EVALUATING A PREDATOR-INDUCED PHENOTYPE IN A MIXED SPECIES CONTEXT

By

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**ABSTRACT**

Phenotypic plasticity, a single genotype producing multiple phenotypes in response to environmental change, is crucial to our understanding ecological and evolutionary processes. Adaptive plasticity describes phenotypic response wherein a subsequent fitness benefit is conferred to the plastic individual. Predator-induced plasticity is a well-studied form of adaptive plasticity. For instance, numerous tadpole species exposed to aquatic predators produce more muscular and brightly colored tail fins, which have been shown to improve survival chances in subsequent predator encounters compared to noninduced individuals. However, predator-induced phenotypes can be costly when expressed in a non-lethal environment. Current understanding of the relative costs and adaptive benefits of predator-induced plasticity is based on intraspecific comparisons. However, multiple species differing in their plastic abilities often co-occur and interact with one another in nature. Few studies have evaluated whether the

adaptive benefits and relative costs of predator-induced plasticity are retained in multi-species assemblages (a more ecologically-relevant setting). We conducted a two-phased experiment to evaluate the adaptive value and relative costs of a predator-induced phenotype in tadpoles of the Pine Woods Treefrog, *Hyla femoralis*, in the presence and absence of a congeneric species, the Squirrel Treefrog (*Hyla squirella*). *Hyla femoralis* and *H. squirella* share ecological settings and close evolutionary ties, yet larval *H. squirella* does not exhibit the same phenotypic response (changes in body/tail morphology) to predation risk as larval *H. femoralis* does. In Phase I (Induction), single-species assemblages were assigned to one of two predator-exposure (induction) treatments: a non-lethal treatment with a caged dragonfly nymph or a control with no predator. After four weeks, *H. femoralis* tadpoles from both induction treatments were photographed for morphometric analysis to quantify any plastic responses ( change in morphology) to perceived predation risk. We found that larval *H. femoralis* morphology significantly differed between induced and noninduced populations. In Phase II (Predation Trials), tadpoles from single (*H. femoralis* only) and mixed-species assemblages (*H. femoralis* and *H. squirella*) were exposed to one of two predation treatments: a lethal, free-swimming predator treatment or a no-predator control. Periodic survival estimates were determined for both assemblages in lethal treatments to quantify possible survival advantages conferred by the inducible phenotype in larval *H. femoralis*. Growth metrics (size at emergence) and a development metric (time to emergence) were collected for both assemblages in control treatments to quantify possible costs associated with induced plasticity. Survival data supports that the adaptive advantage of increased survival in induced *H. femoralis* tadpoles is retained in mixed-species assemblages. Size at emergence in larval *H. femoralis* was not affected by induction treatment or assemblage type. Conversely, time-to emergence was significantly

impacted by induction treatment. Induced *H. femoralis* tadpoles in both single and mixed-species assemblages took longer to reach metamorphosis, indicating predators likely have a stronger effect on developmental timelines than the presence of another tadpole species. This study aims to contribute revelatory insights into the ecology and maintenance of adaptive plasticity in natural, complex community systems.

EVALUATING A PREDATOR-INDUCED PHENOTYPE

IN A MIXED SPECIES CONTEXT




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By

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**INTRODUCTION**

Phenotypic plasticity is now being recognized as a ubiquitous phenomenon that likely underpins many ecological and evolutionary processes, and serves as an interface bridging the two fields (Hendry 2016, Berg and Ellers 2010). Phenotypic plasticity is the ability to produce alternate phenotypes from a single genotype in response to varying environmental conditions. Plasticity can be a major source of trait novelty and have significant effects on the resulting direction, magnitude and duration of future evolutionary trends (Hendry 2016, Fordyce 2006, Levis and Pfennig 2019). Adaptive plasticity occurs when specific environmental cues stimulate phenotypic changes that confer fitness benefits to a plastic individual under those conditions (Ghalambor et al 2007, Innes-Gold et al 2019, McCoy et al 2012). Adaptive plasticity can change the nature of intra- and interspecific interactions in ways that can cascade through food webs, change nutrient flows across trophic levels, and alter community composition (Berg and Ellers 2009, Hendry 2016, Miner et al 2005). For example, non-lethal exposure to predators induced Acute Bladder Snails (*Physa acuta*) to develop thicker, larger shells (in terms of shell mass and shell dimensions) along with predator-avoidance behaviors that lowered predation risk by crayfish relative to noninduced (predator-naïve) Bladder snails (Auld and Relyea 2011). These changes in the strength of ecological interactions as a result of plasticity can also shape the outcomes of evolution by modifying the strength of selection and rates of adaptation in different environments (Hendry 2016). For example, species-level plasticity and polymorphisms in Spadefoot Toads (*Spea spp*) reduces interspecific competition among species in shared habitats and helps maintain species boundaries (Pfennig and Murphy 2000). Specifically, *Spea bombifrons* (the Plains Spadefoot Toad) and *S. multiplicata* (the New Mexico Spadefoot Toad) can both be induced to produce either omnivore or carnivore phenotypes dependent upong the

types of resources available in certain environments. However, when reared together (or co-occurring in the field), *S. bombifrons* produced mainly carnivore morphs while *S. multiplicata* produced mainly omnivore morphs (Pfennig and Murphy 2000). Moreover, the phenotype of each species differentially affected foraging efficiency and growth of the alternate phenotype of the other species (Pfennig and Murphy 2000). Natural selection for alternative, adaptive phenotypes and differential competitive ability of alternative forms in different habitats likely reinforced these tradeoffs allowing these species to coexist within a single environment (Pfennig and Murphy 2000).

Predator-induced plasticity is one of the most well studied forms of adaptive plasticity, as predator encounters are ubiquitous in nature and prey fitness (survival) is paramount (Teplitsky et al. 2005, Tollrian and Harvell 1999). A classic example of predator-induced plasticity involves the development of long, toothed neck spines in water fleas (*Daphnia pulex*) post-exposure to chemical cues emitted by predatory midge larvae (*Chaoborus americanus*) (Lüning 1992). Similarly, chemical cues from aquatic predatory dragonfly nymphs induce *Hyla chrysoscelis* (Cope's Gray Treefrog) tadpoles to develop deep, muscular, reddish, pigmented tails that enhance survival in subsequent predator encounters by increasing escape speed and distracting predatory attacks away from core body tissues (McCollum and Buskirk 1996, Wilbur and Semlitsch 1990). Despite the aforementioned adaptive benefits (i.e. higher survival), predator-induced plasticity can be context dependent, with the inducible phenotype having maladaptive effects in predator-free settings. For instance, predator-induced *H. chrysoscelis* tadpoles suffered higher mortality rates compared to noninduced conspecifics in caged (non-lethal) predator environments (McCollum and Buskirk 1996). This suggested that induced *H. chrysoscelis* tadpoles experienced a fitness cost (higher mortality) due to sources other than actual predation,

and that producing the anti-predator morphology can be costly in the wrong environmental context (McCollum and Buskirk 1996). Induced *H. chryoscelis* tadpoles also took longer to reach metamorphosis (McCollum & Van Buskirk 1996). Delayed metamorhposis is a general pattern observed in several studies documenting slower development in predator-induced tadpoles of many larval anuran species (McCollum and Buskirk 1996, Relyea 2002, LaFiandra and Babbit 2004). These fitness costs in predator-free environments likely stem from allocation of resources toward unecessary anti-predator morphologies versus growth or development, as well as from constant exposure to a stressful, predator cue-filled environment (LaFiandra and Babbit 2004, Buskirk 2000, Gonzalez et al 2011, Fraker et al 2009, Middlemis Maher et al 2013). Anuran larvae of many species have been shown to produce anti-predator tail morphologies in response to perceived risk from predator chemical cues in their aquatic environment (McCollum and Buskirk 1996, Touchon and Warkentin 2008, Lafiandra and Babbit 2004, Kruger and Morin 2020). In other larval anuran studies, predator-induced tadpoles have been shown to tradeoff smaller size at metamorphosis for faster development (McCollum and Leimberger 1997, Ficetola and Bernardi 2006, Skelly and Werner 1990, Wilbur and Fauth 1990, Touchon et al 2015). In addition to morphological and developmental responses to predators, many larval anurans also exhibit behavioral changes, including decreases foraging activity and increases in "hiding" and refuge-seeking behaviors (Relyea 2001, McCoy and Bolker 2008, McIntyre et al 2004).

