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A USEFUL METHOD FOR HOUSING SALAMANDER LARVAE DURING CONTROLLED LABORATORY STUDIES

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Abstract.—**Amphibians are facing unprecedented threats across the globe, prompting biologists to maintain and study captive populations for research, breeding, and rescue purposes. Methods that reduce the effort and cost of maintaining captive animals will be helpful for amphibian conservation. Communal housing of amphibian larvae is often used to maintain hundreds of animals through metamorphosis; however, some research and conservation objectives may necessitate keeping larval amphibians isolated to prevent cannibalism or reduce competition and maximize growth. This is challenging as monitoring water quality on hundreds of individual housing units is costly in effort. We developed a method to maintain salamander larvae in isolation, while keeping 22 individuals per aquarium. This minimized effort to maintain water quality across several hundred housing units. Our method requires inexpensive materials that should be locally available for most researchers. We describe our methods and compare results of survival, body size, and time to metamorphosis for 180 larvae with previous studies to evaluate the effects of our housing design. Larval survival was high (> 90%); however, time to and size at metamorphosis differed from most other laboratory studies, probably due to our feeding regime, not housing. Position effects within and across aquaria were minimal, demonstrating our housing method generates little phenotypic variation across individuals. If researchers or conservation professionals wish to maintain salamander larvae in isolation in the laboratory, our method could reduce the time necessary to provide larval care.**

Key Words.—Ambystoma barbouri; aquaculture; Caudata; egg incubation; larval physiology; propagation; Streamside Salamander

INTRODUCTION

Components of global change, such as disease, habitat destruction, and climate change, threaten amphibians across the planet (Hof et al. 2011). These threats result in the need to maintain, propagate, and study amphibians in captivity for two primary reasons. First, there is a need to understand how environmental factors influence survival and fitness proxies, such as time to metamorphosis or body size. For such research, it is useful to measure variables in the wild and replicate them in a controlled setting, such as mesocosms in the field or laboratory enclosures (Boone and James 2005). For example, researchers might measure water temperatures in breeding streams, collect eggs or larvae, and incubate them at similar temperatures in the laboratory to understand effects of climate change (Oborová et al. 2024; Thulander and Hall 2025). Second, sensitive species may require the establishment of captive colonies to save them from extinction (i.e., assurance colonies), for propagation and reintroduction, or for conservation research and education (reviewed by Harding et al. 2016). Such *ex-situ* management has attracted much attention since the creation of the Amphibian Conservation Action Plan of the International Union for Conservation of Nature (Gascon et al. 2007; Zippel et al. 2011), which identified captive propagation as a critical conservation objective for amphibians. Thus, it is important that both researchers and conservation professionals establish and report effective methods for maintaining amphibians in controlled settings (e.g., Smyers and Rubbo 2001; Tornabene and Hoverman 2018).

Amphibian eggs and larvae are often maintained in groups for logistical reasons. Eggs may be collected in masses and maintained in aquaria until hatching or larvae may be kept in mesocosms, such as large cattle tanks or aquaria in the laboratory (e.g., Petranka 1984; Smyers and Rubbo 2001; Browne and Zippel 2007; Semlitsch and Boone 2009; Oborová et al. 2024). Such communal housing is helpful to maintain large numbers of animals or perform experiments that determine the effects of interactions among individuals, such as competition or predation (Semlitsch and Boone 2009). Communal housing may, however, present experimental or logistical problems for other objectives. Competition for resources inevitably generates among-individual variation in growth rates or time to metamorphosis (Collins and Cheek 1983). Moreover, larvae may injure or even cannibalize one another (Wissinger et al. 2010), particularly larval salamanders, which are more prone to aggressive encounters than tadpoles (Tornabene and Hoverman 2018). Therefore, if a conservation objective is to raise robust larvae for release or future breeding stock, communal housing may not be ideal. Moreover, researchers sometimes aim to isolate the effect of experimental variables, such as food availability, water quality, temperature, or pollutants on larval amphibians, and competition may generate unwanted variation in phenotypes (Tornabene and Hoverman 2018). In these cases, treatments should be applied and evaluated at the level of the individual, not the group.

Researchers who seek to isolate the effects of environmental factors, like toxins, often house animals in isolation (e.g., Rehage et al. 2002), but this presents another set of problems. Water quality, in particular, must be monitored and maintained to achieve high egg and larval survival and viability (Browne and Zippel 2007), which may involve periodically changing water across hundreds of individual containers or constructing filtration systems that filter and replenish water across all enclosures. Additionally, if experimental treatments are applied at the egg or larval stage (e.g., differences in water temperature, salinity, pesticides), these must be applied and maintained for each enclosure. Given that many studies or conservation efforts involve hundreds of animals, these efforts may be prohibitively costly due to the sheer number of person-hours required to perform the work. Therefore, methods that allow individuals to be kept in isolation while reducing the effort to maintain water quality and treatments will be useful.

Tornabene and Hoverman (2018) presented one solution with their description of what they called the Salamander Daisy, which houses larvae in individual containers but within a larger unit like a cattle watering tank. Their device is a set of individual plastic housing units that float within a large tank, allowing water to flow in and out of the containers while keeping the larvae isolated. Thus, larvae are housed individually in these containers, but researchers can monitor the water quality of a few large tanks rather than hundreds of individual units. This device is well-suited for outdoor mesocosm studies or rearing efforts, but we wanted to develop a similar method for conducting controlled laboratory studies. Thus, we were inspired by Tornabene and Hoverman (2018) to create a system to maintain larvae individually in glass aquaria rather than cattle tanks.

We report methods used to conduct a relatively large ecophysiology study of a stream-breeding salamander, the Streamside Salamander (*Ambystoma barbouri*). The Streamside Salamander is native to the U.S. in Kentucky, Ohio, and Indiana, with isolated populations in West Virginia, western Kentucky, and Tennessee. Their life history is similar to other *Ambystoma* species, and a thorough description can be found in Petranka (1998). Briefly, adults remain underground during the warmer months and emerge in late fall or early winter (November to February) to migrate to shallow, intermittent streams for breeding. Eggs are deposited beneath slab rocks or attached to submerged grasses or other debris and hatch within 1–2 mo. Larvae remain in the stream, feeding on zooplankton until metamorphosis, which typically occurs in April or May. Metamorphs move away from streams and remain below ground until sexually mature.

