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THE ART OF WAR: PATTERNS AND MECHANISMS UNDERLYING PREDATOR-INDUCED PLASTICITY OF AMPHIBIANS

by

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University of Pittsburgh, 2013

Organisms often employ phenotypic plasticity as a strategy to cope with variable environments. This is particularly true of predation threats, wherein prey induce defenses to reduce detection or capture by predators. In order to produce appropriate defenses, prey must be able to discern useful information from environmental cues. Despite the pervasive production of inducible defenses, we understand very little of how much useful information is conveyed to organisms in cues, or how the subsequent plastic responses vary within groups of organisms.

To address the need for comparative studies of phenotypic plasticity, we sought to examine morphological and behavioral defenses of five species of *Ambystoma* salamander larvae in response to larval dragonfly (*Anax junius*) chemical cues in a common garden environment. Dragonfly cues induced relatively few morphological changes across species. Likewise, salamanders did not vary in their refuge use during the experiment, though several species reduced their activity in the presence of predators early in development. Our results suggest that behavioral and morphological defenses in *Ambystoma* are highly variable among species and the genus appears to be less plastic than tadpoles and other salamander species.

To understand what types of information prey are capable of responding to in their environment, we raised grey treefrog tadpoles (*Hyla verisciolor*) in the presence of cues isolated from different stages of an attack sequence by larval dragonflies (*A. junius*) or larval dragonflies

consuming different combinations of grey treefrog tadpoles and snails (*Helisoma trivolvis*) across different temporal sequences. When exposed to a predator consuming grey treefrogs, tadpoles reduced their activity, increased their hiding behavior, and induced deeper tails. As we exposed prey to more types of cues from an attack sequence, they also increased tail depth and hiding behavior but did not change their activity. Additionally, treefrog tadpoles generally increased their defense as the biomass of treefrogs consumed increased, regardless of whether heterospecifics were being consumed. Our results suggest that treefrogs can gain cue information from all portions of an attack sequence, and that both temporal patterns of feeding and diet content of predators influence the type and magnitude of induced prey defenses.

TABLE OF CONTENTS

PREFACE ..... **XIII**

1.0 INTRODUCTION **1**

2.0 PREDATOR-INDUCED DEFENSES IN FIVE SPECIES OF *AMBYSTOMA* SALAMANDERS **4**

2.1 INTRODUCTION **4**

2.2 MATERIALS AND METHODS **9**

2.2.1 Evolutionary history **9**

2.2.2 Natural history of the five salamander species **9**

2.2.3 Animal collection and rearing **10**

2.2.4 Experimental design **11**

2.2.5 Statistical analysis **13**

2.3 RESULTS **15**

2.3.1 Responses of *A. barbouri.* **15**

2.3.2 Response of *A. gracile*. **15**

2.3.3 Response of *A. laterale*. **16**

2.3.4 Responses of *A. maculatum*. **16**

2.3.5 Responses of *A. tigrinum*. **18**

2.4 DISCUSSION **18**

..... 28

3.0 THE SMELL OF PREDATION: HOW LARVAL *HYLA VERSICOLOR* RESPOND TO FINE-SCALE DIFFERENCES IN PREDATOR CHEMICAL CUES 29

3.1 INTRODUCTION 29

3.2 MATERIALS AND METHODS 35

3.2.1 Experimental design 35

3.2.2 Statistical analysis 39

3.3 RESULTS 41

3.3.1 Do the prey respond to predators? 43

3.3.2 Do prey defenses increase as prey gain information from a predator attack sequence? 44

3.3.3 Do prey defenses increase as the biomass of consumed conspecifics increases? 45

3.3.4 Do prey respond to temporal variation in predator consumption? 46

3.3.5 Additional responses to the predator treatments. 47

3.4 DISCUSSION 47

3.4.1 Do prey defenses increase as prey gain information from a predator attack sequence? 49

3.4.2 Do prey defenses increase as the biomass of consumed conspecifics increases? 50

3.4.3 Do prey respond to temporal variation in predator consumption? 54

3.5 CONCLUSIONS 56

4.0 OVERARCHING CONCLUSIONS 58

BIBLIOGRAPHY 61

LIST OF TABLES

Table 1. Percent survival for five species of Ambystoma salamanders in the presence and absence of aeshnid predator cues. Values are percent of individuals alive at the end of the experiment from the initial 48 animals 14

Table 2. Mann Whitney U test results for activity and refuge use of five species of Ambystoma salamanders in the presence or absence of aeshnid predator cues. Activity and refuge use measurements were collected on days 7, 14, 21, and 28. For each species, U values are the top row and p values are the bottom row. *Significant at α = 0.05 based on a sequential Bonferroni correction. 17

Table 3. Comparison of animal sizes among this study and two other studies. *Study did not list initial or final mass; values were estimated from figures. 22

Table 4. A comparison of hypotheses regarding the outcome of cue source and content on prey induced defenses. Predictions are rank ordered by strength of induced response. 36

Table 5. Results of a rm-ANOVA on (A) the number of gray treefrog tadpoles observed and (B) the activity of the tadpoles when exposed to 13 predator-cue treatments. Bold fonts indicates statistical significance at α = 0.05. Asterisks indicate Greenhouse-Geisser corrected values used for analyses that violated assumptions of sphericity..... 42

Table 6. Results of a MANOVA on mass and mass-independent morphological dimensions of gray treefrog tadpoles when exposed to 13 predator-cue treatments. Bold font indicates statistical significance at α = 0.05. 43

LIST OF FIGURES

Figure 1. Phylogeny of Ambystoma species of North America North of Mexico, as recognized by the Committee on Standard English and Scientific Names. 8

Figure 2. Refuge use (mean ± SE) for five species of Ambystoma salamanders in the presence (closed circles) and absence (open circles) of aeshnid predator cues. Values are the grand means ± SE of behavioral assays averaged across 3 binary observations (using refuge = 1, out of refuge = 0) for each individual on each of days 7, 14, 21, and 28. 26

Figure 3. Morphological variables for five species of Ambystoma salamanders in the presence (closed circles) and absence (open circles) of aeshnid predator cues. Values are the grand means ± SE of behavioral assays averaged across 3 binary observations (using refuge = 1, out of refuge = 0) for each individual on each of days 7, 14, 21, and 28. 26

Figure 4. Morphological variables for five species of Ambystoma salamanders in the presence (closed circles) and absence (open circles) of aeshnid predator cues.Values are mass-independent treatment means ± SE of animals measured at the end of the experiment on day 28. 28

Figure 5. (A) Number of observed gray treefrog tadpoles and (B) their activity when raised in 13 predator-cue treatments. Data are the least squared means ± SE of 27 observations made over 3 d. Data are grouped by hypotheses; therefore, some treatments are repeated within panels. * indicates significance at α = 0.05; + indicates significance at α = 0.10. 52

Figure 6. Mass-independent morphological dimensions for gray treefrog tadpoles when raised in 13 predator-cue treatments: (A) tail depth, (B) body length, and C) body depth. Data are means ± SE of animals measured at the end of the experiment on day 16. Data are grouped by hypotheses; therefore, some treatments are repeated within panels. * indicates significance at α = 0.05. 53

PREFACE

There are countless people to whom I owe thanks for helping me get to the end of this degree. Starting with technical help, I am grateful to Tim Sesterhenn, Julia Buck, Jason Hoverman, and Jeff Briggler for their assistance in collecting *Ambystoma* eggs in other states. I also want to thank the ESGR for allowing me access to their facilities; Devin Jones, Chris Davis, John Hammond, Rickey Cothran, and Jessica Hua for assistance with field collection; and Maya Groner for her assistance with animal care. Additionally, I owe a debt of gratitude to Jenise Brown, Emily Cholak, and Sasha Rhode for their assistance on countless nights finding breeding treefrogs out in the wetspot in the dark; [undergrads/grads—I don't have my experiment cover sheet on me] for their assistance with behavioral observations; Aaron Stoler, Jessica Hua, Will Brogan, RJ Bendis, Devin Jones, and Rickey Cothran for their feedback on the *Hyla versicolor* manuscript while it was still in preparation.

Personally, I am prone to verbosity and absurd sentiment, so I will keep my acknowledgements brief. My labmates have been invaluable friends and colleagues over the past five years, and I am grateful for their dealings with my madness and their help in keeping me sane and grounded. Jessica and Will have been with me since the start and I am sorry to leave them early, and I literally do not know where I would have ended up in these last few years if it were not for Maya's generosity. My roommates have been an incredible force of good in my life,

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1.0 INTRODUCTION

Organisms rarely live in static environments and instead must deal with contexts that change over time and space. This is particularly true of threats from predators, and prey may rely on inducible defenses to reduce detection or capture by predators (West-Eberhard 1989, Harvell 1990). Inducible defenses are ubiquitous among organisms and have been identified in a wide range of protists, plants, and animals (Bradshaw 1965, Lüning 1992, Karban and Baldwin 1997, Belk 1998, Van Donk et al. 1999, Peckarsky et al. 2001, Kopp and Tollrian 2003, Petrusek et al. 2009). However, despite the pervasive production of inducible defenses, we know relatively little about how plasticity varies within groups of organisms (but see Relyea 2001, Van Buskirk 2002a, Orizaola and Brana 2004, Schmidt and Van Buskirk 2005).