Despite the clear fitness benefits of adaptive phenotypic plasticity, the ability to respond plastically to environmental change is an adaption not universally observed across taxa, even among closely-related species inhabiting similar environments (Fordyce 2006). Numerous ecological and evolutionary studies have evaluated the relative fitness costs and adaptive benefits of predator-induced plasticity whereby induced individuals are competed against noninduced

conspecifics (McCollum and Buskirk 1996, DeWitt 1995, Relyea 2002, Warkentin 1995). In natural settings, however, species exhibiting predator-induced plasticity (heretofore adaptive plasticity) typically do not inhabit ponds limited to conspecifics only, and certainly not conspecifics lacking the induced phenotype.They more commonly co-occur with one or more (sometimes closely-related) species that may not exhibit adaptive plasticity despite sharing similar ecology and/or phylogenetic history (Fordyce 2006, Berg and Ellers 2010). Thus, it remains unclear whether the adaptive fitness benefits of predator-induced plasticity are retained when evaluated in a mixed species setting, played out with two closely-related species that differ in their plastic responses to the same environmental conditions (a shared predator and ecological settings.) Understanding the tradeoffs associated with predator-induced plasticity in mixed species setting could offer new insights and a better understanding of phenotypic plasticity's ecological and evolutionary consequences. Potential tradeoffs with heterospecific competitors could change our interpretation of the adaptive value of an inducible phenotype and the perceived costs.

In this study, we evaluated the adaptive benefits and relative fitness costs of an inducible phenotype in a two-phased experiment involving the Pine Woods Treefrog, *Hyla femoralis*, relative to a related, non-plastic congeneric species, the Squirrel Treefrog (*Hyla squirella*). Larvae of *H. femoralis* have been shown to develop deeper tail fins and increased tail coloration and pigmentation in response to predator metabolites perfused through experimental arenas (LaFiandra and Babbit 2004, McCoy 2007). Converesely, larvae of *H. squirella* are not known to produce any morphological changes in response to predation risk (McCoy and Bolker 2008). In the first phase of our experiment, we quantified phenotypic responses in larval *H. femoralis* to non-lethal predator exposure. In the experiment's second phase, we conducted predation trials,

using free-swimming dragonfly nymphs as potential predators in both single-species (*H. femoralis*) and mixed-species (*H. femoralis*, *H. squirella*) assemblages.

Predator-exposure treatment during the first phase was expected to have a significant effect on tail morphology of *H. femoralis* tadpoles but no effect on *H. squirella* tadpoles (LaFiandra and Babbit 2004, McCoy and Bolker 2008). If a phenotypic response in induced *H. femoralis* tadpoles is adaptive, then higher survival would be expected in both induced single and induced mixed-species assemblages. Specifically, we hypothesized that induced *H. femoralis* tadpoles in mixed-species assemblages would maintain survival levels comparable to the survival advantage observed when comparing induced and noninduced *H. femoralis* tadpole survival in single-species assemblages. We also predicted that the relative fitness costs of the induced phenotype would be maintained in a mixed-species assemblage by induced *H. femoralis* tadpoles. Finally, we predicted that induced *H. femoralis* tadpoles from both induced single and mixed-species assemblages would emerge later as smaller metamorphs compared to noninduced *H. femoralis* tadpoles from noninduced single and mixed-species assemblages.

**METHODS**

<u>*Study System*</u>

Both species of treefrogs used in this study (*Hyla femoralis* and *Hyla squirella*) are common throughout the southeastern United States and cooccur in mixed species assemblages in temporary ponds and ephemeral wetlands. The release of aquatic alarm pheromones and metabolites of digested conspecifics as a result of predation by dragonfly (*Anax spp.*) nymphs induces larval *H. femoralis* to develop modified anti-predator tail morphologies (LaFiandra and Babbit 2004). Tadpoles exposed to predator chemical cues often develop brighter, reddish and pigmented tail fins, in addition to deeper, wider tail fins, and shorter body lengths and tail fin lengths compared to noninduced individuals (Richardson 2006, Touchon and Warkentin 2008, LaFiandra and Babbit 2004, Kruger and Morin 2020). Variation in tail coloration, pigmentation, tail and body shape/size is thought to reflect the intensity and duration of exposure, as well as the source of the predator chemical cue (whether pheromones or metabolites) and surrounding competitor densities experienced by experimental individuals (Touchon and Warkentin 2008, Teplitsky et al 2005, Relyea 2004, McCoy 2007).

*Hyla femoralis* and *H. squirella* occur in monophyletic sister groups in the family Hylidae (Figure 1) **(**Wiens et al. 2010). Both species of treefrog coexist in and utilize similar ecological settings and are exposed to shared assemblages of predator species. However, *H. squirella* tadpoles do not exhibit the same plastic morphological responses to predator chemical cues (McCoy and Bolker 2008). Larval *H. squirella* have been shown to exhibit behavioral plasticity typical of tadpoles in response to chemical predator cues, such as reduced foraging and decreased rates of activity (McCoy and Bolker 2008).

## Collection of Eggs & Predators

On July 13th, 2020 we collected six amplectant adult pairs of *H. femoralis* and three amplectant adult pairs of *H. squirella*. Capture sites for both species were located within a three-mile radius of each other in Craven County, North Carolina. Individual pairs were placed in containers with API® dechlorinated tap water and left in place in the field to allow for overnight oviposition in predator-free water. All pairs mated successfully, and adults were released at their sites of capture the following morning. Clutches were transported back to the laboratory and maintained in their collection containers for 48 hours, with gentle changes of dechlorinated tap water as needed to remove debris, maintain good water chemistry, and ensure sufficient oxygenation. All eggs (of both species) hatched by July 18th (~Gosner stage 20; after yolk absorption), and developing tadpoles were fed finely-ground rabbit chow ad libitum.

Twenty-six *Anax junius* (Common Green Darner dragonfly) nymphs (F4-F0 instars) were collected from a retention pond in Greenville, NC (35.590661, -77.319253) on July 20th, 2020. *Anax junius* is a common tadpole predator in fish-free ponds throughout the southeastern US and is known to induce morphological and behavioral phenotypic plasticity in several species of larval treefrogs (Tennessen 2019, Kruger and Morin 2020, Peacor 2006).

## Experimental Design

The six clutches of *H. femoralis* were combined, as were the three *H. squirella* clutches, to mix lineages and ensure genetic diversity among experimental treatments. This experiment was conducted between July 14th – November 26th, 2020 using 52 (total) 10-gallon aquaria maintained in an approved animal care facility (IACUC AUP #D363). Each aquarium was prepared on July 18th with roughly 30 liters of dechlorinated tap water, two plastic aquarium plants, and submerged plastic tubing for aeration. Phase I (Induction) of this experiment began

on July 19th.  Tadpoles of each species were transferred from capture containers to randomly

assigned experimental aquariums. Each aquarium was then randomly assigned to either control

(empty cage, no predation risk) or non-lethal (caged predator, predation risk) induction

treatments.

Our experiment was conducted with two-phases in a complete randomized-blocked

design. Phase I (Induction) consisted of two species (separate assemblages of larval *H. femoralis*

and *H. squirella*) crossed with two induction treatments that were replicated 12 times (Figure 2).