We collected eggs from natural nests, incubated them at a range of temperatures, and maintained larvae in the laboratory through metamorphosis. Importantly, we wanted to isolate the effect of egg incubation temperature on larval survival, body size, and time to metamorphosis, so we needed to house larvae individually. Our objective is to describe our methods with sufficient detail for their reproduction and evaluate their effectiveness by comparing our results with those of other studies that measured larval survival, time to metamorphosis, and body size at metamorphosis for *A. barbouri*.

MATERIALS AND METHODS

***Summary of empirical study*.**—We designed our housing unit and refined our rearing protocols while investigating the effects of water temperature on development of embryonic *A. barbouri* (Thulander and Hall 2025). We collected eggs from across the range of *A. barbouri* and incubated them at one of five temperatures in a split-clutch design (5°, 10°, 20°, 22°, 25° C). Because we were examining the effect of egg incubation temperature on survival and growth of larvae and metamorphs, we needed to house larvae and metamorphs individually to isolate the effect of temperature. As expected, the warmer incubation temperatures (20°, 22°, and 25° C) were detrimental and induced unnaturally low larval and metamorph survival (0–61%) and morphological abnormalities. Thus, here we leverage novel analyses using data from our 5° and 10° C incubation treatments as these temperatures are within the range of those experienced by eggs in the wild (Thulander and Hall 2025).

Egg collection and incubation.—We distributed 211 eggs from 29 clutches into the 5° C and 10° C incubation treatments (2–4 eggs per clutch, per incubation treatment). We collected eggs from one site in Ohio, two sites in Kentucky, and eight sites in Tennessee (Thulander and Hall 2025). Per genetic analysis, our collection sites represented four lineages (Hubbs et al. 2022). There were three distinct lineages in Tennessee, which will henceforth be called South, Central, and North. These are geographically separated from populations in Kentucky and Ohio, which will henceforth be grouped and called KY/OH. To ensure eggs experienced laboratory conditions for most of development, we only collected eggs that were at Harrison stage 12 or earlier (mean stage = 6.4; standard deviation = 3.3; Duellman and Trueb 1994).

After returning to the laboratory, we assigned each egg to a Harrison stage using a dissecting microscope or a jeweler's loop. After they were staged, we placed eggs individually into 59.1 ml (2 oz) glass jars that were filled with tap water treated with API® Tap Water Conditioner (Mars Fishcare North America Inc., Chalfont, Pennsylvania, USA). We placed jars in an IPP55plus incubator (Memmert, Eagle, Wisconsin, USA) set to either 5° C or 10° C.

Larval housing, care, and phenotypes.—At hatching, we photographed larvae beneath a dissecting scope alongside a ruler so we could later measure total body length using the software ImageJ 1.53t (Schneider et al. 2012). We maintained larvae (n = 180) in a common garden at room temperature in our laboratory (mean = 19.4° C; standard deviation = 1.5° C). Two windows provided an ambient photoperiod. Larvae were housed in custom built units. Our description of their construction is in metric units; however, imperial units and tools (e.g. drill bits) were used during construction. Units were composed of sections of 7.62 cm (3 in) diameter polyvinyl chloride (PVC) pipe (23 cm long, 7.75 cm internal diameter) with screen mesh bottoms (Phifer Pool and Patio fiberglass flyscreen, Phifer Inc., Tuscaloosa, Alabama, USA) secured with aquarium silicone (Fig. 1; see Table 1 for a complete materials list). Due to the size of the tube and the amount of water we maintained in our aquaria, this equated to about 0.75 L of water within each housing unit. To construct housing units, we purchased 3 m (10 ft) sections of PVC pipe from a local hardware store, cut them to our desired length with a miter saw, and drilled 0.95 cm (0.375 in) holes, centered in the diameter of the tube and 0.95 cm (0.375 in) from one end of the tube using a drill press (Fig. 1). We used a sander to smooth the edges where the PVC was cut and secured fiberglass fly screen to the end of the tube opposite the holes using 100% silicone (Fig. 1). Silicone covered the entire end of the tube leaving no gaps through which larvae could escape. Both PVC and cured silicone are safe for aquatic animals and are commonly used to construct custom housing units and pumps for aquaria in zoos and research facilities (Koike 2013).

Drilling the holes allowed us to slide 0.64 cm (1/4 in) dowel rods (30.5 cm; 12 in, in length) through a set of three PVC tubes and rest them on the top edge of a 75.7 L (20-gallon) aquarium (Fig. 1). This suspended the tubes above the bottom of the tank allowing circulation of water up into the tube. We fit 23 tubes per aquarium leaving a gap (1 tube missing) for a Fluval® C2 113.6 L (30-gallon) power filter (Rolf C. Hagen Inc., Mansfield, Massachusetts, USA) with aquarium bio filter sponge (Fig. 1). One PVC tube did not have a mesh bottom and allowed access for an aerator (Uniclife Air Pump; Shenzhen Hidom Electric Co., Ltd., Guangming, Shenzhen, China) to oxygenate the water. We supplied a plastic plant to each tube for structure and placed a jar lid (5.5 cm diameter, 1 cm height) upside down in the bottom of each tube. The jar lids were the tops for the 59.1 ml glass jars used to incubate eggs (see above) and helped to hold food within the housing unit long enough to be eaten by the salamander. Otherwise, small food items (e.g., hatchling brine shrimp) would drift through the mesh and be inaccessible. The lids were small enough that they did not fully cover the screen mesh and allowed for water exchange between the tank and the housing unit (Fig. 1). We affixed larval ID numbers to each PVC tube; however, we also affixed a schematic to each tank that demonstrated the location of larva to ensure individual locations could be identified (Fig. 1). To accommodate the number of larvae for the study, we outfitted 12 aquaria with our housing units