While broad comparisons of plasticity are useful in helping us generalize across organisms, the cues that actually induce defenses within individuals are equally important. To employ phenotypic plasticity as a strategy to cope with variable environments, organisms must be able to discern useful information from within a potentially noisy environment of multi-sensory cues (Kats and Dill 1998, Burks and Lodge 2002). Prey regularly rely on chemical cues that are produced during predation events to establish the risk level of their environment and respond accordingly (Nolte et al. 1994, Dicke and Grostal 2001, McCarthy and Fisher 2008). If they are unable to assess this information with speed and accuracy, there is a high likelihood that prey will develop inadequate defenses (DeWitt et al. 1998, Donaldson-Matasci et al. 2010) at the

risk of death (Harvell 1990, Skelly 1992, Kats and Dill 1998, Dicke and Grostal 2001).

There is an abundance of chemical information available to prey in their environment, and many have access to information in cues not directly intended for them. Considering the volume of potential indirect cues available in any chemically noisy environment, there is a substantial opportunity for prey to modify their defenses based on information they were never meant to receive (Magalhães et al. 2005, Wisenden and Chivers 2006, Dalesman et al. 2007a, Schaefer and Ruxton 2012). However, we rarely understand how much useful information is conveyed to organisms in these indirect cues (Bourdeau 2010, Donaldson-Matasci et al. 2010).

In amphibians, larval anurans have been widely used in the study of inducible defenses and we have a good understanding of what defenses they generally induce in response to predators (Skelly 1994, Anholt and Werner 1995, Lima 1998, Relyea and Werner 1999, Relyea 2001, 2003, Van Buskirk et al. 2003, Benard 2004, Kishida and Nishimura 2005). However, we have far fewer data on induced defenses in salamanders (Storfer and Sih 1998, Van Buskirk and Schmidt 2000, Orizaola and Brana 2003) including comparative studies of their defenses (Orizaola and Brana 2004, Scmidt and Van Buskirk 2005). There is also a significant body of data which indicates that tadpoles also have some ability to fine-tune their defenses based on the cues in their environment (Wilson and Lefcort 1993, Schoeppner and Relyea 2009a,b,c).

To address the need for both comparative studies of phenotypic plasticity and more data about how salamander defenses differ from those of anurans, we sought to examine morphological and behavioral defenses of five species of *Ambystoma* salamander larvae in response to the chemical cues of larval dragonflies (*Anax junius*). To better understand what types of indirect information prey are capable of responding to in their environment, we raised grey treefrog tadpoles (*Hyla verisciolor*) in the presence of cues isolated from different stages of an attack sequence by larval

dragonflies (*A. junius*) or larval dragonflies consuming different combinations of grey treefrog tadpoles and snails (*Helisoma trivolvis*) across different temporal sequences.

2.0 PREDATOR-INDUCED DEFENSES IN FIVE SPECIES OF *AMBYSTOMA* SALAMANDERS

2.1 INTRODUCTION

While predator-induced plasticity has been demonstrated in wide range of organisms, relatively few data exist to compare differences among species. In studies of predator-induced plasticity in amphibians, larval anurans have been widely examined, but there are fewer data for larval salamanders. We sought to examine morphological and behavioral defenses in five species of *Ambystoma* salamander larvae. We raised five species of larval mole salamanders (*A. barbouri, A. gracile, A. laterale, A. maculatum, A. tigrinum*) in a common garden environment and exposed them to predator cues from larval dragonflies (*Anax junius*). Salamanders did not vary in their refuge use during the experiment, but *A. gracile, A. laterale,* and *A. tigrinum* reduced their activity in the presence of predators early in development. Dragonfly cues induced relatively few morphological changes across species: *A. barbouri* developed relatively large heads and deep tails, *A. gracile* and *A. laterale* developed relatively shorter heads, and *A. maculatum* developed relatively wider heads and shorter tails. Our results suggest that behavioral and morphological defenses in *Ambystoma* are highly variable among species and they appear to be less plastic than tadpoles and other salamander species.

"Eat or be eaten," is a fundamental tradeoff for organisms, a problem which many prey mediate through inducible defenses. Inducible defenses are phenotypically plastic responses that organisms develop in response to predators to avoid detection, capture, or consumption (West-Eberhard 1989, Harvell 1990). This predator-induced plasticity exists in a wide range of organisms, including both terrestrial and aquatic plants (Bradshaw 1965, Karban and Baldwin 1997, Van Donk et al. 1999), protists (Kopp and Tollrian 2003), and numerous species of animals (Lüning 1992, Belk 1998, Peckarsky et al. 2001, Petrusek et al. 2009). The ubiquitous nature of inducible defenses has produced a dramatic range of antipredator strategies that have allowed researchers to make generalizations and predictions of what defenses organisms will produce and under what conditions.

In amphibians, larval anurans have been widely used in the study of inducible defenses and we have a good understanding of what defenses larval anurans employ in regard to predator identity, predator density, and predator diet. For example, behavioral responses typically consist of reduced activity and increased refuge use or hiding (Skelly 1994, Anholt and Werner 1995, Lima 1998, Relyea and Werner 1999, Benard 2004). Morphological responses are often characterized by relatively shorter bodies and deeper tailfins (Relyea 2001, 2003b, 2004; Van Buskirk et al. 2003, Kishida and Nishimura 2005). There are exceptions to this pattern, most notably *Rana pirica* tadpoles, which develop an enlarged, bulgy body and a deep tailfin in the presence of cues from *Hynobius* salamanders (Kishida and Nishimura 2004, Kishida et al. 2006). Predators can also induce changes in life history traits, including time to and size at metamorphosis (Werner 1986, Skelly and Werner 1990, Bendard 2004, Relyea 2007).

Like tadpoles, aquatic larval salamanders employ antipredator strategies to deal with predation pressure from both heterospecific predators and cannibalistic conspecifics. Predators

include a variety of invertebrates, fish, reptiles, tadpoles, and both conspecific and heterospecific salamanders (Petranka 1998, Holbrook et al. 2004, Burley et al. 2006, Urban 2008, 2010, Pearson and Goater 2009, Hopkins and Migabo 2010, Wilson and Hopkins 2011). Although aquatic larval salamanders commonly become intermediate or top predators in aquatic communities later in ontogeny (Davic and Welsh 2005, Hopkins et al. 2011), they can suffer high predation rates early in development (Stenhouse 1985, Peterson et al. 1991).

Many studies on inducible defenses in larval salamanders focus on embryonic and very early larval development (Sih and Moore 1993, Moore et al. 1996, Orizaola and Brana 2004, Warkentin 2011) although several have examined behavioral and morphological defenses in older larvae. Studies of behavioral responses to predators find that salamanders respond to predators in many of the same ways as larval anurans; salamanders decrease activity and increase refuge use (Storfer and Sih 1998, Van Buskirk and Schmidt 2000, Garcia and Sih 2003, Orizaola and Brana 2003, Hoffman et al. 2004, Yurewicz 2004). Some species also exhibit spatial and temporal avoidance of predators (Mathis et al. 2003, Garcia et al. 2009). Those that move in the presence of predators are most likely to swim away from the source of the predator cue (Wells and Harris 2001) or to avoid hypoxic conditions associated with reduced movement (Iwami et al. 2007). With this information we can begin to make predictions about the antipredator behavior other salamander species may use.

Studies of morphological responses to predators suggest antipredator responses among species are highly variable. In crested newts, cues from aeshnid dragonflies induce multiple changes in the larvae, inducing relatively shorter and deeper tailfins, wider and deeper heads, and deeper bodies (Van Buskirk and Schmidt 2000,Schmdit and van Buskirk 2005, Van Buskirk 2009). In the Ezo salamander (*Hynobius retardatus*), aeshnid dragonflies induce deeper tail fins

(Hangui et al. 2009) and larger gills (Iwami et al. 2007). In mole salamanders, aeshnid dragonfly and *Dytiscus* beetles induce relatively deeper tailfins, shorter bodies, and longer tail muscles (Storfer and White 2004, Yurewicz 2004). Moreover, Texas (*A. texanum*), streamside (*A. barbouri*), and long-toed (*A. macrodactylum*) salamanders can induce color changes that aid in camouflage and defenses based on hiding behavior when exposed to fish or other predatory newts (Garcia and Sih 2003, Garcia et al. 2009).

There is no clear pattern of life history defense responses from aquatic larval salamanders. Evidence indicates that some species will reduce time and/or size at metamorphosis (*A. talpoideum* and *A. maculatum*, Semlitsch 1987; *Eurycea wildrae*, Beachy 1997), particularly with embryonic predator exposure (*T. helveticus*, Orizaola and Brana 2005), whereas others grow faster and or metamorphose larger in the presence of predators (*Eurycea wildrae*, Beachy 1997; *T. alpestris*, Van Buskirk and Schmidt 2000). Additionally, predator presence reduces the incidence of paedomorphism (Jackson and Semlitsch 1993).

Given that data on larval salamander defenses against predation are limited to only a few genera and are often incomplete for any single species, we sought to examine a full complement of morphological and behavioral defenses in the streamside (*A. barbouri*), Northwestern (*A. gracile*), blue-spotted (*A. laterale*), spotted (*A. maculatum*), and tiger (*A. tigrinum*) salamanders. In doing so, we tested the following hypotheses: (1) species should induce the same defensive traits and respond to predator cues by altering their traits in the same direction, and (2) species will vary in their magnitude of predator defenses.