Phase I's replication scheme was two times larger than Phase II to allow for maintenance of

tadpole density across the experiment. Phase II (Predation) consisted of two tadpole experience

treatments (induced vs. noninduced) crossed with two species assemblage treatments (single or

mixed) and two predation treatments (control no-predator or lethal predator) replicated five times

for a total of 40 experimental units.

### *Phase I: Induction*

For the induction phase (Phase I), separate tadpole species assemblages were maintained

and randomly assigned to one of two induction treatments: a non-lethal (caged *A. junius* nymph)

treatment or a control (empty cage, no predator), which served as a basis for comparison (Figure

2). Tadpoles from non-lethal induction treatments will be referred to as the induced population in

future discussion, and those from control treatments as the noninduced population. Phase I was

conducted over a period of four weeks from July 19th – August 16th, 2020. To maintain

comparable tadpole densities across phases (I and II),  the Phase I replication scheme was

repeated twice. All Phase I aquaria received single-species assemblages of 30 tadpoles per

aquarium on July 19th.  On day 3 of the experiment, deceased tadpoles, assumed to have

succumbed from handling stress, were removed and replaced. Any incidents of tadpole mortality after day 3 were attributed to treatment effects.

After a 24-hour acclimation period, each non-lethal induction treatment received a single, caged *A. junius* nymph, and control tanks received an empty cage with no predator. Cages were constructed from Rubbermaid® "Take-along" 6.4 oz containers (19.4 cm (H) x 17.7 cm (W) x 16.2 cm (D)); rectangular holes were cut out of each container wall and covered with a fiberglass mesh to allow chemical cues of predation risk to diffuse throughout treatment aquaria. Dragonfly nymphs in non-lethal induction treatments were fed three conspecific tadpoles (from separately-maintained feeder stocks, Gosner stages 21-25) of the same species as the focal species in the tank every other day for 28 days (Gosner 1960). During predator feedings, cages were temporarily removed from experimental tanks and individual predator condition was assessed. Nymphs were replaced as needed (due to metamorphosis or death). Empty predator cages in control induction tanks were also temporarily removed and placed back into their respective tanks in conjunction with the predator-feeding events for the non-lethal induction tanks to maintain the same disturbance regime. Tadpoles across treatments were fed ad libitum a 3:1 ratio of finely-ground Kaytee Timothy Complete® rabbit chow and TetraMin® fish flakes three times a week (LaFiandra and Babbit 2004). All aquaria (48 experimental units + four extra treatment tanks) remained uncovered and unfiltered but received partial water changes performed manually by removing and replacing 1/3 of the tank volume twice a week throughout Phase I. Waste accumulation was monitored daily; dipnets and turkey basters were used to remove waste as needed.

*Phase II: Predation*

To investigate the effects of larval environment (Induction) and species assemblage on survival and growth, tadpoles from all 52 tanks (48 Phase I induction tanks + 4 "back-up" treatment tanks) were transitioned into either single- or mixed-species assemblages in one of two predation treatments: a lethal treatment, containing a free-swimming *A. junius* predator, or a no-predator control treatment (Figure 2). On August 28th 2020, tadpoles from each induction treatment and species group (induced *H. femoralis*, induced *H. squirella*, noninduced *H. femoralis*, noninduced *H. squirella*) were consolidated into four temporary housing containers, combining individuals from the same treatment and species prior to random assignment to Phase II tanks. Tadpoles from the four additional Phase I treatment tanks were added as needed to achieve appropriate tadpole densities for Phase II. Phase I water was drained from aquaria into two clean 125-liter Rubbermaid® trash cans, separating non-lethal treatment water from control treatment water. Aquaria were then individually rinsed with tap water and refilled with a ratio of 7:23 liters of Phase I water and fresh dechlorinated water. Phase I non-lethal treatment tanks were transitioned to becoming lethal treatment tanks in Phase II, each being filled with the "non-lethal" and fresh dechlorinated water mixture. This was done to control for effects that lingering chemical predator cues from non-lethal tanks may have on tadpole survival during Phase II. Similarly, Phase I control tanks were transitioned to Phase II control tanks, filled with the control-fresh water mixture. All predator cages were removed, and Phase I nymphs were used as lethal predators in Phase II.

In Phase II, tadpoles of both species from each induction treatment were randomly assigned to one of two assemblage treatments and one of two predation treatments. All Phase II tanks housed initial densities of eight tadpoles total; single-species assemblages contained eight

*H. femoralis* tadpoles whereas mixed-species assemblages contained four *H. femoralis* tadpoles and four *H. squirella* tadpoles. Lethal treatment tanks were populated with a single free-swimming dragonfly nymph that could feed freely on tadpoles whereas nymphs were absent in control treatment tanks.Phase II tadpole feeding and aquarium cleaning schedules were the same as Phase I.

The number of tadpoles surviving from all lethal treatment tanks (of both species assemblages and induction types) was recorded every three hours for the first 48 hours and 12-hour intervals thereafter until no surviving tadpoles remained. The predation trials lasted over seven days, until 100% mortality was observed across all lethal treatment tanks.

To quantify the potential costs of plasticity we recorded a series of growth and development metrics for metamorphosing froglets from the control treatments. Metrics were gathered for each metamorph upon emergence. We defined emergence as the time point at which a metamorph was visibly breathing air and possessed developed forelimbs (~Gosner stage 42, Gosner 1960). Time-to emergence was recorded for each metamorph as a metric of development, and quantified as the number of days from the onset of Phase I to metamorph emergence.  Mass (g) and snout-vent-length (SVL) (cm) at emergence were recorded for each metamorph as metrics of size and correlates of fitness (Albecker and McCoy 2019). This period of gathering growth and development metrics on emerging metamorphs lasted 89 days. Froglets were released at parental capture periodically throughout the emergence period. Any control tadpoles that had not metamorphosed by 90 days were photographed, removed from their tanks, and released at parental capture sites.

To quantify phenotypic responses induced by Phase I treatments, high-resolution photos of individual tadpoles were taken and analyzed for subsequent geometric morphometric analyses (Zelditch et al. 2012). At the end of the four-week induction period, five haphazardly-selected tadpoles were captured from each of the 52 induction treatment tanks for digital photography. If a tank contained less than five tadpoles (due to mortality over the course of Phase I), the remaining tadpoles were photographed. Tadpoles were individually and temporarily anesthetized with a pre-mixed solution of equal parts (0.2 g/L) MS-222 and sodium bicarbonate as a buffer, mixed with dechlorinated tap water (Touchon and Warkentin 2008). Individual anesthetized tadpoles were then placed in a "photography depot" created with a small Ziploc® sandwich container (5.72 cm (H) x 15.54 cm (W) x 15.54 cm (D)) lined with styrofoam backing as a stage (Touchon and Warkentin 2008). Anesthetized tadpoles were immersed in a shallow amount of the MS-222/sodium bicarbonate/dechlorinated water solution within the photography depot and secured in a standardized position. Digital photos were taken using a Canon® EOS 40D camera and a 90 mm macro lens mounted on a tripod approximately one meter above the tadpole stage. Photos were manually focused and taken with a F/6.3 aperture and $1/160^{th}$ second exposure time. Strategically placed dissection pins were used to orient and hold each anesthetized tadpole in place for dorsal and lateral view imaging. After photos were taken, tadpoles were allowed to recover in clean dechlorinated tap water, and returned to their respective treatment tanks. No tadpole mortality resulted from this process. Total processing time for an individual tadpole, from sampling to returning to treatment tanks, was ten minutes on average.