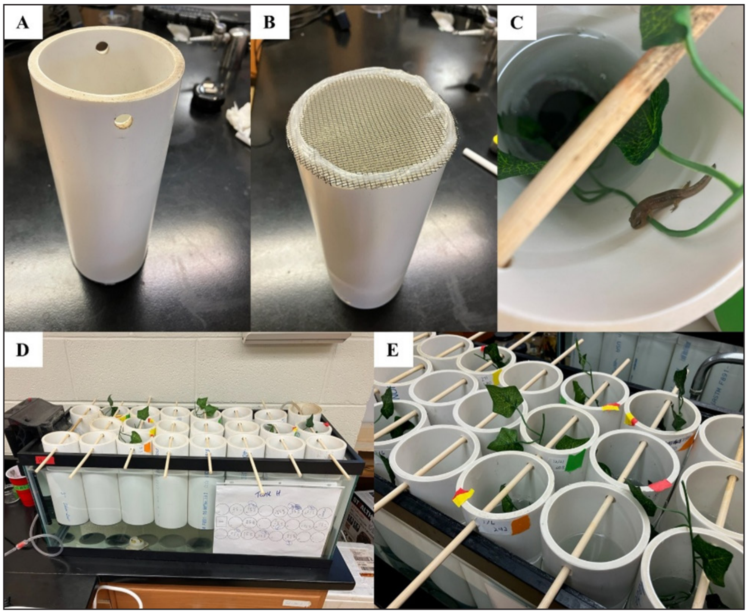

Figure 1. Method for rearing larval salamanders in the laboratory. (A) Individual housing unit showing the top. (B) individual housing unit showing the bottom. (C) A metamorph salamander within a housing unit. Note the black jar cap at the bottom of the unit. (D) and (E) are a complete set of housing units maintained within a 70-L (20 gallon) aquarium. (Photographed by Joshua Hall).

(264 units, 22 units per aquarium × 12 aquaria).

We fed larvae a mixture of live brine shrimp and frozen-thawed blood worms using methods that controlled the amount of food across larvae and enhanced efficiency during feeding. We hatched brine shrimp eggs according to the instructions of the supplier, rinsed hatchlings with fresh water, transferred them to a plastic cup, and added the hatchlings to larval housing units using a 1-ml plastic dropper. Blood worms were purchased in frozen cubes and separated into smaller chunks that were placed into mini-ice cube trays (1 × 1 × 1 cm cubes) and frozen with fresh water. This allowed us to quickly drop cubes into larval housing units for feeding. We measured a subset of 1 ml brine shrimp solutions and cubes of blood worms to estimate the amount of food provided to larvae. We fed larvae every Monday, Wednesday, and Friday (MWF), and we increased the quantities of food as they grew. We fed each larva 2 ml of brine shrimp during the first 30 d (average dry weight per feeding = 6.3 ± 0.652 mg). For the next two weeks we fed them 1 ml of brine shrimp concentrate and one cube of blood worms (average dry weight per bloodworm cube = 2.5 ± 0.62 mg) to wean them onto blood worms. After weaning, we fed them two cubes of blood worms. At 60 d, we fed them three cubes, and after 90 d we fed them six cubes of blood worms. Adding frozen cubes likely decreased water temperatures for a short time after feedings; however, *A. barbouri* larvae should be robust to such changes as they experience relatively broad daily temperature fluctuations in the wild (see Fig. B.8 in Thulander 2024).

Table 1. Materials required to construct one set of 22 larval salamander housing units along with cost (in U.S. dollars as of December 2024). Most items were purchased at local hardwood, craft, or pet stores, but some items were purchased from online suppliers for convenience. Tools required, but not listed, include a miter saw, drill press, caulk gun, power drill, and power sander. See main text for instructions and Figure 1 for photographs. Metric dimensions are provided but materials were purchased and constructed using imperial units.

Material description	Unit price (USD $)	Quantity	Total cost
75 L (20-gallon) aquarium (77 cm L × 33.4 cm D × 33 cm H)	59.99	1	59.99
3 m section of 7.6 cm (3 inch) PVC pipe	37.96	2	75.92
Aquarium aerator (Uniclife air pump)	15.19	1	15.19
Aquarium filter (Fluval C2, 113.6 L (30 gallon)	34.01	1	34.01
Small insect fiberglass screening, 0.91 × 2.1 m roll	7.98	1	7.98
Tube of silicone (298 ml)	10.98	1	10.98
Artificial plant (vine)	8.99	2	17.98
Dowel rods (0.6 × 30.5 cm, plastic); 12 per pack	8.99	1	8.99
		Total cost	$231.04

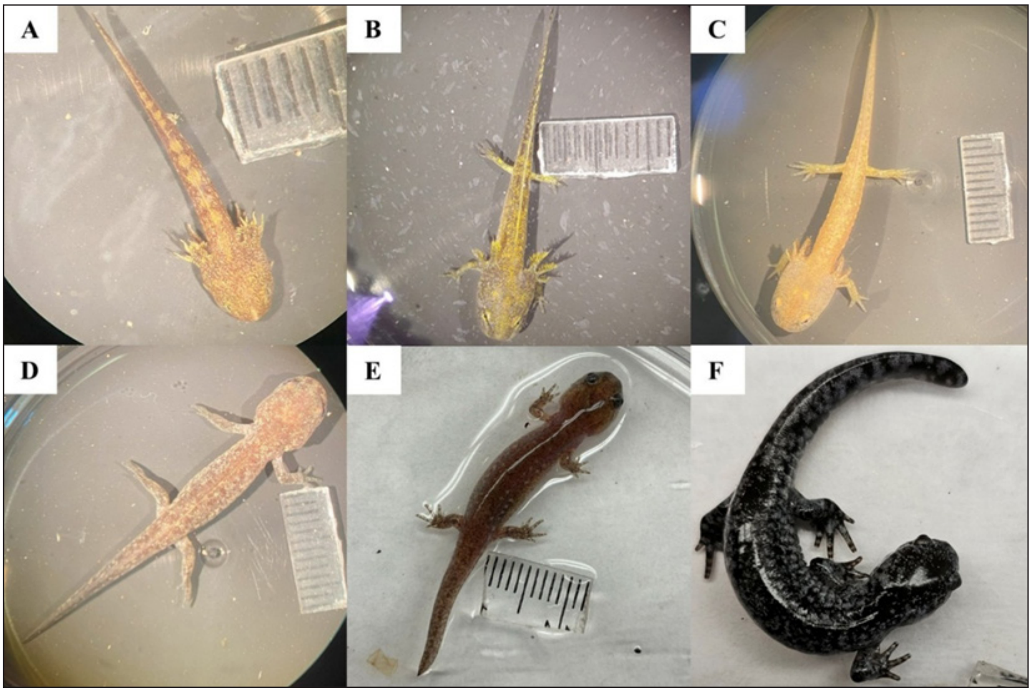

Figure 2. Development of Streamside Salamanders (*Ambystoma barbouri*) in the laboratory. (A-C) Progression through larval stages, (D) resorption of gills at metamorphosis, (E) growth from metamorphosis, and (F) growth to 90 d post-metamorphosis. (Photographed by Julia Thulander).