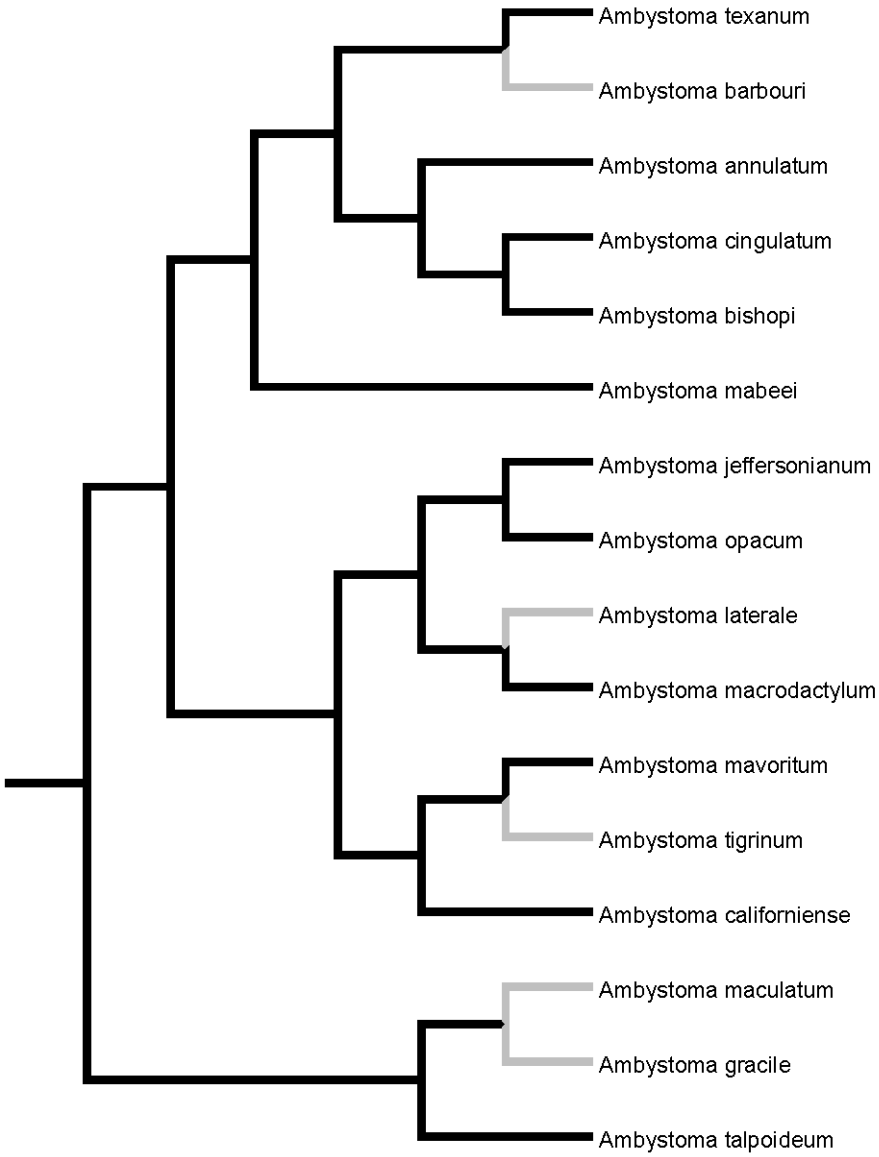

Figure 1. Phylogeny of Ambystoma species of North America North of Mexico, as recognized by the Committee on Standard English and Scientific Names. Summarized from Shaffer et al. (1991), Shaffer and McKnight (1996), and Pauly, Piskurek, and Shaffer (2007). Tree is not drawn to scale.

2.2 MATERIALS AND METHODS

2.2.1 Evolutionary history

Based on mitochondrial DNA, data suggest that Ambystomatidae first diverged from Salamandridae roughly 145 MYA, very early in the Early Cretaceous, and from their sister taxon Dicamptodontidae approximately 100 MYA, late in the Early Cretaceous (Zhang and Wake 2009). However, divergence times for individual species are much harder to discern. *A. maculatum* and *A. tigrinum* first appear in the fossil record in the Lower Pliocene (Holman 1975), suggesting an earlier divergence time. Mitochondrial data from Bi and Bogart (2010) suggest that *A. tigrinum* diverged from *A. laterale* and *A. barbouri* in the Oligocene, approximately 23.5 MYA, while *A. laterale* and *A. barbouri* themselves diverged in the Miocene, approximately 20 MYA. Unfortunately, the evolutionary history of *Ambystoma* remains ambiguous due to conflicting results among studies utilizing different combinations of molecular and morphological data (Petranka 1998; Fig. 1).

2.2.2 Natural history of the five salamander species

The five species of *Ambystoma* we selected are similar in their larval natural history (Petranka 1998 and references therein). Semi-permanent and ephemeral ponds in quarries, pastures, and woodlands are the primary breeding sites for *A. gracile*, *A. laterale*, *A. maculatum*, and *A. tigrinum*, although *A. gracile* and *A. tigrinum* may also be found in roadside ditches and lakes. While *A. barbouri* prefers to breed in the pools, runs, and riffles of 1st and 2nd order streams, they

too can be found opportunistically in quarry and farm ponds. Breeding period varies depending on latitude and climate, but typically *A. barbouri* and *A. maculatum* breed between December and April, *A. gracile* and *A. tigrinum* from February to April, and *A. laterale* from March to May. Egg laying mode varies among the species, with *A. gracile*, *A. maculatum*, and *A. tigrinum* laying egg masses, *A. barbouri* laying eggs in a single monolayer, and *A. laterale* laying eggs singly or in small clusters (Petranka 1998, and references therein).

The size of individual eggs varies between 1.5 mm to nearly 4.0 mm. *A. barbouri* and *A. tigrinum* consistently have the largest eggs, while *A. laterale* have the smallest. The eggs of all five species hatch within 1 to 2 months, depending mostly on temperature, but the length of the larval period varies considerably among species. *A. barbouri* metamorphose in 1.5 to 2.5 months, *A. laterale*, *A. maculatum*, and *A. tigrinum* metamorphose in 2 to 3 months, with *A. tigrinum* taking up to 5 months in some cases, and *A. gracile* requires 12 to 14 months. Size at metamorphosis is also variable, and is known to be plastic in *A. maculatum*, but *A. tigrinum* are expected to be the largest by both snout-vent length and mass (Petranka 1998, and references therein).

2.2.3 Animal collection and rearing

Newly laid egg masses from each of the five salamander species were collected from ponds and shipped overnight or transported to the Pymatuning Laboratory of Ecology in Linesville, PA. To maximize genetic diversity, eggs were collected from at least 10 different females for each species, and we assume these females will be representative of the entire population. *A. tigrinum* eggs were collected from the Edwin S. George Reserve (ESGR), Livingston County, MI on 10

April 2009. *A. gracile* eggs were collected from Twin Sisters Pond, OR on 21 April 2009. *A. barbouri* eggs were collected from Franklin County, TN on 9 March 2009. *A. maculatum* eggs were collected from Mallard and Rail Road Ponds in Crawford County, PA between 29 March and 2 April 2009. *A. laterale* eggs were collected from the ESGR on 1 April 2010. For all five species, the eggs were hatched and raised in outdoor mesocosms before the experiments began. All salamanders were fed zooplankton *ad libitum* prior to the start of the experiment and throughout the experiment.

2.2.4 Experimental design

The experiments were conducted under laboratory conditions at the University of Pittsburgh's Pymatuning Lab of Ecology. For each species, we used a randomized block design consisting of a predator-cue treatment and a no-predator control, each replicated 48 times for a total of 96 experimental units. The experimental units were 1-L plastic containers containing 600-mL filtered well water and a 25 mm x 38 mm piece of PVC pipe split in half to serve as refuge. The containers for each species were set up on separate shelving units and each shelf was considered a block that contained 32 experimental units, which were divided evenly between the two treatments. The animal room was kept at 22°C with a 14-hr light/10-hr dark cycle.

Experimental units were filled with carbon- filtered, UV-irradiated well water prior to the start of the experiment and a single salamander larva was added to each container the following day (*A. barbouri:* 27 April 2009, *A. gracile:* 5 May 2009, *A. tigrinum:* 5 May 2009, *A. maculatum:* 4 June 2009; *A. laterale:* 8 May 2010). Salamanders were fed zooplankton *ad libitum* and the water in the container was changed every 4 d. All experiments were terminated after 28 d; this duration is long enough to allow substantial growth and the expression of

morphological defenses while short enough to avoid initiating metamorphosis (Yurewicz 2004, Currens at al. 2007, Pearson and Goater 2009).