Ninety-two *H. femoralis* (50 from non-lethal induction treatments and 42 from control induction treatments) and 86 *H. squirella* tadpoles (46 non-lethal induction treatments and 40

from control induction treatments) were photographed. As noted, larval *H. squirella* do not develop anti-predator tail morphologies in the presence of non-lethal predators(McCoy and Bolker 2008); thus, we did not obtain morphometric data from images of *H. squirella* tadpoles. Standardized landmarks for geometric morphometrics were chosen to compare lateral views of 92 *H. femoralis* tadpoles, using slightly-modified protocols outlined in Buskirk (2009). Twenty-one lateral landmarks (Figure 3) were selected using the image analysis program ImageJ 1.53a (NIH), with an installed PointPicker plug-in (Thévenaz 2003).

### *Statistical Analyses*

All statistical analyses were performed using the R statistical programming environment (v 4.0.3). Geometric morphometric analysis of landmarks was performed in the package geomorph (Adams et al. 2013). A permutational ANOVA was used to analyze variance observed in the Procrustes distances from mean tadpole shape as a function of treatment using the package RRPP (Collyer and Adams 2018). Analyses of linear generalized linear models (GLM) were conducted using the MASS package (Venables and Ripley), and generalized linear mixed effect models (GLMM) were performed using the package lme4 (Bates et al. 2015). The Anova function within the car package was utilized (Fox et al. 2013) to test hypotheses surrounding the effects of fixed effects on response metrics. In cases where we were unable to obtain reliable estimates for random effects standard deviations, we used Bayesian mixed models (BLMER) using the package blme (Chung et al. 2013). Specifically, we used the default weak Wishart prior distribution to obtain estimates and confidence metrics for random effects. Analyses of growth metrics (SVL, mass, time-to emergence) and survival included random effects for tank and block to account for autocorrelation for individuals housed within the same treatment tanks and blocks.

For our geometric morphometric analysis of landmark data, we utilized the standardized procedures of a Generalized Procrustes Analysis (GPA) in order to superimpose lateral landmark coordinates from 92 *H. femoralis* specimens onto a standardized shape plane that corrects for differences in size, shape, and orientation between specimens. This GPA was performed via the function gpagen within package geomorph (Adams et al. 2013). The results of this standardization provides a list of Procrustes coordinates that describe average, overall tadpole shape. We then conducted a Principal Component Analysis (PCA) on the Procrustes-standardized coordinates to better visualize and understand variation in *H. femoralis* morphology between treatments. PCA was performed using the function gm.prcomp within package geomorph (Adams et al. 2013). One extreme outlier was identified (likely due miscalibration during image analysis) and was removed. The top five principal axes of our PCA contained 66.47% of the variation in *H. femoralis* morphology. Plots depicting changes in morphospace from mean consensus shape to the target induction treatment morphologies were created using the functions plotRefToTarget within package geomorph (Adams et al. 2013, see Figure 4).

To determine if there were differences in *H. femoralis* survival based on induction treatment and/or assemblage type, we used a binomial GLMM to analyze the proportion of individuals surviving over time in each treatment combination (induced or noninduced x /single- or mixed-species assemblage). For this analysis, block was included as a random effect.

To determine if either (or both) assemblage type and/or induction treatment affected size at metamorphosis, we compared SVL using a Bayesian mixed model with Gaussian error distribution on logged transformed measurements of SVL with tank number as a random effect.

To investigate the effects assemblage type and/or induction treatment had on emergence mass, we compared emergence masses using a linear mixed effects model with a Gaussian error

distribution on logged transformed measurements of emergence mass with tank number as a random effect. One individual outlier specimen was identified during this analysis and omitted based on high Cook's distance (>0.10) .

Lastly, to determine the effects assemblage type and/or induction treatment had on time-to emergence, we compared emergence timelines among treatments using a Bayesian linear mixed model with a Gaussian error distribution on logged-transformed measurements of time-to emergence with tank number as a random effect.

# RESULTS

## *Morphometric Analysis*

Permutational ANOVA results indicate that Procrustes distances from mean, overall *H. femoralis* morphology significantly differed between induction treatments ($R^2 = 0.040$, F = 3.694, p = 0.002).  Our PCA utilizing the standardized Procrustes shape coordinates for twenty-one lateral tadpole landmarks reported 59.12% of the variation in landmark coordinates being explained by the first four principal component axes: PC1 = 23.99%, PC2 = 14.37%, PC3 = 12.13%, PC4 = 8.63%.  The remaining 34 components accounted for < 8% of morphological variation.

Loadings onto PC1 (23.99%) indicated changes in a tail fin depth landmark. (Figure 4, Table 1). Shape changes away from the mean in a positive direction (positive loadings) indicated the posterior ventral edge of the tail fin (2/3rds down tail length) moving anteriorly and more medial in direction, along the ventral edge. Thus, PC1 predominantly captures changes in tail fin depth, with the widest portion of induced tail fin occurring more medially and anteriorly compared to noninduced, control tail fins. This explains the anterior medial movement of the tail fin's ventral edge reflected by positive loadings on PC1. Loadings onto PC2 (14.37%) also indicate changes in the same tail fin landmark, the posterior ventral edge of the tail fin (2/3 of the way down the tail) (Figure 4, Table 1). Positive loadings onto PC2 indicate the position of the posterior ventral edge of the tail fin moving laterally, away from the mid-tail fin region and therefore increasing tail depth. Variation in tail fin length was captured on PC3 (12.13%). Tail tips lengthen and move posteriorly away from the tadpole body with positive PC3 loadings. Negative loadings reflect the tail tip moving anteriorly, shortening the tail fin (Figure 4, Table 1). PC4 (8.63%) reflects variation in a cephalic landmark (center of partially-open mouth), likely

indicative of changes in head size. Positive loadings on PC4 indicate lateral movement of the center of the partially-open mouth, possibly indicating a widening of the tadpole head capsule; conversely, negative loadings indicate medial movements, which suggest a narrowing of the head (Figure 4, Table 1). These PCA results, in summary, suggest that induced *H. femoralis* tadpoles exhibited deeper, longer tail fins and slightly narrower head capsules in relation to overall tail/body size (Figure 4, Table 1). Noninduced *H. femoralis* tadpoles exhibited shallower, shorter tail fins and wider head capsules, comparatively.

### *Survival*

The effects of induction treatment and assemblage type on *H. femoralis* survival varied over time ($\chi^2 = 14.619$, p = 0.0022). Induced (non-lethal, predator-exposed) *H. femoralis* in single-species assemblages had the highest probability of surviving (0.502 : 0.497, 0.507) followed by noninduced *H. femoralis* in single species cohorts (0.496 : 0.490, 0.502) (*Probability of survival estimate : 95% confidence interval with 2.5% and 97.5% values). Induced *H. femoralis* survival remained somewhat level in mixed-species assemblages (0.491 : 0.483, 0.499). Finally, the lowest survival probabilities were observed in the noninduced *H. femoralis* mixed-species assemblages (0.483 : 0.478, 0.487) (Figure 5).

### *Size*

*H. femoralis* tadpoles metamorphosed at similar snout-vent lengths from induced vs. noninduced treatments ($\chi^2 = 0.262$, p = 0.608) as well as from the mixed vs single-species assemblages ($\chi^2 = 0.209$, p = 0.648; Figure 6). *H. femoralis* tadpoles also emerged at similar body masses (upon metamorphosis) across both induction treatments ($\chi^2 = 0.0007$, p = 0.979) and assemblage types ($\chi^2 = 0.239$, p = 0.625; Figure 7).

*Development*

Induced *H. femoralis* took longer to reach metamorphosis ($\chi^2$ = 4.189, p = 0.041; Figure 8), taking 23% percent longer to emerge compared to noninduced *H. femoralis*. However, there were no detectable differences in time-to emergence as a function of assemblage type ($\chi^2$ = 0.053, p = 0.818).