To maintain water quality, we performed pH, ammonium, nitrate, and nitrite tests weekly on one haphazardly chosen tank and each tank had a 20% water change once per week. If the test results showed unfavorable water quality, we ran tests on all 12 tanks and corrected water quality as needed. We vacuumed uneaten food from the tanks once per month or as needed. We checked larvae 3–5 times per week and removed dead larvae immediately when discovered.

We removed larvae every 30 d and photographed them beneath a dissecting microscope to evaluate progression toward metamorphosis, which we defined as the complete resorption of gills (Fig. 2). Near metamorphosis (Fig. 2), we monitored them daily to assign a date at metamorphosis. Metamorphs can easily climb out of the PVC tubes (Fig. 1); therefore, when larvae approached metamorphosis, we placed the lid of a 90 mm petri dish over the PVC pipe, which fit snugly and prevented escape. At metamorphosis (Fig. 2), we removed larvae from their PVC tubes, blotted them dry with a paper towel, placed them on a mass balance to measure body mass at metamorphosis (to nearest 0.001 g, mass balance BCE641-1S, Sartorius Lab Instruments, Göttingen, Germany), and moved them to terrestrial housing.

We placed 159 metamorphs individually into plastic containers (Sterilite© 27.94 × 16.764 × 6.858 cm; Sterlite Corp., Townsend, Massachusetts, USA), filled with enough treated water to produce 1–2 cm depth at one end when tilted on a wooden dowel. We provided moist paper towels and cardboard as substrate and hides. We fed metamorphs every MWF to create an *ad libitum* environment. They received a variety of invertebrates: Porcelain Woodlouse (*Trichorhina tomentosa*), European Nightcrawlers (*Dendrobaena hortensis*), and Powder Blue Isopods (*Porcellionides pruinosus var*. Powder Orange). These feeders are relatively rich in vitamins A and B, and calcium, which are the primary limiting nutrients of captive amphibians (Wright and Whitaker 2001). We provided worms and isopods with a variety of vegetables to enhance their nutrient content. Moreover, we supplemented worm diet with Uncle Jim's Worm Food (Uncle Jim's Worm Farm, Spring Grove, Pennsylvania, USA) and isopod diet with fish flakes (Tetra, Blacksburg, Virginia, USA). We cleaned the Sterilite© containers once per month or as needed by replacing all terrarium materials. Finally, at the end of 90 d (Fig. 2), we weighed each juvenile to estimate early-life body size.

Statistical analysis.—The goal of this study was to describe our housing design and evaluate its effects on important traits such as body size and time to metamorphosis. Thus, we used our analyses to generate estimates of traits for comparison with previous studies and evaluate position effects within and across aquaria. Additionally, our analyses included the fixed effects of incubation temperature (5° C versus 10° C), population of origin, and their interaction to account for these important factors. Because our populations are distinct genetic lineages, we used population of origin (South, Central, North, KY/OH) as a fixed effect. Additionally, factors with fewer than five levels are inappropriate to use as random effects; therefore, including population as a fixed effect is preferred (Harrison et al. 2018). We include the interaction between population and temperature because populations may exhibit differential response to thermal variation. To evaluate position effects due to our housing design, we included Tank ID (n = 12 tanks) and tank location (n = 22 locations) as random effects. Tank locations represent the location of each housing unit within each tank. These locations were repeated across tanks. For example, locations one and two were always in the first row, adjacent to the filter, while locations 21 and 22 were in the last row adjacent to the aerator, which was in location 23. Position effects may occur because some larvae were closer to the aerator or filter than others and may have benefited from greater oxygenation due to perturbation of the water. We also included clutch ID as a random effect to account for genetic or maternal effects.

We analyzed egg, larvae, and juvenile survival with Generalized Linear Mixed Effects Models (GLMEM; 1 = survive, 0 = died). We also used GLMEM to analyze total body length at hatching, time to metamorphosis, mass at metamorphosis, and mass at 90 d post metamorphosis. If a random effect was uninformative (i.e., produced a variance of zero), we removed it from the model. Therefore, if tank ID and tank location are uninformative in the analysis, it indicates these variables did not influence the phenotype. For informative random effects, we calculated the Intraclass Correlation Coefficient (ICC) to evaluate the importance of the random effect. If the interaction between temperature and population was not significant ($P > 0.05$), we removed it from the model. We performed all analyses in R version 4.3.2 (R Core Team 2023) using the lmertest and lme4 packages for General and GLMEM (Bates et al. 2015; Kuznetsova et al. 2017). We assessed significance of fixed effects with an Analysis of Variance (ANOVA) with $\alpha = 0.05$, and we used the emmeans package to perform post hoc comparisons among groups, adjusting P values for multiple comparisons using the false discovery rate method (Lenth et al. 2018). In the presence of a significant interaction term, we did not evaluate all pairwise comparisons (n = 28 possible comparisons) as some comparisons are not biologically meaningful (e.g., comparing 5° C treatment from one population with the 10° C treatment of another population). Rather, we compared incubation treatments within populations and populations within treatments. We used the sjPlot package to calculate r^2, residual variance, variance of the random effect, and the intraclass correlation coefficient on the random effects (Lüdecke 2018). We created figures using ggplot2 (Wickham 2016). We evaluated model assumptions by visually inspecting residuals.