The predator cue was generated by housing 10 aeshnid dragonfly larvae (*Anax junius*) in a 13-L plastic tub containing 9 L of filtered well water. For each species, the dragonflies were fed a diet of the focal salamander species. Each dragonfly was placed in an individual 450-mL plastic cup covered with fiberglass screen and floated in the plastic tub. This allowed chemical cues to mix freely in the water while preventing the dragonflies from cannibalizing one another. Each dragonfly was fed a single larval salamander of the focal species every other day. Since the mass of each dragonfly's diet increased as feeder animals grew, we monitored the total mass of prey consumed by all 10 predators in a tub and varied the volume of water changed daily in the predator-cue tub to keep the cue concentration constant at 1.7 mg/mL in each experimental unit throughout the experiment. This concentration was selected to maximize antipredator responses, based on the anuran risk-response curves produced by Schoeppner and Relyea (2008). To apply the treatment, a volume of water was removed from each experimental unit using a 60-mL plastic syringe and replaced with an equal amount of predator-conditioned water from the dragonfly tub (i.e. for the predator-cue treatment) or filtered water in a tub that was allowed to sit several hours until it came to room temperature (i.e. for the no-predator control).

We recorded salamander behavior (moving or not; using the refuge or not) on days 7, 14, 21, and 28. Each individual was observed three times per observation day prior to feeding and adding predator cues. We recorded the mass from a subset of individuals at least once per week to determine the growth rate of each treatment throughout the experiment.

On day 28 of each experiment, the surviving animals were euthanized in MS-222 and preserved in a 10% formalin solution. We later took photos of the preserved animals, including

the dorsal, lateral, and ventral views. Using Image J, we calculated the following linear measurements from landmarks following Van Buskirk and Schmidt (2000): maximum head width, head length, maximum head depth, body length, tail length, tailfin depth, and tail muscle depth.

2.2.5 Statistical analysis

The data consisted of behavioral and morphological response variables. Our behavioral data were averaged for each individual on each observation day. Because these data were non-parametric, and there is no way to test for a time-by-treatment interaction with a non-parametric test, we used a Mann-Whitney U test at each time point with a sequential Bonferroni correction to test for predator effects.

Because linear dimensions of organisms increase with the mass of organisms, we needed to determine the relative shape of the salamanders independent of their mass. To do this, we began by log transforming the mass and linear dimensions of each individual to improve the linearity of the mass-dimension relationships. To produce mass-independent morphology for each species, we conducted a MANCOVA with the log-transformed morphological dimensions as dependent variables, log-transformed mass as the covariate, and the predator treatment as a fixed factor. This analysis determines whether the slopes of the relationships are similar between treatments, which is an important assumption for mass-adjusted data. We found that several dimensions had slopes that visually appeared to differ between the predator and no-predator treatments despite a non-significant interaction in the MANCOVA. These traits were analyzed using the Wilcox method to look for ranges over which slopes were significantly different (Quinn and Keough 2002, Wilcox 1987). We found that all slopes did not significantly different

from one another at any point across their entire range, so we used the residuals and estimated marginal means saved from the MANCOVA to calculate size-independent measurements for each individual. For each species, we then ran a MANOVA with log-transformed mass and the mass-independent morphological dimensions as the dependent variables.

Table 1. Percent survival for five species of Ambystoma salamanders in the presence and absence of aeshnid predator cues. Values are percent of individuals alive at the end of the experiment from the initial 48 animals

Treatment	***A. barbouri***	***A. gracile***	***A. laterale***	***A. maculatum***	***A. tigrinum***
No Predator	98%	100%	94%	92%	85%
Predator Cue	98%	96%	98%	90%	81%

Due to a low amount of mortality during the course of the experiment (Table 1), sample sizes varied a bit among species. There is also variation in sample size between behavioral and morphological traits of a given species. All individuals who lived through the experiment were included in the behavioral analyses. However, we excluded any individuals from the morphological analysis if their tail was damaged in the process of preservation or imaging and one replicate of *A. barbouri* that exhibited developmental abnormalities.

2.3 RESULTS

For each species, we begin by describing the effects of predator cues on salamander activity and refuge use at each observation time. Following this, we describe the effects of predator cues on relative morphology and mass.

2.3.1 Responses of *A. barbouri.*

When we examined the behavior of *A. barbouri* we found that predator cues had no effect on activity or refuge use at any point in ontogeny (Table 2, Figs. 2-3). Our analysis of relative morphology revealed a significant multivariate effect of predator cues ($F = 3.4$, $df = 8, 83$, $p = 0.002$). Subsequent univariate analyses indicated that predator cues induced wider and longer heads ($p \leq 0.026$), shorter bodies ($p = 0.023$), and deeper tails ($p = 0.024$; Fig. 4). There was no effect of predator cues on mass ($p = 0.133$).

2.3.2 Response of *A. gracile*.

The behavior of *A. gracile* was affected by predator cues. Activity was significantly affected by predator cues during the first week of exposure (Table 2, Fig. 2). Refuge use not affected by predator cues (Table 2, Fig. 3). In the third week there was a pattern of increased refuge use with predator cues; however, this difference was no longer significant after Bonferroni correction.

Our MANOVA on morphology revealed a significant multivariate effect of predator cues ($F = 189.5$, $df = 8, 83$, $p < 0.001$). Subsequent univariate analyses indicated that predators

induced shorter heads ($p = 0.003$) and shorter bodies ($p = 0.018$). There was no effect of predator cues on mass ($p = 0.250$; Fig. 4).

2.3.3 Response of *A. laterale*.

For *A. laterale*, predator cues induced changes in behavior. Exposure to predator cues induced lower activity during week 1 and week 2, but not during week 3 or week 4 (Table 2, Fig. 2). There was no effect of predator cues on refuge use (Table 2, Fig. 3).

Our MANOVA on the morphological responses revealed a significant multivariate effect of predator cues ($F = 2.7$, $df = 8, 76$, $p = 0.009$). Subsequent univariate analyses indicated that predators induced shorter heads ($p = 0.003$; Fig. 4). There was no effect of predator cues on mass ($p = 0.808$).

2.3.4 Responses of *A. maculatum*.

Predator cues reduced salamander activity throughout the experiment, but the significant reductions in activity during week 1 and week 2 were no longer significant after Bonferroni correction (Table 2, Fig. 2). Predator cues had no effect on refuge use throughout the experiment (Table 2; Fig. 3).

The analysis of morphology detected a significant multivariate effect of predator cues ($F = 2.4$, $df = 8, 76$, $p = 0.020$). Subsequent univariate analyses indicated that predator cues induced wider heads ($p = 0.005$) and shorter tails ($p < 0.001$; Fig. 4). There was no effect of predators on final mass ($p = 0.250$).

Table 2. Mann Whitney U test results for activity and refuge use of five species of Ambystoma salamanders in the presence or absence of aeshnid predator cues. Activity and refuge use measurements were collected on days 7, 14, 21, and 28. For each species, U values are the top row and p values are the bottom row. *Significant at α = 0.05 based on a sequential Bonferroni correction.

	Activity day 7	**Activity day 14**	**Activity day 21**	**Activity day 28**	**Refuge use day 7**	**Refuge use day 14**	**Refuge use day 21**	**Refuge use day 28**
A. barbouri	996	1045	963	1086	1032	1081	1034	1104
n = 94	0.343	0.635	0.255	0.885	0.228	0.559	0.241	1.000
A. gracile	897	1068	947	985	1049	1015	929	1037
n = 94	0.002*	0.625	0.114	0.199	0.437	0.167	0.022	0.265
A. laterale	749	685	818	974	1010	1034	1057	1033
n = 92	0.003*	0.002*	0.051	0.499	0.146	0.307	1.000	0.542
A. maculatum	776	860	841	825	905	926	903	925
n = 87	0.032	0.044	0.215	0.129	0.535	0.591	0.314	0.779
A. tigrinum	528	683	670	499	758	692	799	799
n = 80	0.005*	0.031	0.177	0.001*	0.144	0.060	1.000	1.000

2.3.5 Responses of *A. tigrinum*.

Predator cues induced changes in the activity of *A. tigrinum.* Animals showed reduced activity when exposed to predator cue during week 1 and week 4, but no significant response during week 2 or week 3 (Table 2, Fig. 2). There was no effect of predator cue on refuge use (Table 2, Fig. 3). In the analysis of morphological responses, we found no multivariate effect of predator cues ($F = 0.8$, $df = 8, 62$, $p = 0.556$; Fig. 4).

2.4 DISCUSSION

We found unique suites of antipredator responses among the five species of larval *Ambystoma.* The most prevalent behavioral pattern was the tendency for the larvae to reduce their activity in the presence of predator cues early in ontogeny. There was no obvious, consistent pattern of antipredator refuge use by *Ambystoma*. Morphologically, we found little consistency among *Ambystoma* species; each induced a different subset of traits when raised with predator cues. Across all species, head length was the most likely trait to show significant changes in the presence of predators; predator cues induced one species to have relatively longer heads and two species to have relatively shorter heads.

In terms of activity, three of the species showed significant reductions in activity early in ontogeny when predator cues were present, but they increased their activity over time as they grew. Given the constant predator cue, the patterns of induced behavioral defenses across time may be due to prey perceiving predators as less risky over ontogeny (Lima and Dill 1990, Lima and Bednekoff 1999, Mirza et al. 2006, Ferrari and Chivers 2009; but see Laurila et al. 2004).

However, there is much more evidence to suggest that this change in antipredator behavior over time may simply be due to changes in prey strategies. One strategy many amphibians employ is a reduction in behavioral defenses over time as they reach a size threshold, or a size at which predators are unable to handle them, to escape predators (Van Buskirk and Schmidt 2000, Relyea 2003, Urban 2007a, Ferrari et al. 2009, 2010). Regardless of time, smaller prey tend to show greater magnitudes of antipredator behavior than larger conspecifics (Kats et al. 1994, Mathis et al. 2003), which would be consistent with the behavior and size of our animals in early versus late ontogeny. The fact that *A. tigrinum* once again reduced their activity in the presence of predators in the final week of the experiment supports this notion, though it is not clear what might cause this use of antipredator behavior later in ontogeny.

