$ = 0.93). Thus, the statistically observed increases in the average and total volumes in the second capture event are related to just a few large prey items.

Table 2. Mean (± SD) diet parameters among stream salamanders after repeated recapture and gastric lavage stomach flushing, results were back-transformed to scale of the data.

| | All | Adults | Larvae |
|---|---|---|---|
| Days Since 1st Capture | 29.47 (± 1.57) | 29.12 (± 1.57) | 30.74 (± 1.58) |
| # Prey Items 1 | 2.42 (± 1.83) | 2.47 (± 1.87) | 2.27 (± 1.71) |
| # Prey Items 2 | 2.79 (± 1.73) | 3.03 (± 1.66) | 2.07 (± 1.86) |
| # Prey Types 1 | 2.12 (± 1.77) | 2.26 (± 1.76) | 1.66 (± 1.70) |
| # Prey Types 2 | 2.44 (± 1.68) | 2.66 (± 1.65) | 1.79 (± 1.64) |
| Average Prey Vol 1 | 4.15 (± 4.79) | 3.67 (± 4.85) | 6.45 (± 4.61) |
| Average Prey Vol 2 | 8.01 (± 3.83) | 8.58 (± 3.42) | 6.28 (± 5.74) |
| Total Prey Vol 1 | 9.33 (± 5.16) | 8.49 (± 5.50) | 13.07 (± 4.19) |
| Total Prey Vol 2 | 20.42 (± 3.89) | 23.19 (± 3.59) | 13.13 (± 5.07) |
| Shannon Diversity 1 | 0.952 (± 0.33) | 0.983 (± 0.34) | 0.842 (± 0.25) |
| Shannon Diversity 2 | 1.002 (± 0.35) | 1.032 (± 0.37) | 0.899 (± 0.24) |
| Body Condition 1 | 0.0216 (± 0.015) | 0.0255 (± 0.016) | 0.0171 (± 0.005) |
| Body Condition 2 | 0.0234 (± 0.015) | 0.0275 (± 0.016) | 0.0186 (± 0.005) |

## Discussion

It is important for researchers to understand how various study methods and techniques may directly influence the study population, particularly when the methods are relatively invasive. In this study, our data provide the first field-based assessment of gastric lavage stomach flushing on subsequent feeding behavior of stream salamanders. Overall, we found no negative effects on the future ability of either larval or adult salamanders to obtain prey. In our study, we surveyed streams four times over a 3-mo period and had 41 recapture events from 260 marked salamanders (15.77% recapture rate). This recapture rate is within the range expected in comparison to previous studies and suggests our study and approach did not impact survival. Cecala et al. (2009) for example, reported a recapture rate of 29.04% for larval PR however the streams were sampled 14 times from May 2006 to April 2007, which likely explains the higher recapture rate. Additionally, Bailey et al. (2004) reported that plethodontid salamander recapture probabilities in southern Appalachia ranged between 0.20–0.30 after 3 years of sampling, illustrating a relationship between sampling effort and recapture rates and probabilities. Beyond survival effects, our study also demonstrates that gastric lavage is a safe assay that does not impose long term costs on foraging or digestion and assimilation of prey.

We observed no negative effects of recapture and flushing on any of the diet parameters, suggesting foraging behavior was not impacted. In this study, we recaptured and flushed individuals approximately every 29 days and found no relationship between the days since first capture and the subsequent number of prey, number of prey types, average prey volume, total prey volume, Shannon diversity, or body condition. However, it is unknown how quickly (following full recovery from anesthesia and release) salamanders will begin to forage again, especially due to their primarily nocturnal behavior. In our study, the shortest time between the second stomach flushing was 18 days. By comparison, Patto (1998) captured, obtained stomach contents via gastric lavage, and uniquely toe clipped 97 *Hylodes asper* (Anura: Leptodactylidae) before release. Twenty animals were successfully recaptured and stomach flushed a second time over an 18-d period. Patto (1998) reported prey items in 88% of the frogs, suggesting no significant effects of stomach flushing on the recapture and subsequent prey consumption on the study species. Solé et al. (2005) reported the consumption of offered termite prey by captive anurans just two hours after lavage, the authors then kept the anurans in captivity for a month before releasing. Taken together, these results suggest amphibian foraging behavior is not unduly affected by gastric lavage.

Although some amphibians in captivity appear to be able to accept food shortly after gastric lavage recovery, there are still implications of the immediate loss of captured prey items from stomach flushing in the wild sample population. The minimum amount of time suggested to wait before flushing again or the absolute maximum number of times researchers should flush an individual in a season are not well tested and are very likely dependent on the season, species, and age class. For example, Maiorana (1978) reported complete digestion of prey in terrestrial salamanders to take approximately four days, therefore, prey items found via gastric lavage potentially represent prey that would have been assimilated over several days after feeding. However, salamander prey digestion has also been shown to be temperature dependent (Fontaine et al. 2018); therefore, researchers have an obligation to consider how their methods influence the foraging behavior and energy balance of their study species. This concern is of particular importance during differing seasons and life stages when energy demand is highest (i.e., breeding season or times of reduced resources). While our study does not necessarily provide clarity on this concern because the study took place over a single season, our results suggest both adults and larvae foraging behavior are not unduly affected.

In our study, there was a disproportionate number of large volume prey items found in the second capture events of salamanders, which may be due to differences in the abundance or emergence of large prey types later in the season. Larval EC (68.74–83.28 $mm^3$) were found in the stomach contents of two larval GP and an adult DF during second capture events. Additionally, during second capture events, an adult stonefly species (Plecoptera: Perlidae; 110.06 $mm^3$) and an adult Bark Centipede (Chilopoda: Scolopocryptopidae: *Scolopocryptops sexspinosus*; 127.30 $mm^3$) were found in stomach contents of two separate adult DW. At our study sites, the eggs of EC did

not hatch until early June, and stonefly metamorphosis was noted in June–July. Further, these three prey types were only found in stomach contents during second capture events. Since plethodontid salamanders display a euryphagous feeding strategy (Jaeger 1981), differences in prey availability are more likely to contribute to observed differences in diet composition between sampling periods. However, our results highlight the importance for the inclusion of additional diet parameters such as Shannon diversity, number of prey items, and number of prey types consumed. In this study, despite differences in the average and total consumed prey volumes, the overall Shannon diversity, number of prey items, and number of prey types were not found to significantly change between capture events as those differences were driven by just a few individual prey items.

Overall, this study illustrates the efficacy of gastric lavage stomach flushing, in combination with a Maximum Strength Orajel® anesthetic solution, on stream salamanders. Diet studies are critical to understanding the roles of salamanders in ecosystem processes and community dynamics (Davic and Welsh 2004; Jouquet et al. 2006; Lavelle et al. 2006; Walton 2013). As amphibians continue to decline globally, it is imperative to consider and understand the possible method-based repercussions of each study on a population. Gastric lavage stomach flushing is a popular method which can provide reliable stomach content data with relative ease, requires easily obtained and inexpensive materials, and can be used on numerous species in the same region. Future studies should focus on evaluating the method on previously unreported species as well as species lacking dietary information.

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