Finally, we reviewed the literature to find other studies that measured the same phenotypes for *A. barbouri* in the laboratory or field so we could compare our rates of larval survival, time to metamorphosis, and body size at metamorphosis with other housing methods. We did not perform a systematic review. We consulted Petranka (1998) to find literature and searched the reference sections in relevant papers. We also performed a search with scholar.google.com using relevant keywords (*Ambystoma barbouri* OR *Ambystoma texanum* AND larvae OR egg OR development). Older studies list *A. barbouri* as *A. texanum* as these species were originally synonymous. We found eight relevant studies that were conducted either in the laboratory (n = 6) or field (n = 2). One study used communal housing in the laboratory (n = 10 larvae per housing unit) whereas others housed larvae individually in glass or plastic jars. From each study, we recorded mean values of larval survival, time to metamorphosis, and size to metamorphosis across populations or groups to compare with our data.

Most studies did not experimentally manipulate the larval environment, which allows for meaningful comparison across studies. Two studies, however, were ecotoxicology studies, but we only collected data from the control groups to allow for comparison with our data. We did not extract estimates of variation (e.g., standard error, standard deviation, ranges) because we did not intend to use these values in a statistical analysis but rather qualitatively compare results to determine if our method for maintaining larva produced similar results. Many studies incubated eggs at warmer temperatures than those we used (e.g., 15°–17.5° C) or allowed eggs to incubate in natural nests. Because we observed a strong effect of incubation temperature on most traits, to best compare our results with the literature, we used model estimates from our 10° C egg incubation treatment only. This temperature is more like those used in other studies and better reflects average nest temperatures in the wild than the 5° C treatment (Thulander and Hall 2025). Specifically, we used coefficients from our final models to estimate mean trait values for each of our four populations (South, Central, North, KY/OH) for the 10° C treatment. We report these values as a range for each trait and qualitatively compare them with values from the literature.

Results

Effect of temperature and population on survival and phenotypes.—We observed high survival across all life stages (Fig. 3, Table 2). The lowest rates of survival were at the egg stage (80–91% across populations) and survival was comparatively higher for larvae (90–91%) and metamorphs (94–100%). We did not observe any effects of population or egg incubation temperature on survival at any life stage (Fig. 3, Table 2).

Length at hatching was consistently greater for larvae from the 10° C treatment across all populations although there was no significant population by temperature interaction (Fig. 3, Table 3). Moreover, length varied across populations such that KY/OH larvae were larger at hatching than those from the South population, but other differences were not significant (Fig. 3, Table 3). Unlike hatchling length, we observed a significant interaction between population and temperature for time to metamorphosis, body mass at metamorphosis, and juvenile body mass (Fig. 3, Tables 3, 4). Time to metamorphosis was similar among populations for individuals incubated at 10° C, except that those from TN South took longer than those from TN North (Fig. 3). Incubation at 5° C, however, induced substantial variation among populations such that all TN populations had greater time to metamorphosis than those from KY/OH and TN South and TN Central exhibited greater time to metamorphose than TN North (Fig. 3). Moreover, all TN populations exhibited significantly greater time to metamorphosis when incubated as eggs at 5° C than 10° C, but temperature did not significantly affect

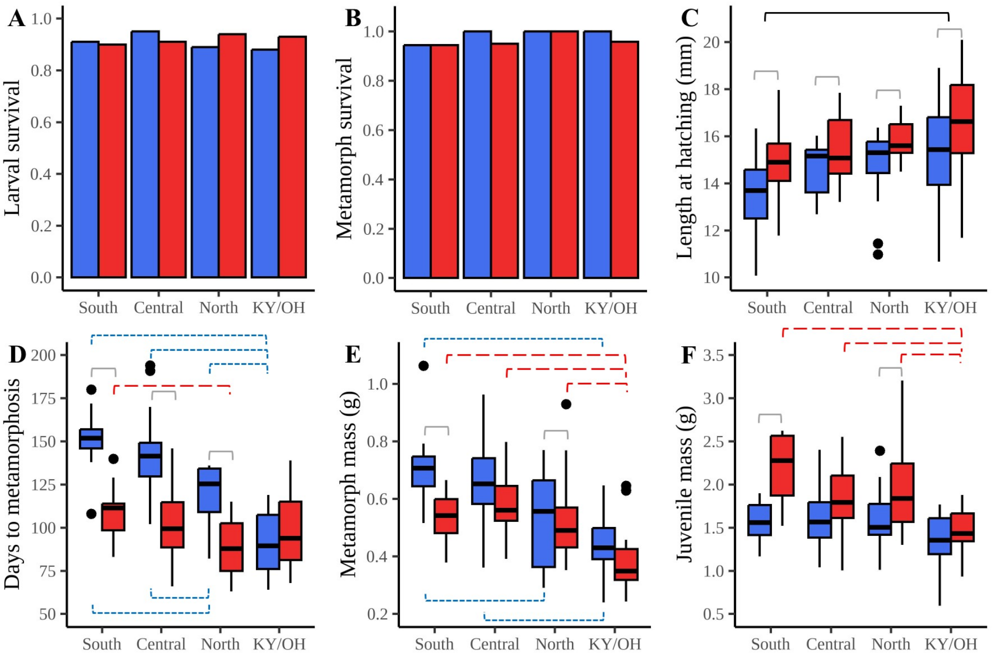

Figure 3. Survival and phenotypes of Streamside Salamanders (*Ambystoma barbouri*) across incubation temperature and populations. Blue and red bars and boxes denote 5° C and 10° C incubation treatments, respectively. Panels A and B demonstrate survival of larvae and metamorphs, respectively. Panels C, D, E, F illustrate among-population body length at hatching, time to metamorphosis, size at metamorphosis, and size 90 d post-metamorphosis, respectively. Brackets within panels denote significance (alpha = 0.05) among groups after false discovery rate correction. Solid black brackets demonstrate population-level differences, solid gray brackets demonstrate temperature differences within populations, blue, short-dashed brackets demonstrate population differences for the 5° C treatment, and red, long-dashed brackets show population differences for the 10° C treatment. Closed, black dots show outliers.

Table 2. Model summary results for egg, larval, and metamorph survival of Streamside Salamanders (*Ambystoma barbouri*) from Tennessee (TN), USA. *P* values for pairwise comparisons are not adjusted for multiple comparisons (see graphs for adjusted estimates of statistical significance). The reference (i.e., intercept) value is from the KY/OH population incubated at 5° C. Abbreviations and symbols are n = sample size, σ^2 = residual variance, τ_{00} = variance of the random effect, ICC = intraclass correlation coefficient, df = degrees of freedom, and SE = standard error.