The reduction in defensive behavior is sometimes driven by increased foraging behavior in the presence of predators to reach a refuge size (Urban 2007b, c). However, we did not find evidence of increased growth in *Ambystoma* exposed to predator cues. The lack of size differentiation when exposed to predator cues suggests that reaching a predator size threshold is not a ubiquitous defensive strategy in the genus. Since future fitness is not affected by size at metamorphosis for some species (O'Laughlin and Harris 2000, Orizaola and Brana 2004; but see Semlitsch et al. 1988, Scott 1994), there is no long-term consequence of adopting an alternative strategy which results in smaller metamorphic size or slower growth. Thus, growing into a size refuge may not be a general salamander antipredator defense.

Whereas we exposed salamanders to chemical cues of predators, other studies have used caged predators, which potentially provide visual predator cues as well. Simultaneous cues may result in the induction of different defenses than those based on single cues (Burks and Lodge 2002, Chivers et al. 2001), so defenses such as refuge use may not be induced by chemical cue

alone in larval salamanders. Species may not show *any* behavioral response if, for example, they employ other strategies that make anti-predator defenses unnecessary or less effective for survival (i.e., color change; Benard and Fordyce 2003, Dayton and Fitzgerald 2011). From an experimental perspective, the concentration of our chemical cues was based on the response curves of the highly plastic tadpole *Hyla versicolor* (Schoeppner and Relyea 2008). Similar curves do not exist for salamanders, and it is possible that the concentration in our study did not produce defenses as strong as those seen in previous studies.

Based on the literature, an increase in relative head length, as we observed in *A. barbouri*, does not appear to be adaptive, but may be a consequence of other developmental changes. However, a decrease in relative head length, as we measured in *A. gracile* and *A. laterale*, can come with a cost. Orizaola and Brana (2004) found that *Triturus* newts exposed to predator cues during embryonic development hatched at earlier developmental stages and with shorter, shallower heads for their size relative to predator-naïve newts. The authors suggested that the short heads of these larvae, among other traits, might represent a non-adaptive developmental cost of plasticity since reduced head length is not expected to improve survival against predators, though their study did not test for these costs. However, in other gape-limited predators shorter heads may represent a feeding cost due to increased handling and ingestion time, or maximum ingestible prey size (Forsman and Lindell 1993, Vincent et al. 2005, Vincent and Mori 2007). Experimentally, high food conditions in the lab (e.g., *ad libitum*) which lead to high growth rates may result in shorter heads in gape-limited animals when growth of morphological dimensions are decoupled from one another and other dimensions develop quicker compared to the head of the organism (Bonnet et al. 2001). Smaller heads may also negatively impact escape swimming performance in salamanders when coupled with large tails if the force generated by the tail is lost

to the animal rotating due to the head being insufficiently stabilizing against water resistance (Fitzpatrick et al. 2003). However, in other anguilliform swimmers, head length has not been found to be important to morphological changes related to locomotion (Mehta et al. 2010 and references therein, Ward and Mehta 2010.

Because changes in tailfin and body morphology are generally common in amphibians and are thought to improve avoidance and escape from predators, we expected larval *Ambystoma* to commonly exhibit predator-induced increase in tailfin depth and body depth, and a decrease in tail length and body length (Doherty et al. 1998, Landberg and Azizi 2010). Past studies in lab and mesocosm settings have observed induced morphological defenses in *A. tigrinum* (shorter tails and bodies, deeper tailfins), *A. maculatum* (deeper tailfins), and *A. laterale* (shorter and deeper bodies) in response to predators (Storfer and White 2004, Yurewicz 2004). Though we found changes in tailfin depth, body length, and body depth in other species, the individuals of those same three species in our study induced different suites of morphological defenses. Storfer and White (2004) individually reared *A. tigrinum*, as in our study, but otherwise the two experiments carried out very differently. In their experiment, animals were raised in a greater volume of water, their diets were set rather than *ad libitum*, and the predators were present in cages in each experimental unit. Moreover, these animals were from a lab-bred pair of Arizona tiger salamanders (*A. t. nebulosum*), and genetic differences between them and our population *(A. t. tigrinum*) may explain some of the differences in the results of the two studies. The experimental design of Yurewicz (2004) resembled ours more closely, controlling the mass of prey consumed by predators and feeding the *Ambystoma* zooplankton *ad libitum*. However, the experiment was conducted outdoors in large pools containing groups of 10 animals. Given that the animals used by Yurewicz (2004) were likely from the same population, or a population

close to the population that we used, genetic differences are unlikely to be a factor in the different outcomes observed. In addition, both previous studies used caged predators rather than predator cues alone. These differences in experimental protocols may have contributed to the variation in outcomes among these studies.

Furthermore, it is possible that salamanders are simply more sensitive to predators during early embryonic development than during larval development (Fuiman and Magurran 1994, Orizaola and Brana 2004, 2005; Vonesh and Bolker 2005, Gomez-Mestre and Warkentin 2007, Mandrillon and Saglio 2008), in which case our data may reflect a time outside the window of opportunity. Developmental windows have been experimentally identified for a handful of species and, in those cases, if organisms can develop defenses at all they are typically weaker (Harvell 1990, Orizaola and Brana 2005, Lehman and Campbell 2007, Mandrillion and Saglio2008). If such a developmental window exists, then we would expect fewer induced defenses in our study.

Table 3. Comparison of animal sizes among this study and two other studies. *Study did not list initial or final mass; values were estimated from figures.

Study	**Species**	**Initial mass**	**Duration of predator exposure (d)**	**Final mass (mg)**
Shaffery and Relyea	*A. tigrinum*	41.9 ± 1.4	28	850.2 ± 12.6
	A. laterale	37 ± 2.9	28	257.6 ± 4.8
	A. maculatum	42.9 ± 3	28	268 ±8.5
Storfer and White (2004)*	*A. tigrinum*	hatching	35	750
Yurewicz (2004)	*A. laterale*	41 ± 2	32	374 ± 22
	A. maculatum	72 ± 2	32	312 ± 26
	A. tigrinum	60 ± 3	32	489 ± 34

To address the hypothesis of sensitive time windows, we can compare our results to similar studies. Looking at differences in experimental timing and duration (Table 3), Storfer and White (2004) started their experiment with animals earlier in development than ours and ran the experiment for a week longer, but our *A. tigrinum* achieved a larger final size. The animals in Yurewicz (2004) entered the experiment larger than ours and her experiment ran 4 d longer. After 32 d, her *A. laterale* and *A. maculatum* were larger than ours, but her *A. tigrinum* were roughly half the size of ours. It is difficult to make comparisons with only two other studies that roughly approximate our methods and focal predator, particularly with such dramatically different growth trajectories and experimental designs among studies. Based on the overlap in developmental time among these experiments, it appears we did not miss any developmental windows. However, given the available data, we find that evidence for potential differences in developmental trajectories remains ambiguous and warrants further study.

Cannibalism may offer an alternative explanation for our limited morphological predator defenses. Many species of *Ambystoma* become cannibalistic under a variety of conditions, including conspecific density (Petranka 1998, Wildy et al. 2001, Mott and Maret 2011). Cannibals grow quicker than their noncannibals (Wakahara 1995, Brodman 2004). This early cannibalistic morphology at the start of the experiment could change overall morphology or time to induce anti-predator defenses (Collins et al. 1993, Maret and Collins 1994). Despite attempts to keep our culture pools at low densities prior to the start of the experiment, we did note the development of cannibals reducing the populations in the pools of feeder animals over time. We cannot rule out the possibility that density or the early development of cannibalism across both treatments may have altered morphology or developmental trajectory. However, morphological defenses are known to be reversible in some species (Van Buskirk 2002a, Relyea 2003a, Kishida

and Nishimura 2004, 2006, Orizaola et al. 2012). If indeed cannibalism affected the focal salamanders, then the lack of induced morphology may reflect a time lag resulting from reversing cannibal-induced changes before inducing appropriate new defenses.

Though we did not collect genetic data, it is possible that genetic constraints explain the patterns of plasticity that we see. Assuming that plasticity exists in some species of *Ambystoma*, it is possible that our species, or the populations we sampled, may lack the genes necessary to produce plasticity in particular traits (Bradshaw 1965, DeWitt et al. 1998, Pigliucci 2001). Moreover, if any of the *Ambystoma* studied have fixed defenses, we would likely not detect this with our methodology, assuming that they all definitely use defenses (West-Eberhard 1989, Scheiner 1993). Alternatively, any of our species may have historically lacked the genetic variation necessary for plasticity to evolve in the first place, though without a phylogenetic analysis we cannot say this with confidence (Schlichting and Pigliucci 1993, Pigliucci 2005).

Our results suggest that species of *Ambystoma* salamanders differ in their induction of antipredator defenses and they are somewhat less morphologically plastic than observed in past studies. Specific morphological responses to predators, when they exist, vary among species in both magnitude and direction. Additionally, our data indicate that while behavioral defenses are more consistent among species, not all species respond to predators with activity reductions or increased refuge use. While these defenses appear to be most important early in exposure, most of these salamanders apparently stopped relying on them the longer exposure continued. clearly, more data are required to make conclusions about the patterns of *Ambystoma* inducible defenses, both in terms of specific defenses and interspecific variation.