**DISCUSSION**

Individual-level expression of phenotypic plasticity can be mediated by a multitude of biotic and abiotic factors. Climatic extremes, complex species interactions, nutrient dynamics, genetic variation and anthropogenic activity can all interact and influence the propensity and magnitude of phenotypic responses exhibited by individuals in response to changing environmental conditions. Environmentally-induced changes in a phenotype can be adaptive when it enhances individual fitness by increasing probability of growth, survival, and/or reproductive activity. Non-adaptive or maladaptive plasticity can occur when there is a loss in fitness due to phenotypic "mismatches" between individuals and the environment. Quantifying and inferring the adaptive value of a particular phenotypic response is typically measured relative to conspecifics with an alternate phenotype. However, a phenotypic trait's adaptive value can be, and most likely is, context dependent. Therefore, there is a need to broaden the range of ecorelevant conditions under which adaptive plasticity is assessed to more precisely quantify and accurately understand the adaptive value of a particular trait. This study aims to investigate the adaptive value of a well-studied predator-induced defense in larval treefrogs in the context of interspecific interactions with a non-plastic competitor and shared predator.

Predators significantly shape ecosystem dynamics and can drive interspecific interactions by altering community composition via prey consumption and indirect effects on prey traits (McCoy et al. 2012, Peacor and Werner 2001, Pressier et al. 2007). Avoiding depredation is a universal selective pressure for prey species. Predator-induced plasticity has been shown to be adaptive relative to noninduced conspecifics in numerous field and empirical studies (McCollum and Buskirk 1996, Warkentin 1995, Auld and Relyea 2011, Lüning 1992). However, most experiments on predator-induced plasticity have been conducted in an

"ecological vacuum" -- not accounting for other biotic and abiotic factors that may influence an inducible phenotype's net adaptive value. Here, we expand on the existing body of plasticity-related research to include the presence of non-plastic heterospecifics as an ecological factor that may augment benefits or exacerbate costs associated with phenotypic induction. We showed that *H. femoralis* tadpoles with predator-induced morphology performed better than noninduced conspecifics even in a mixed species assemblage with a non-plastic heterospecific present.

*Hyla femoralis* tadpoles exposed to chemical cues of dragonfly nymph predation developed a different morphology than tadpoles reared in a predator-free environment. Predator-exposed *H. femoralis* tadpoles developed longer, deeper tail fins compared to tail fins of predator-free *H. femoralis* (Table 1, Figure 4). The presence of deeper tail fins is consistent with phenotypic responses observed in other studies of *H. femoralis* and other related species of Hyla (LaFiandra and Babbit 2004, McCollum and Buskirk 1996, Richardson 2006). We acknowledge our observation of increasing tail fin lengths in induced *H. femoralis* is in direct contrast with the findings of LaFiandra and Babbit (2004), who reported the shortening of tail fins in induced *H. femoralis* treatments. This observed divergence in tail length from our induced *H. femoralis* populations can perhaps be attributed to differences in morphometric analytical methods. Some studies utilize allometric measurements of tadpole body and tail morphology, converting measurements of tail length, body length, tail depth, etc., to more overarching/universal measures of "body size" and "tail shape" (Richardson 2006, LaFiandra and Babbit 2004, Touchon and Warkentin 2008). We propose that perhaps our approach of utilizing standardized landmarks and a geometric morphometric analysis may more precisely capture individual-level variation in body and tail morphology, yielding a more robust analysis of phenotypic responses to larval environment (Sherratt et al. 2018). Additionally, Kruger and Morin (2020) found that

the timepoint at which morphometrics are gathered is significant. Tail fin depth measurements decreased overtime in induced *Hyla andersonii* (Pine Barrens Treefrog) tadpoles, suggesting that observed experimental variation in tail morphology may reflect the developmental timepoints at which researchers are chosing to obtain morphometric measurements (Kruger and Morin 2020).

Induced *H. femoralis* that responded to the chemical cues of predation experienced higher survivorship in subsequent encounters with lethal predators compared to predator-naïve tadpoles (Figure 5). However, induced *H. femoralis* tadpoles also took longer to reach metamorphosis than did control tadpoles when no lethal predator was present (Figure 8). This "individual investment" in the development of the anti-predator morphology may have indeed incurred a slight cost to development, prolonging metamorphosis in exchange for enhanced survival in a stressful larval environment. Such phenotypic fitness tradeoffs are commonly noted and may prevent canalization of the phenotype and loss of phenotypic plasticity (LaFiandra and Babbit 2004, McCollum and Buskirk 1996, Relyea 2004, Levis and Pfennig 2019).

Importantly, we also observed that fitness benefits of the predator-induced phenotype in *H. femoralis* were also realized in mixed-species assemblages with *H. squirella*. Predator-induced *H. femoralis* experienced enhanced survival in subsequent encounters with predators when morphologically non-plastic *H. squirella* was also present (Figure 5). In fact, there was a greater difference in survival probability observed when comparing noninduced vs. induced *H. femoralis* survival (difference of .008) in mixed-species assemblages, compared to the difference in survival probability observed in single-species assemblages (.006). This observation suggests that adaptive benefits of the predator-induced phenotype were not only maintained against predator-naïve conspecifics but were also apparent with congenerics and a shared predator. Interestingly, the presence of *H. squirella* did not affect time-to metamorphosis for *H. femoralis*

(Figure 8). This result suggests that the adaptive value of predator-induced tadpole phenotypes may be advantagious over noninduced conspecifics as well as over non-plastic, mixed species assemblages in a shared predator community—typical of most ephemeral pond ecosystems. These results bolster the generality of previous studies focused on evaluating the relative costs and benefits of predator-induced plasticity between induced versus noninduced conspecifics within a single species context.

Although we expected there might be differences in tadpole size associated with induced verus noninduced phenotypes for *H. femoralis,* we did not observe significant differences in our study. However, our results are consistent with other indoor aquaria experiments (Relyea 2001, Beck 1997, see Figures 6 and 7) and likely stem from high resource availability and highly stable conditions, typical of aquarium experiments. Regular feeding and frequent water quality maintenance provided suitable conditions for tadpoles to achieve optimal size prior to metamorphosis. There were, however, differences in time-to metamorphosis for *H. femoralis* tadpoles exposed to predator chemical cues (Figure 8). Predator-induced tadpoles took longer to reach metamorphosis than noninduced tadpoles, which likely stems from diverting energy and resources towards anti-predator morphologies early in development (LaFiandra and Babbit 2004, Buskirk 2000, Gonzalez et al. 2011). Stressful larval environments often disrupt developmental timelines and time to metamorphosis in anuran larvae (Wilbur 1987, Pfennig and Murphy 2000, Relyea 2003). The presence of a congener (*H. squirella*) did not affect time-to metamorphosis in *H. femoralis* (Figure 8), suggesting that predator-prey interactions had a larger influence on fitness correlates than did the presence of a competitor. Although this study was not designed to assess non-morphological responses to predators by *H. squirella,* comparing phenotypic

responses and fitness tradeoffs between the two species could be an important subject for follow-up studies.