Response	Fixed Effect	Estimate	SE	*z*-value	*P*-value
Egg survival					
n = 211	Intercept (5° C, KY/OH)	2.10	0.69	3.04	0.002
conditional r^2 = 0.437	Temperature (10° C)	-0.09	0.45	-0.21	0.832
marginal r^2 = 0.057	TN Central	0.97	1.06	0.91	0.361
	TN North	-0.25	1.00	-0.26	0.799
	TN South	1.12	1.12	1.01	0.315
	Random effect (Clutch ID) (σ^2 = 3.29, τ_{00} = 2.22, ICC = 0.40, n = 29)				
Larval survival					
n = 180	Intercept (5° C, KY/OH)	2.11	0.49	4.34	< 0.001
	Temperature (10° C)	0.19	0.53	0.36	0.717
	TN Central	0.41	0.74	0.55	0.630
	TN North	0.17	0.74	0.23	0.853
	TN South	0.02	0.68	0.03	0.973
Metamorph survival					
n = 159	Intercept (5° C, KY/OH)	4.64	1.32	3.50	0.002
	Temperature (10° C)	-1.18	1.17	-1.01	0.314
	TN Central	-0.23	1.44	-0.16	0.503
	TN North	16.62	3123	0.01	0.995
	TN South	-1.07	1.25	-0.85	0.393

time to metamorphosis for KY/OH (Fig. 3).

Body mass at metamorphosis was similar among TN populations from the 10° C treatment, but each of these groups were significantly larger in size than those from KY/OH (Fig 3, Table 4). TN populations from the 5° C treatment exhibited some variation in body mass with TN South being larger than TN North. Additionally, KY/OH population was significantly smaller than TN South and TN Central (Fig. 3). Moreover, in general, metamorphs were more massive when incubated at 5° C than 10°C, but this was only a significant difference for TN South and TN North populations (Fig. 3).

Finally, for the 5° C treatment, all populations exhibited similar juvenile body mass; however, the KY/OH population had a significantly smaller body mass than all three TN populations from the 10° C treatment (Fig. 3, Table 4). Juvenile body mass trended larger for individuals incubated at 10° C compared with those incubated at 5° C, but this was only significant for TN South and TN North populations (Fig. 3, Table 4).

Effect of tank ID, tank location, and clutch ID on survival and phenotypes.—Clutch ID contributed to variation in egg survival but did not significantly affect survival at later stages (Table 2). Moreover, we did not observe any effect of tank ID or tank location on either larval or metamorph survival (Table 2). Clutch ID contributed to variation in all larval and metamorph traits (Tables 3, 4), but we did not observe any effect of tank location on any traits, nor was tank ID informative for most traits. The exception is that tank ID influenced the time to metamorphosis (Table 3).

Comparison of traits with the literature.—All previous laboratory studies experienced high rates of larval survival similar to the results of our study (all > 90%); however, survival in the field was much lower (1–6 %; Table 5). We observed times to metamorphosis that ranged from 88.8 (± 5.8 standard error) d to 108.3 (± 5.4) d and body masses at metamorphosis from 0.37 (± 0.03) g to 0.59 (± 0.03) g across populations. Only one of six laboratory studies (Venesky and Parris 2009) observed time

Table 3. Model summary results for length at hatching and time to metamorphosis of Streamside Salamanders (*Ambystoma barbouri*). *P* values for pairwise comparisons are not adjusted for false discovery rate (see figures for adjusted estimates of statistical significance). Abbreviations and symbols are n = sample size, σ^2 = residual variance, τ_{00} = variance of the random effect, ICC = intraclass correlation coefficient, df = degrees of freedom, and SE = standard error.

Response	Fixed Effect	df	Estimate	SE	*t*-value	*P*-value
Total length of hatchling larvae (mm)						
n = 179	Intercept (5° C, KY/OH)	23.3	15.46	0.43	36.24	< 0.001
conditional r^2 = 0.526	Temperature (10° C)	149.5	1.12	0.21	5.43	< 0.001
marginal r^2 = 0.203	TN Central	21.5	-0.90	0.63	-1.42	0.169
	TN North	22.1	-0.94	0.67	-1.40	0.176
	TN South	20.9	-1.91	0.66	-2.91	0.004
	Random effect (Clutch ID) (σ^2 = 1.86, τ_{00} = 1.26, ICC = 0.41, n = 28)					
Time to metamorphosis (days)						
n = 159	Intercept (5° C, KY/OH)	39.63	91.48	4.35	21.02	< 0.001
conditional r^2 = 0.642	Temperature (10° C)	130.85	6.96	4.92	1.42	0.159
marginal r^2 = 0.562	TN Central	45.92	52.37	6.59	7.95	< 0.001
	TN North	43.44	27.14	7.10	3.82	< 0.001
	TN South	44.85	60.48	6.79	8.91	< 0.001
	TN Central: (10° C)	131.26	-47.21	7.45	-6.34	< 0.001
	TN North: (10° C)	131.69	-36.77	8.06	-4.56	< 0.001
	TN South: (10° C)	132.33	-50.59	7.69	-6.58	< 0.001
	Random effect (Clutch ID) (σ^2 =304.5, τ_{00} = 61.9, ICC = 0.17, n = 27)					
	Random effect (Tank ID) (σ^2 = 304.5 τ_{00} = 6.1, ICC = 0.02, n = 12)					

to metamorphosis that overlapped with our results (Table 5). All other lab studies and both field studies observed shorter times to metamorphosis ranging from 31 to 75 d across studies and populations (Table 5). One of six laboratory studies (Venesky and Parris 2009) and both field studies (Petranka 1984; Petranka and Sih 1986) observed masses at metamorphosis that overlapped with our data (Table 5); however, the other five laboratory studies observed body mass at metamorphosis that was substantially larger: 0.95 to 1.4 g (Table 5).