Taking a phylogenetic approach to studying salamander inducible defenses may be a useful best step. A growing body of theory (Bradshaw 1965, Schlichting and Levin 1986, West-

Eberhard 1989, Agrawal 2001, Nussey et al, 2005) and empirical evidence (Pigliucci et al. 1999, Pollard et al. 2001, Pigliucci 2005, Relyea 2005a) suggest that plasticity can evolve and may play a significant role in evolution (Agrawal 2001, Price et al. 2003, West-Eberhard 2003, Pigliucci et al. 2006, Ghalambor 2007, Crispo 2008). Despite an increasing number of comparative plasticity studies, the dearth of phylogenetic analyses limits our ability to draw conclusions about patterns of adaptation and the evolution of plasticity (Garland and Adolph 1994, Doughy 1996, Pigliucci 2003, Relyea 2005b, Gomez-Mestre and Warkentin 2007). Phylogenetic signal in plasticity and plastic traits among *Ambystoma* and other salamanders may explain the interspecific variation observed within the literature while giving us greater insight into the evolution of these defenses.

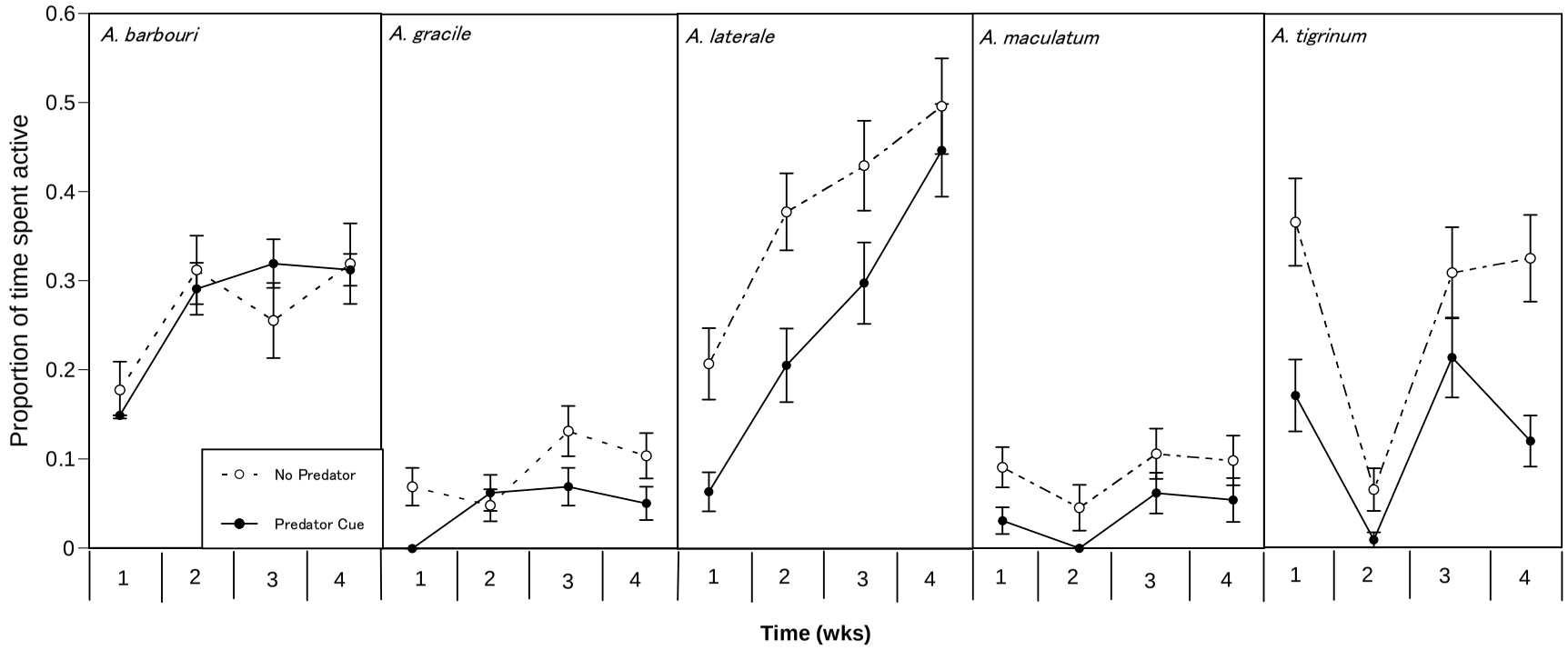

Figure 2. Refuge use for five species of Ambystoma salamanders in the presence (closed circles) and absence (open circles) of aeshnid predator cues. Values are the grand means ± SE of behavioral assays averaged across 3 observations (using refuge = 1, out of refuge = 0) for each individual on each of days 7, 14, 21, and 28.

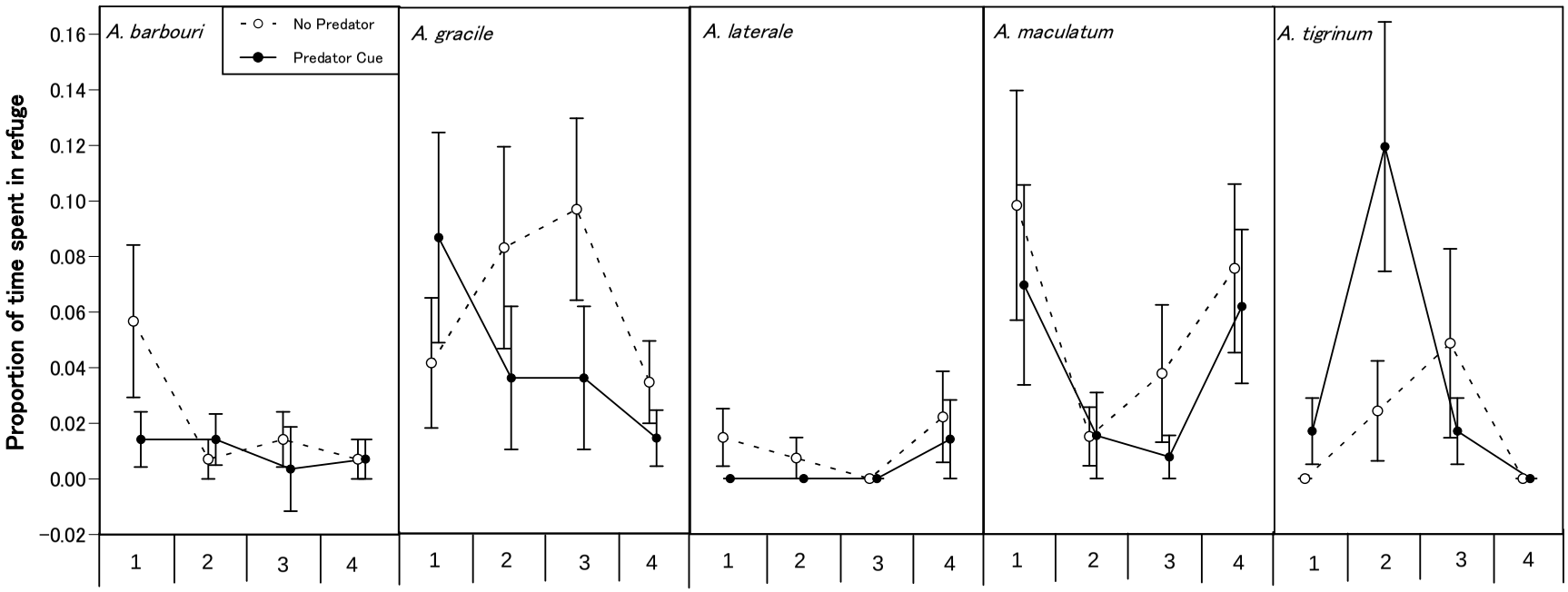

Figure 3Refuge use (mean ± SE) for five species of Ambystoma salamanders in the presence (closed circles) and absence (open circles) of aeshnid predator cues. Values are the grand means ± SE of behavioral assays averaged across 3 binary observations (using refuge = 1, out of refuge = 0) for each individual on each of days 7, 14, 21, and 28. Time on the x-axis is noted in number of weeks.