This work highlights the value of expanding ecologically-relevant contexts under which adaptive plasticity is evaluated and quantified. Indeed, our results suggest that the adaptive value of predator-induced phenotypes in lethal environments may be underappreciated, and their costs might be overestimated without considering theses costs and benefits relative to other competitor species that share habitat. Given that induced *H. femoralis* survival in mixed-species assemblages remained relatively high over time (compared to survival of all other *H. femoralis* in lethal treatments), one may argue that the adaptive benefit of increased survival in induced *H. femoralis* in a multispecies context with a common predator outweighed any subsequent growth or development costs. This study offers a broader perspective to the onset and maintenance of adaptive plasticity in natural systems. We suggest that assessing the adaptive value of anti-predator morphology in mixed species assemblages provides a more robust exploration and more accurate understanding of adaptive plasticity and its evolutionary maintainance in complex, dynamic communities.
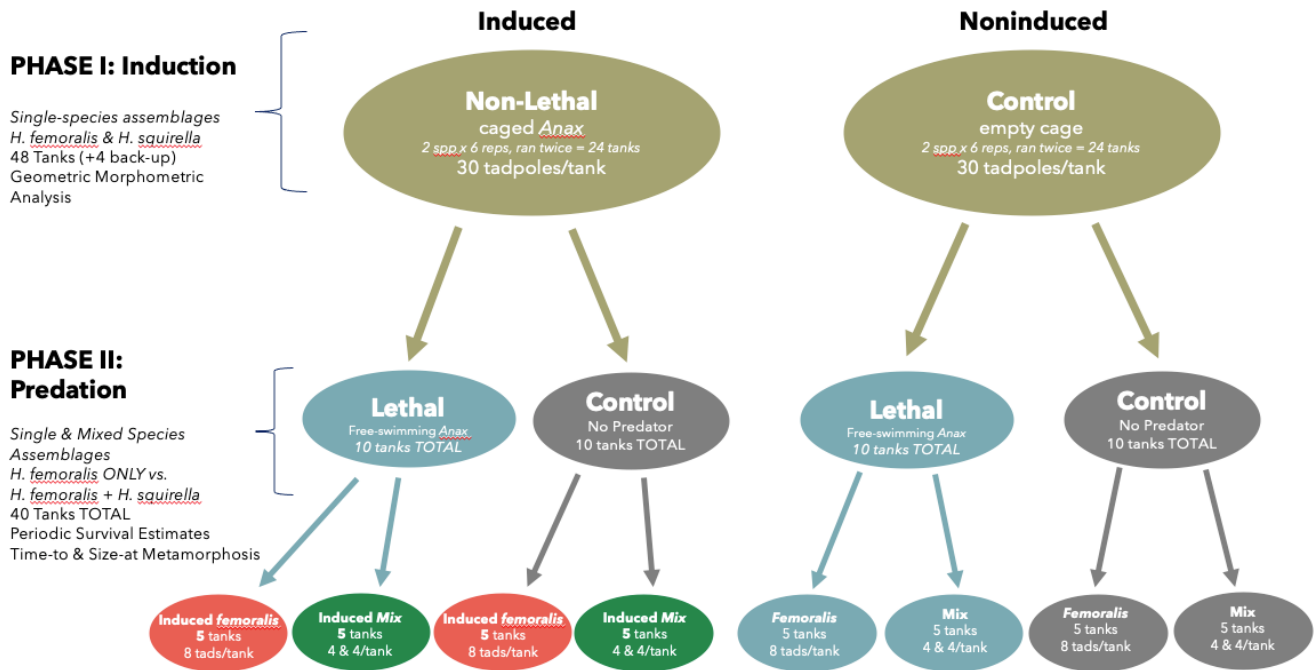
**Figure 1 :** Phylogeny of the hyline tribe Hylini based on maximum likelihood analysis of combined nuclear and mitochondrial genes. Numbers adjacent to nodes indicate bootstrap values > 50% (Wiens et al 2010).

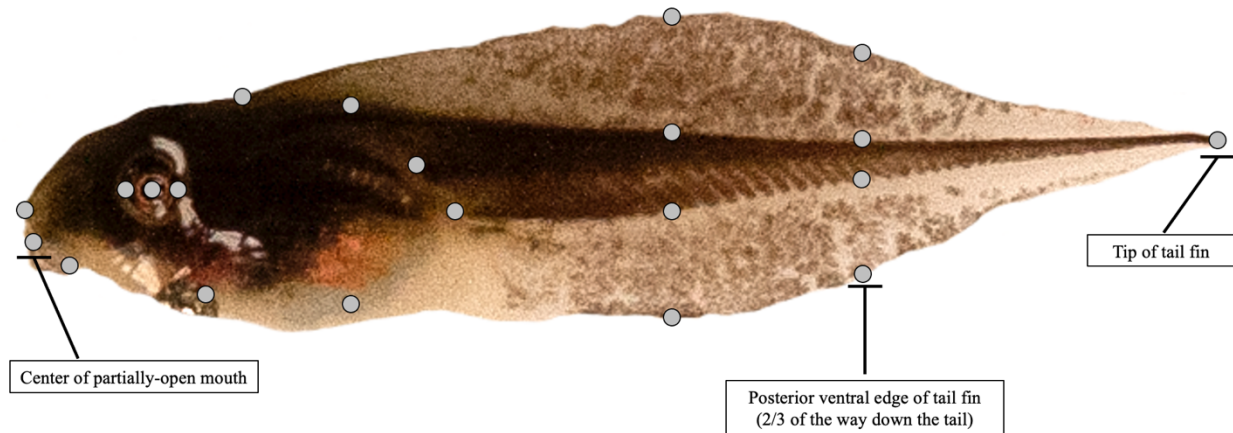**Figure 2**: Illustration of experimental design and replication scheme.

**Figure 3**: Diagram of all twenty-one selected lateral landmarks on an *H. femoralis* tadpole. Landmarks referring to major principal components (from the conducted PCA on Procrustes shape coordinates) are labeled with morphological descriptions.
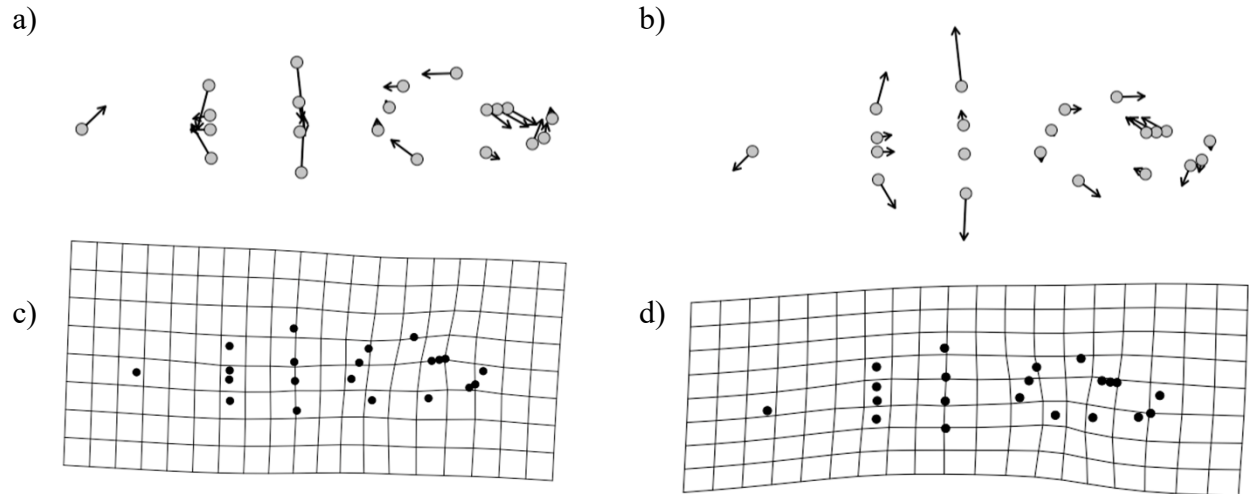
a)

b)

c)

d)

**Figure 4:** a) Overall *H. femoralis* consensus shape (gray dots) with vectors pointing in directions of growth towards the noninduced (control) morphology. b) Overall *H. femoralis* consensus shape with vectors pointing in directions of growth towards the induced (non-lethal) morphology. c) Deformation grid plot of noninduced (control) *H. femoralis* morphology.
 d) Deformation grid plot of induced (non-lethal) *H. femoralis* morphology. Grid squares are warped in morphospace as landmarks oriented on *H. femoralis* consensus shape (determined by Procrustes-corrected coordinates) move toward the target morphology associated with induction treatments.