Discussion

Amphibians are facing numerous human-caused threats and populations are declining at a rapid, global scale (Stuart et al. 2004; Collins 2010). The need for laboratory methods that adequately care for each portion of the life cycle is great, and sharing methods will help meet conservation and research objectives while keeping costs low. We developed a simple method for housing larvae in isolation in a controlled laboratory setting and used it to conduct a large-scale ecophysiology study that required housing 290 larvae (180 used in our analyses). Our method utilized readily available, inexpensive materials and minimized care and maintenance costs by allowing us to monitor 12 aquaria rather than individual housing units. We observed high survival across all life stages; however, time to and size at metamorphosis differed substantially from many previous studies. These differences probably relate to our feeding regime rather than our housing method (see below). The research and conservation communities that specialize on amphibian larvae, and caudate larvae in particular, can benefit from employing our design if housing larva in isolation is an important objective.

Although our objective in the current study is to describe our novel method, we observed some notable biological effects. Though population and temperature had no effect on survival at any life stage, both, and often their interaction, influenced body size and time to metamorphosis. There is a range of temperatures across which development proceeds efficiently (the Optimal Thermal Range; Hall et al. 2024), but temperatures warmer or cooler reduce body size, performance, and embryo survival (Noble et al. 2018; Gatto and Reina 2022). Our 10°

Table 4. Model summary results for mass at metamorphosis and 90 d later for Streamside Salamanders (*Ambystoma barbouri*). *P* values for pairwise comparisons are not adjusted for false discovery rate (see figures for adjusted estimates of statistical significance). Abbreviations and symbols are n = sample size, σ^2 = residual variance, τ_{00} = variance of the random effect, ICC = intraclass correlation coefficient, df = degrees of freedom, and SE = standard error.

Response	Fixed Effect	df	Estimate	SE	*t*-value	*P*-value
Mass at metamorphosis (g)						
n = 158	Intercept (5° C, KY/OH)	39.0	0.44	0.03	15.55	< 0.001
conditional r^2 = 0.589	Temperature (10° C)	131.0	-0.06	0.03	-2.16	0.032
marginal r^2 = 0.460	TN Central	40.8	0.24	0.04	5.67	< 0.001
	TN North	38.3	0.09	0.05	1.90	0.070
	TN South	41.2	0.27	0.05	6.05	< 0.001
	TN Central: (10° C)	130.4	-0.02	0.04	-0.49	0.625
	TN North: (10° C)	130.1	0.07	0.05	1.50	0.135
	TN South: (10° C)	131.4	-0.11	0.05	-2.41	0.017
	Random effect (Clutch ID) (σ^2 = 0.01, τ_{00} = 0.00, ICC = 0.24, n = 27)					
Mass at 90 days post metamorphosis (g)						
n = 139	Intercept (5° C, KY/OH)	39.83	1.34	0.09	15.57	< 0.001
conditional r^2 = 0.51	Temperature (10° C)	113.12	0.13	0.09	1.36	0.176
marginal r^2 = 0.361	TN Central	45.20	0.29	0.14	2.15	0.033
	TN North	37.64	0.26	0.14	1.86	0.065
	TN South	49.42	0.25	0.15	1.65	0.101
	TN Central: (10° C)	114.10	0.13	0.14	0.90	0.370
	TN North: (10° C)	113.04	0.18	0.15	1.23	0.222
	TN South: (10° C)	121.44	0.51	0.16	3.24	0.002
	Random effect (Clutch ID) (σ^2 = 0.10, τ_{00} = 0.03, ICC = 0.23, n = 27)					

C treatment was likely in this range as it resulted in relatively large body size at hatching, shorter time to metamorphosis, and larger body size at the juvenile stage. These positive outcomes, compared with the 5° C treatment, probably emerge from adaptation to stream temperatures that exhibit means of 8.3°–12.5° C during the nesting period (Thulander and Hall 2025). Only rarely do nest temperatures reach a low of 5° C. The population and population by temperature effects were likely due to genetic variation as both mitochondrial and nuclear DNA support these groupings as distinct population

Table 5. Comparison of larval and metamorph data of Streamside Salamanders (*Ambystoma barbouri*) among studies. If a range is given it represents a range of means across groups (e.g., populations). For larval housing temperature, means are provided, and ranges are the range of mean temperatures across treatments or experiments within the study. Room temperature indicates larvae were kept at ambient temperature, but details were not reported. Stream temperatures were not reported for the field studies.

Study	Study location	Housing type	Larval temperature (°C)	Larval survival (%)	Days to metamorphosis	Mass (g) at metamorphosis
Present study	Laboratory	Individual	19.4	90–91	89–108	0.37–0.59
Venesky and Parris 2009	Laboratory	Individual in 4.2 L jars	Room temperature	100	91–99	0.38–0.47
Petranka and Sih 1987	Laboratory	Individual in 1 L jars	20.3–22.4	-	31–36	0.95–1.08
Petranka et al. 1987	Laboratory	Individual in 1 L jars	19.1	-	61	1.21
Rehage et al. 2002	Laboratory	Individual in 1 L jars	21–25	100	40	1.4
Rohr et al. 2003	Laboratory	Communal; 10 per 3.7 L glass bowl	15	95–100	> 50	-
Petranka and Sih 1986	Field	Natural streams	-	1–4	45–75	0.5–1.1
Petranka 1984	Field	Natural streams	-	1–6	49–63	0.57–0.73

units (i.e., evolutionary significant units; Hubbs et al. 2022). Effects of temperature appear more pronounced for Tennessee populations than those from the core range of Kentucky and Ohio, and this may reflect the relatively broad genetic variation that exists within Tennessee compared with the larger, contiguous northern range (Hubbs et al. 2022). These thermal effects on time to metamorphosis and body size likely have an important influence on survival and reproduction in the wild and potentially in captive populations.

Time to metamorphosis is a critically important trait for all amphibians, but particularly those that breed in ephemeral waters like *A. barbouri* because animals must transform and leave the water before the hydroperiod ends (Relyea 2007; Rudolf and Rödel 2007; Richter-Boix et al. 2011). Additionally, a short time to metamorphosis could be useful for captive breeding programs as it hastens the time to maturity, allowing animals to rapidly move through the larval stage where rates of mortality are relatively high (Petranka 1984; Kissel et al. 2019). Of course, this assumes ideal captive conditions as a short larval period may indicate poor water quality or stress and result in reduced body size. Size at metamorphosis is also a critical life-history trait as larger metamorphs are preyed on less often than smaller larvae and they have a wider variety of prey available due to a larger head size (Semlitsch 1987; Urban 2007; Gvoždík and Smolinský 2015). Moreover, a large size at metamorphosis may correlate with larger size in adulthood, which may equate to greater fecundity for females included in breeding programs (Semlitsch 1987; Wise and Jaeger 2021). For propagation and release programs, rapidly producing large metamorphs may increase the number of large, healthy individuals for release. Thus, methods that reduce the time to metamorphosis and maximize size at metamorphosis may be particularly useful.