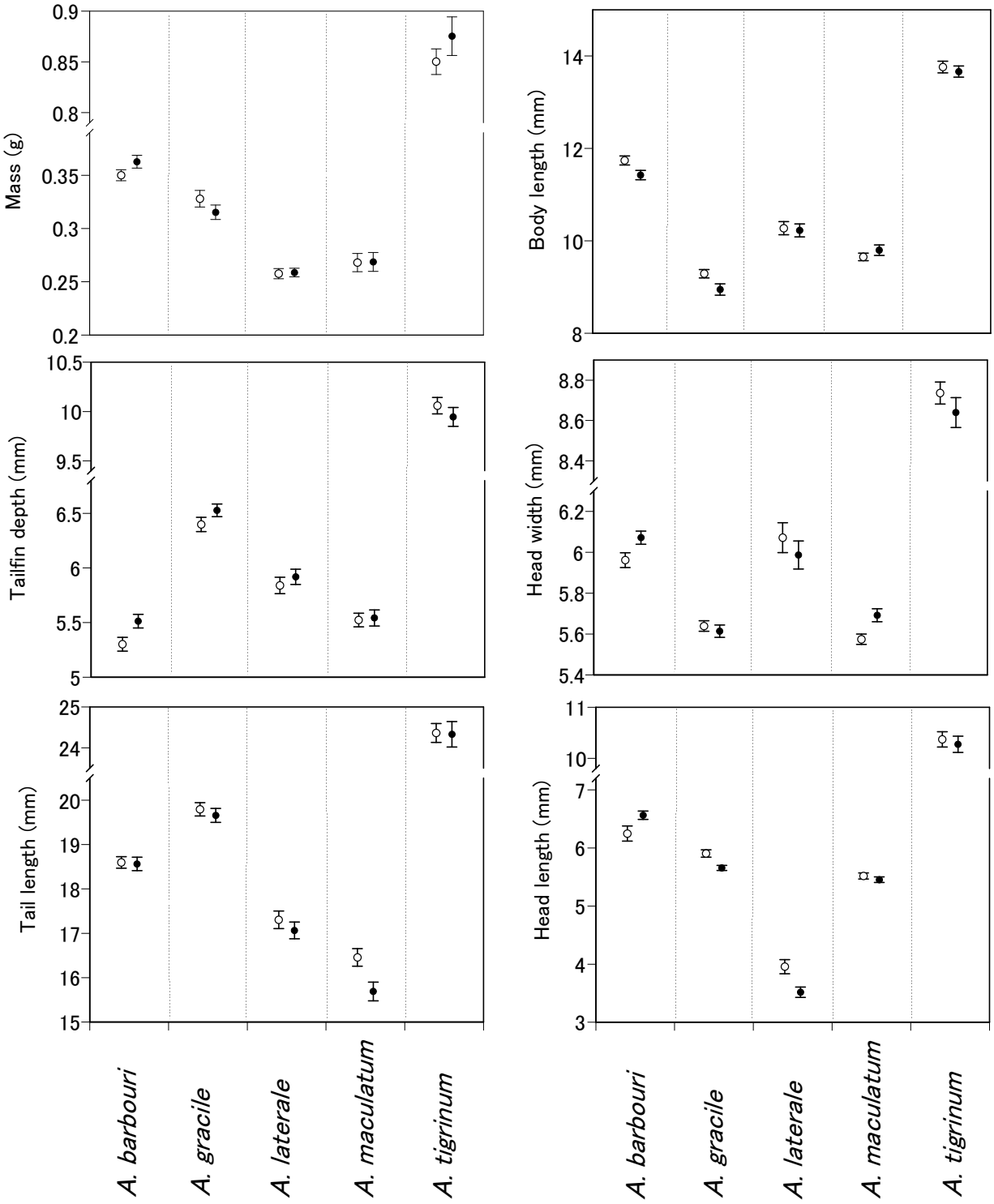

Figure 4. Morphological variables for five species of Ambystoma salamanders in the presence (closed circles) and absence (open circles) of aeshnid predator cues.Values are mass-independent treatment means ± SE of animals measured at the end of the experiment on day 28.

3.0 THE SMELL OF PREDATION: HOW LARVAL *HYLA VERSICOLOR* RESPOND TO FINE-SCALE DIFFERENCES IN PREDATOR CHEMICAL CUES

3.1 INTRODUCTION

Prey use chemical cues from predation events to establish the risk level of their environment and respond by altering their phenotypes. Though we have an extensive knowledge of how prey respond to predators, we still have a poor understanding of the chemical cues involved and from where they emerge during a predation event. We sought to elucidate what portion of an attack sequence induces specific defenses and the role that predator diet plays in defense induction. Using grey treefrog tadpoles (*Hyla verisciolor*), we raised animals in the presence of cues isolated from different stages of an attack sequence by larval dragonflies (*Anax junius*) or larval dragonflies consuming different combinations of treefrog tadpoles and snails (*Helisoma trivolvis*) across different temporal sequences. When exposed to a predator consuming grey treefrogs, tadpoles reduced their activity, increased their hiding behavior, and induced deeper tails. As we broke down the attack sequence into component parts and exposed prey to more types of cues from that sequence, they also increased tail depth and hiding behavior but did not change their activity. Additionally, treefrog tadpoles generally increased their defense as the biomass of treefrogs consumed by dragonflies increased. However, when the tadpoles experienced predators that consumed a mixed prey diet or temporal variation in the composition of their diet, tadpoles produced short bodies and hiding behaviors that resembled the phenotypes

induced when the predators only consumed snails. When tadpoles experienced predators consuming conspecific tadpoles on a temporally varying schedule, tadpole defenses appeared to track the average biomass consumed. Our results suggest that treefrogs can use cue information from all portions of an attack sequence to induce defenses, and that both temporal patterns of feeding and diet content of predators influence the type and magnitude of induced prey defenses.

Many organisms employ phenotypic plasticity as a strategy to cope with variable environments. For these plastic responses to be adaptive, organisms must discern information about their environment via cues from within a potentially noisy environment (Kats and Dill 1998, Burks and Lodge 2002). From the milieu of multi-sensory cues, organisms need to parse critical information related to numerous activities including mating, foraging, and predator avoidance. To some extent, the information provided by cues in the environment is a by-product of species interactions upon which individuals can eavesdrop, rather than information intentionally conveyed to a receiver (Dicke and Grostal 2001, Magalhães et al. 2005, Wisenden and Chivers 2006, Dalesman et al. 2007a, Blanchet et al. 2010, Schaefer and Ruxton 2012). Considering the number of potential cues available in any environment, there is a substantial opportunity for prey individuals to modify their phenotypes based on information they weren't meant to receive. However, in many cases we don't have a sense of the complexity of information that is conveyed to organisms by these cues (Bourdeau 2010, Donaldson-Matasci et al. 2010). With this in mind, we need to assess the degree to which organisms detect and utilize available information in their environment.

One place we can focus our attention is to how prey use environmental information about their predators and respond to these cues with inducible defenses. The study of inducible defenses has provided many insights on the environmental cues that organisms use to match their

phenotype to different environments. In aquatic species, prey commonly rely on water-borne chemical cues that are produced during predation events to assess the risk of predation in their environment and respond accordingly (Nolte et al. 1994, Dicke and Grostal 2001, McCarthy and Fisher 2008). Risk assessment is a complicated process that requires organisms to make sense of cues emitted by predators and by prey (both conspecific and heterospecific prey) over time and space and embedded within the chemical background of the environment (Watts 1991, Brown 2003, Stankowich and Blumestein 2005, Fraker 2008, Ferrari et al. 2010). Failure to accurately interpret this information can result in a phenotypic mismatch (DeWitt et al. 1998, Donaldson-Matasci et al. 2010). Since defenses are costly and an accurate assessment of risk is critical to survival (Harvell 1990, Clark and Harvell 1992, Skelly 1992, Kats and Dill 1998, Dicke and Grostal 2001), selection should favor prey that can fine-tune their defenses based on the cues that provide the most reliable information about their environment (Harvell 1990, Skelly 1992, Kats and Dill 1998).

Chemical cues emitted during predation events can potentially include chemicals emitted by the predator, chemicals emitted by the prey (Wisenden 2000, Ferrero et al. 2011), or prey chemicals that are modified as predators digest the prey (Stabell et al. 2003). During an attack sequence, every step could potentially produce different chemical cues, each of which could provide the prey with information about predation risk in their environment (assuming they can detect each cue; Wisenden and Chivers 2006). Chemical cues released by prey prior to or during an attack, commonly referred to as alarm cues, are thought to function like the alarm calls of birds and mammals that inform other prey of a predator's presence (Chivers et al. 1996, Chivers and Smith 1998, Summey and Mathis 1998, Wisenden et al. 2004, Jacobsen and Stabell 1999, Mirza and Chivers 2008, Kaliszewicz and Uchmanski 2009). Chemical cues released by

predators, generally referred to as kairomones, are also common (Tollrian and Harvell 1999); by detecting these kairomones, prey can potentially distinguish predator identity, predator density, and even combinations of predator species (Harvell 1990, Turner et al. 1999, DeWitt et al. 2000, Relyea 2003, 2004, Hoverman and Relyea 2007). The process of digestion can release chemical cues that induce different defenses than either predator cues or prey cues (Chivers et al. 1996, Jacobsen and Stabell 1999, 2004). What we need to know is how cues from different stages in the predation process affect the anti-predator responses of prey and whether this pattern is generalizable across multiple taxa. To date only a few studies have simultaneously examined the impact of the cues released during different stages of a predation event on prey defenses (LaFiandra and Babbitt 2004, Schoeppner and Relyea 2005, Richardson 2006, Dalesman et al. 2007b, Schoeppner and Relyea 2009a).