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| Induction Treatment | Principal Component Axis | Mean PC Score | Morphological Changes (per direction/sign of PC score) | Overall Shape Indication |
|---|---|---|---|---|
| Non-lethal | PC1 | 0.00502 | Posterior ventral edge of tail fin (2/3 down the tail) moving anteriorly, medially | Deeper tail fin |
| | PC2 | 0.00355 | Posterior ventral edge of tail fin (2/3 down the tail) moving laterally, down and away from the mid-tail fin region | Deeper tail fin |
| | PC3 | 0.00667 | Tip of tail fin lengthening posteriorly | Longer tail fin |
| | PC4 | -0.00099 | Center of partially-open mouth moving medially | Narrowing of the head capsule |
| Control | PC1 | -0.00585 | Posterior ventral edge of tail fin (2/3 down the tail) moving posteriorly, laterally | Shallower tail fin |
| | PC2 | -0.00414 | Posterior ventral edge of tail fin (2/3 down the tail) moving medially, towards the mid-tail fin region | Shallower tail fin |
| | PC3 | -0.00780 | Tip of tail fin shortening anteriorly | Shorter tail fin |
| | PC4 | 0.00115 | Center of partially-open mouth moving laterally | Widening of head capsule |

**Table 1:** Table of mean (average) PC scores per treatment group, along with morphological characteristics/descriptions for the top four principal axes describing 59.12% of the variation in *H. femoralis* morphology. PC scores generated using Procrustes shape coordinates.
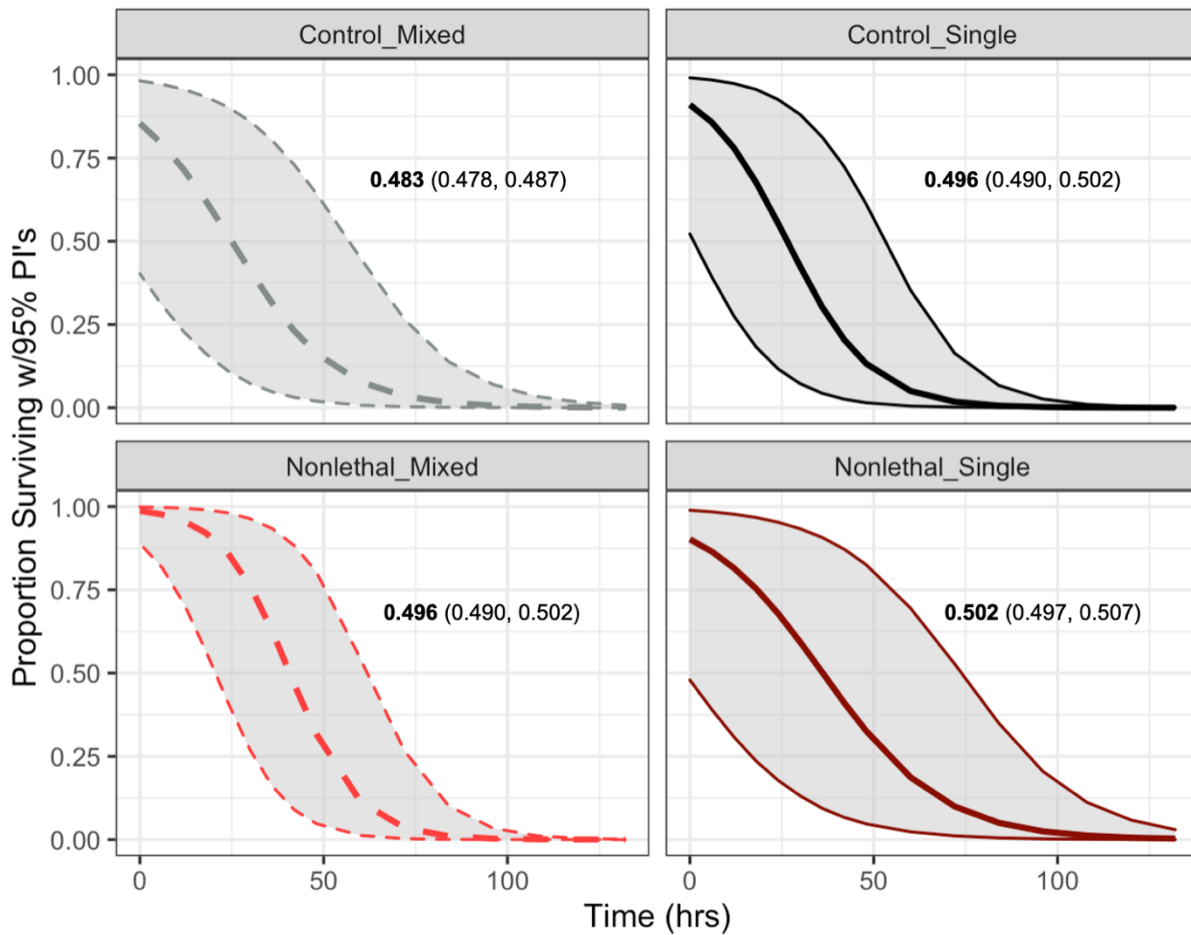
**Figure 5 :** Proportion of *H. femoralis* surviving overtime across all Phase II Predation treatment combinations (induction treatment x assemblage type). Model coefficient estimates for survival probability displayed with 95% prediction intervals and confidence envelopes.
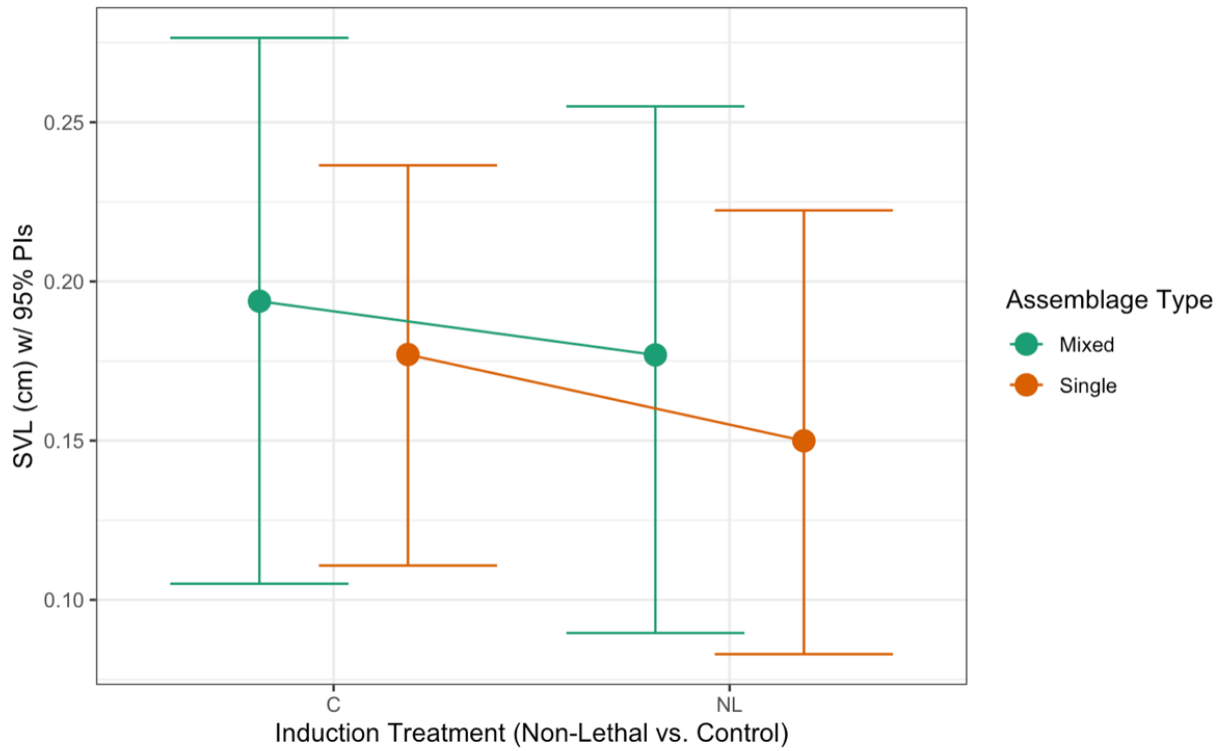