We compared larval survival, time to metamorphosis, and body size at metamorphosis to published studies as these variables are commonly measured and are important proxies for viability in both captive and wild populations. Although survival across all life stages was high in our experiment, our time to and size at metamorphosis only marginally aligned with previous research. Indeed, many previous studies reported times to and sizes at metamorphosis that were as much as 1/3 shorter, and twice as large as ours, respectively. We do not think these differences are due to our housing method, *per se*, but rather our feeding regime. There is a relation between size and time to metamorphosis such that in the presence of abundant food, animals grow rapidly and undergo metamorphosis early, while, when food is scarce, growth rates are slower, and the larval period is longer (Petranka and Sih 1986). The study with a feeding regime most like ours reported time to and size at metamorphosis similar to our data (Venesky and Parris 2009). Other studies provided *ad libitum* feeding (Petranka and Sih 1987; Petranka et al. 1987; Rehage et al. 2002; Rohr et al. 2003). Notably, these employed sample sizes much lower than ours (24–90 total larvae per study). The exception is that Rohr et al. (2003) housed 560 larvae, but these were kept communally (10 larvae per container). In all these studies, larvae were housed in jars or plastic containers that were 1–5 L, and water quality was maintained via regular water changes (not filtration). For example, Rohr et al. (2003) replaced water in their 56 communal housing units every other day. Such effort is required for *ad libitum* feeding without a filtration system as uneaten food can reduce water quality. This may limit sample sizes. Indeed, we were able to use nearly three times the number of housing units as any of these studies. Our intent was to provide a common garden for larvae that tightly controlled the amount of food across individuals. Therefore, we did not apply an *ad libitum* feeding at the larval stage; however, we are confident that increased feeding rates (e.g., daily) or quantities could easily be provided with our design, and this would shorten the larval period and increase size at metamorphosis. Importantly, we maintained many of these research animals (n > 80) in captivity to adulthood. The relatively small size we observed at metamorphosis seems to have little long-term effect on *A. barbouri*, as these animals have achieved body masses of 12–20 g as adults. These body masses are equal to or greater than what is observed for breeding adults in the wild (7–12 g; unpubl. data).

We used mixed effects models to evaluate the potential for clutch ID, tank ID, and tank location to influence important larval and metamorph traits. Our results were encouraging because the larvae location within a tank was not informative in any models. This indicates there were no position effects within our aquaria. Tank ID was uninformative in most models which indicates environmental variation across aquaria was minimal. The exception was that tank ID explained variation in time to metamorphosis. Temperature and photoperiod each influence time to metamorphosis (Neptune and Benard 2024) and may have differed across tanks due to their position in the laboratory. Regardless, the Intraclass Correlation

Coefficient was very low (0.02) indicating little variation was explained by tank ID.

There are several advantages to our housing design. First, all the materials are relatively inexpensive and usually available at local hardware, craft, and pet or hobby stores. No specialized equipment was required. Second, these materials are durable and could be used across multiple studies over many years. Third, the method is adaptable to various species and research objectives. Polyvinyl chloride comes in an array of diameters and sizes that can readily be obtained at most hardware stores. Combining this size variation with an array of aquarium sizes, one could feasibly design an assortment of housing units that vary in their diameter and height as well as various numbers of units per aquarium. Finally, components of the aquatic environment can be controlled across multiple housing units simultaneously. For example, we were able to monitor water quality of only 12 tanks rather than about 300 individual units. We can envision multiple scenarios where this would be useful, such as in toxicology or hydroperiod studies where chemical or water level treatments can be applied to each tank rather than each housing unit. Additionally, this setup could also be useful to quantify specific competitive interactions by housing larvae in variable but controlled densities of the same or different species (e.g., 1, 2, or 3 larvae per unit).

We must, however, point out some notable disadvantages. First, our design allowed for more vertical than horizontal space which might not be ideal for all purposes or species. Second, because small food items (e.g., daphnia; brine shrimp hatchlings) may slip through the flyscreen, it may be challenging to measure quantities of food consumed, maintain truly *ad libitum* conditions, or provide different feeding treatments within the same tank. For these objectives, larger prey is required (e.g., blood worms). Additionally, chemical signals released by larva may transmit through the tank, influencing behavior and physiology; however, tank filtration should minimize this effect. Third, the dowel rods make periodically removing larvae cumbersome. Some dexterity is required to maneuver a net or other device to capture larvae around the rods. Finally, because the tubes were tall and opaque, performing daily checks on health and survival was a challenge, and we often had to use a flashlight to peer down into the housing units. If desired, researchers could substitute clear PVC pipe to enhance visibility of behavior, and food availability. Additionally, using light-colored jar lids or platforms to catch food would enhance visibility. Our jar lids were black, which decreased visibility when larvae were sitting in the lids. Despite these disadvantages, our research was a success, and we look forward to using these housing units in the future.

In conclusion, we present a useful method for individually housing larval salamanders in a controlled, laboratory setting. Our method consists of readily available, inexpensive materials that are durable and will last through many experiments. We found little evidence for experimental effects of our housing design (e.g., tank ID, location) and important phenotypes such as survival, development time, and body size were in line with some previous studies using different methods in the laboratory or field. We encourage researchers to use this housing design when it is preferable to isolate larvae for conservation or experimental purposes. We also encourage researchers to report useful methods they develop as we have done. Amphibian conservation faces many challenges, but we can work together as a community, sharing useful ideas for collection, husbandry, and study of threatened species.

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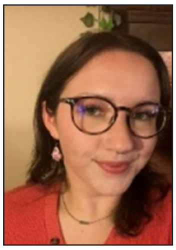

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