**Figure 6 :** Growth response of *H. femoralis* Snout-Vent Length's (SVL, in centimeters) to both main effects of induction and assemblage treatments. Estimates from Bayesian linear mixed models with 95% prediction intervals.
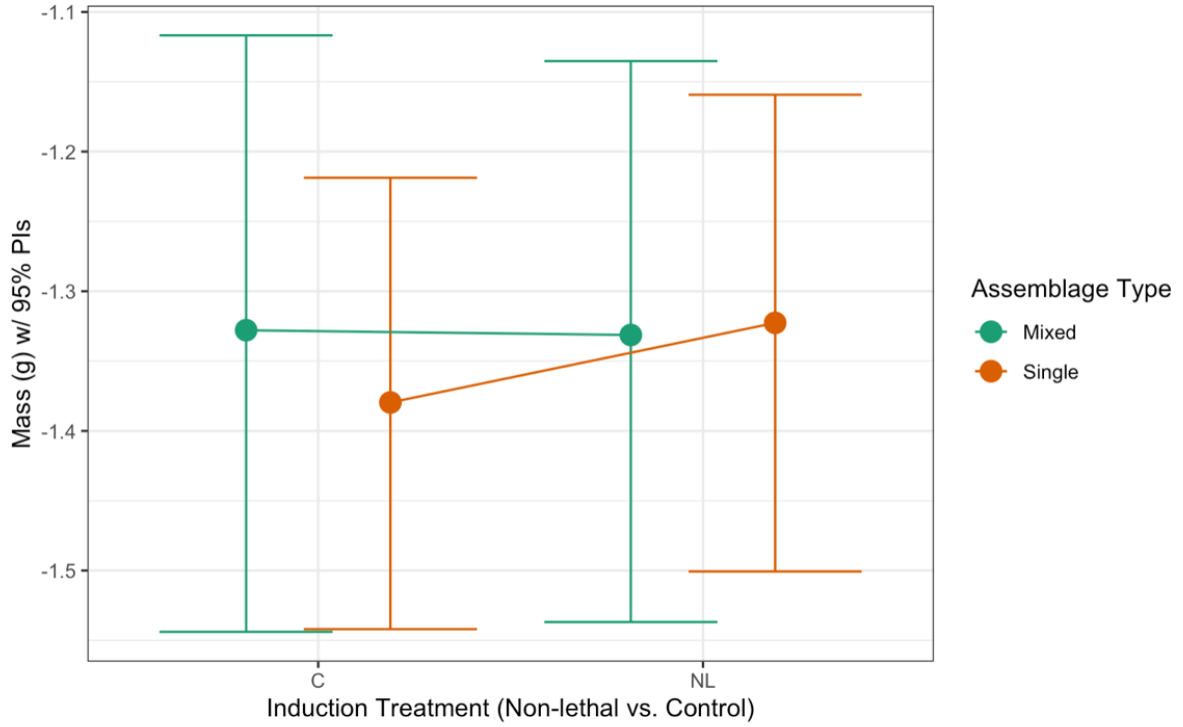
**Figure 7 :** Growth response of *H. femoralis* emergence-day mass (in grams) to both main effects of induction and assemblage treatments. Estimates from linear mixed models with 95% prediction intervals.
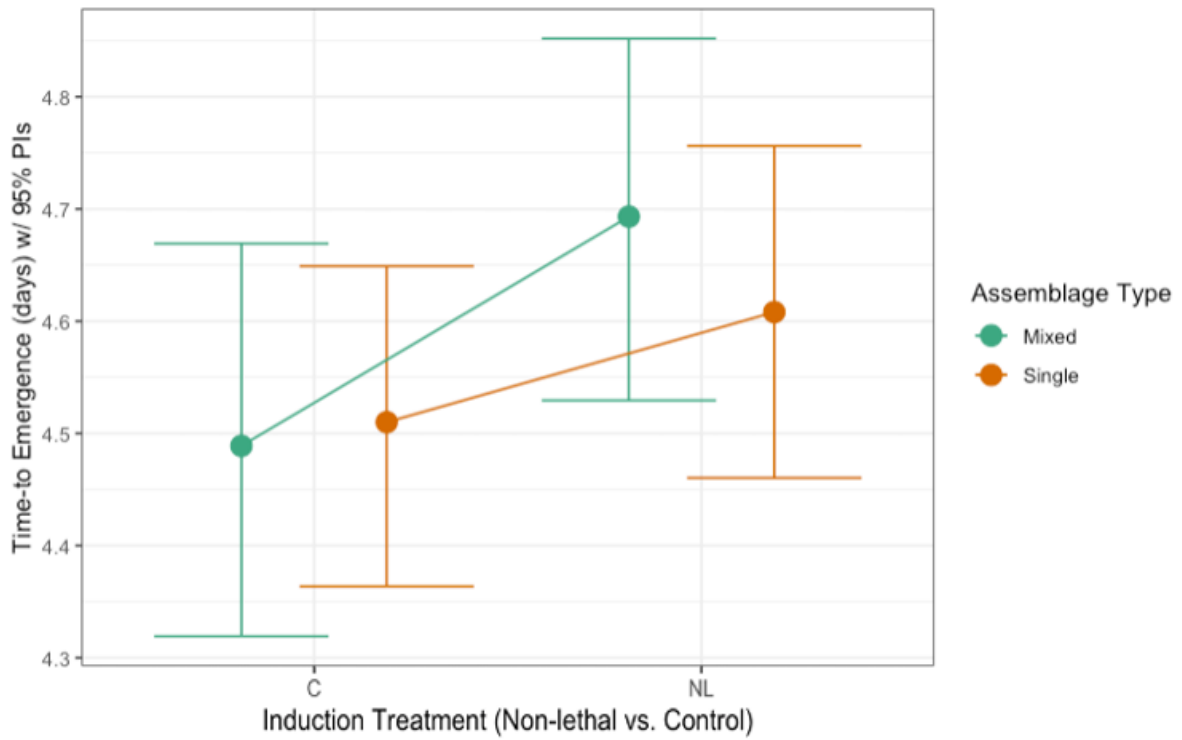
**Figure 8 :** Developmental response of time-to emergence (in days) to both main effects of induction and assemblage treatments. Estimates from Bayesian linear mixed models with 95% prediction intervals.

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**ECU**

June 26, 2020

Michael McCoy, Ph.D.
Department of Biology, ECU

Subject: Protocol D363, original approval date 04/27/2020

Dear Dr. McCoy:

The amendment to your Animal Use Protocol entitled, "Context Matters: Evaluating the Adaptive Benefits of Phenotypic Plasticity in a Community Context using Local *Hyla* " (AUP#D363) was reviewed by this institution's Animal Care and Use Committee on 06/25/2020. The following action was taken by the Committee:

   "Approved as submitted"

**\*\*Please contact Aaron Hinkle prior to any hazard use\*\***

A copy of the protocols is enclosed for your laboratory files.  Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol.  Modifications of these procedures cannot be performed without prior approval of the ACUC.  The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.**

Sincerely yours,

*B McRae*

Sue McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/GD

enclosure