When prey assess their environments, they face the challenge of detecting the relevant alarm cues and kairomones in a complex environment containing many other chemical cues that potentially add noise to the chemical environment. For example, many prey defenses track quantitative indicators of predation risk, such as the number of predators present or the biomass of prey that a predator consumes (Helfman 1989, Bouskila and Blumstein 1992). In aquatic species, an increase in prey consumption by a predator commonly induces prey defenses that increase asymptotically (Van Buskirk and Arioli 2002, Mirza and Chivers 2003a, Schoeppner and Relyea 2008). However, such studies have always focused on predators consuming a single species of prey, yet most predators do not consume a single prey species over extended periods of time (Kats and Dill 1998, Bolnick et al. 2007). If prey only track the consumption of conspecifics and they do not respond to predators simultaneously eating heterospecifics, then the induced defenses of prey should be identical with and without the consumption of heterospecific

prey. Alternatively, the inclusion of heterospecific prey could result in a noisier chemical environment and making it more difficult for prey to respond appropriately (Chivers and Smith 1998, Burks and Lodge 2002). To our knowledge, no studies have attempted to characterize prey responses specific to increases in conspecific biomass with and without the consumption of heterospecifics.

If the consumption of alternative prey adds noise to a prey's environment in ways that affect the induction of defenses, then we should also find these effects when prey experience temporal variation in in predation cues (Schultz et al. 2004, Chivers et al. 2008, Pettorelli et al. 2011). Though the specifics vary among organisms, there is a general consensus that temporal variation in predator presence or attack is an important variable of predation risk that affects defensive decisions (Lima and Bednekoff 1999, Sih et al. 2000). Most investigations of timing seek to identify the specific effects of timing *per* se on induced defenses (Sih and McCarthy 2002, Laurila et al. 2004, Foam et al. 2005, Mirza et al. 2006, Creel et al. 2008), and do not consider interactions between this timing and variation in other factors such as predator identity, diet, and risk level (but see Ferrari and Chivers 2009). In nature, the timing of predator attacks and what prey they are consuming almost certainly vary simultaneously, but we have virtually no idea what effect this has on prey defenses. Given the importance of both timing and dietary cues on preys' ability to induce correct plastic defenses, we need to assess how temporal variation in the consumption of conspecific and heterospecific prey affects the production of inducible defenses.

Larval anurans are an ideal system to address these questions regarding prey responses to chemical cues. In aquatic environments, they rely primarily on chemical communication (Kats and Dill 1998, Burks and Lodge 2002, Miner et al. 2005, Houk 2009). Tadpoles show strong

responses to cues from predation events via behavioral and morphological defenses. Behaviorally, larval anurans typically move less and hide more, and this plasticity commonly comes at a cost of slower growth and smaller size at metamorphosis (Skelly 1992, Anholt and Werner 1995, McCollum and Van Buskirk 1996, Laurila et al. 2004). Morphologically, tadpoles typically induce relatively deep tailfins, short bodies, and other assorted changes which improve their escape chances or make them harder to consume (Laurila et al. 1997, McCollum and Leimberger 1997, Relyea 2001, Van Buskirk 2002b, Relyea and Hoverman 2003). In addition, tadpoles can discriminate among different predator diets; they exhibit strong responses to conspecifics being eaten by predators but little or no responses to distantly related prey, such as snails, being eaten by predators (Schoeppner and Relyea 2009a, c). The lack of response to predators consuming snails represents an experimental opportunity because it allows us to manipulate both the total amount of a predator's diet and the portion of the predator's diet that is composed of conspecific tadpoles.

Using the grey treefrog (*Hyla versicolor*), we sought to address the extent to which prey use information from predation events to fine-tune their antipredator defenses by testing the following hypotheses: (1) Prey defenses are induced by cues produced when predators consume and digest prey (an established yet necessary first step); (2) Prey defenses increase as prey gain more types of information from a predator attack sequence; (3a) Prey defenses increase as the mass of consumed conspecific prey increases; (3b) Given no response to cues from consumed heterospecifics (e.g., snails), prey defenses increase with increased mass of consumed conspecifics even if total biomass is held constant by adding heterospecifics ; and (3c) Prey defenses in response to predators consuming a given biomass of consumed conspecific prey are similar to prey defenses in response to predators consuming a given biomass of consumed

conspecific prey plus an equal biomass of heterospecifics; (4) Given no response to cues from heterospecifics, prey will respond to temporal variation in predator consumption of conspecifics alone in the same way that they respond to temporal variation in predator consumption of conspecifics and heterospecifics combined.

3.2 MATERIALS AND METHODS

3.2.1 Experimental design

We used a completely randomized design with 13 treatments: a no-predator control (No Predator), a starved-predator control (Starved Predator), 100 mg of crushed tadpoles daily (Crushed 100T), a predator only chewing 100 mg of tadpoles daily (Chewed 100T), a predator only digesting 100 mg of tadpoles daily (Digested 100T), a predator chewing *and* digesting 100 mg of tadpoles daily (Consumed 100T), a predator consuming 100 mg of snails daily (Consumed 100S), a predator consuming 100 mg of tadpoles or 100 mg of snails on alternate days (Consumed 100T/100S), a predator consuming 100 mg of tadpoles every other day (Consumed 100T/0S), a predator consuming 50 mg of tadpoles and 50 mg of snails daily (Consumed 50T:50S), a predator consuming 25 mg of tadpoles and 75 mg of snails daily (Consumed 25T:75S), a predator consuming 50 mg of tadpoles daily (Consumed 50T), and a predator consuming 25 mg of tadpoles daily (Consumed 25T). The 13 treatments were replicated 5 times for a total of 65 experimental units. We compared particular subsets of these treatments to address our specific hypotheses and predictions (Table 4).

The experimental units were 100-L outdoor mesocosms set up on tables at the University

of Pittsburgh's Pymatuning Laboratory of Ecology. On 15 and 16 June 2010, pools were filled with well water and covered in 60% shade cloth to prevent colonization by insects and other amphibians. Two weeks before the start of the experiment (16 June 2010), pools were inoculated with 100 g of leaf litter (mostly *Quercus spp.*), 5 g of rabbit chow as an initial nutrient source, and an aliquot of zooplankton and algae that had been collected from three local ponds, screened for invertebrate predators, and mixed together.

On 2 July, a predator cage was added to each experimental unit. The predator cages were constructed of black plastic drain pipe with nylon window screen attached to both ends. Experimental units assigned a treatment that involved a predator contained a late instar *Anax junius* nymph in the cage. All other treatments contained an empty cage. The cages allowed predator cues to diffuse out into the mesocosm but prevented the predators from preying on our focal tadpoles.

Grey treefrog tadpoles (*Hyla versicolor)* were collected as 14 egg masses between 17 and 24 June and hatched in wading pools that had been filled with well water and covered with a 60% shade cloth lid to prevent predators from colonizing. While held in the wading pools, the tadpoles were fed rabbit chow *ad libitum*. On 1 July, tadpoles were selected haphazardly from a mixture of the 14 clutches and added to the pools. The tadpoles were early in development (Gosner stage 24-25; Gosner 1960) with an initial mass of 15 ± 1 mg (mean ± SE). To assess the possibility of mortality due to handling, 20 tadpoles were placed in a 7-L plastic tub (24-hr survival = 100%).

Table 4. A comparison of hypotheses regarding the outcome of cue source and content on prey induced defenses. Predictions are rank ordered by strength of induced response.

Hypothesis	Predictions

H1: Prey defenses are induced by cues produced when predators consume and digest prey	No Predator < Starved Predator < Consumed 100S < Consumed 100T
H2: Prey defenses increase as prey gain more information from a predator attack sequence	No Predator < Crushed 100T < Chewed 100T < Digested 100T < Consumed 100T
H3a: Prey defenses increase as the biomass of consumed conspecific prey increases	No Predator < Consumed 25T < Consumed 50T < Consumed 100T
H3b: Given no response to cues from consumed heterospecifics (e.g., snails), prey defenses increase as the biomass of consumed conspecifics increases while the total biomass consumed is held constant by adding heterospecifics	No Predator < Consumed 25T:75S < Consumed 50T:50S < Consumed 100T
H3c: Prey defenses in response to predators consuming a given biomass of consumed conspecific prey are similar to prey defenses in response to predators consuming a given biomass of consumed conspecific prey plus an equal biomass of heterospecifics	Consumed 25T = Consumed 25T:75S and Consumed 50T = Consumed 50T:50S
H4: Given no response to cues from heterospecifics, prey will respond to temporal variation in predator consumption of conspecifics alone the same way that they respond to temporal variation in predator consumption of conspecifics and heterospecifics combined	No Predator < Consumed 100T/100S = Consumed 100T/0S < Consumed 100T

The next day we applied the predator cue treatments. Starved predators had not been fed for 5 d prior to addition into the mesocosms, and they were replaced with new starved predators after being in the experiment for 5 d. We applied the Crushed 100T treatment by adding 100 mg of tadpoles (killed via cranial concussion) to 30 mL of filtered well water. The crushed animals were homogenized individually using a mortar and pestle. The Chewed 100T and Digested 100T treatments were carried out by allowing predators in the Chewed 100T pools to consume the entire 100 mg of tadpoles. Once the tadpoles were consumed, we transferred the predators, while still in their cage, to the Digested 100T pools and left them there until the next feeding. An empty cage was left in the Chewing 100T treatment pools. Predators in any of the snail treatments were fed *Helisoma trivolvis* snails. The dragonflies only consume the soft parts of a snail, removing the body and leaving behind a clean, undamaged shell, so we calculated the total mass of snails for feeding based only on the mass of soft body parts (estimated from shell length-body mass regressions; Turner 2008, E. Cholak, *personal communication*). Across all treatments, the predators consumed their prey diet within 1 d. Any predators that died during the experiment were replaced with new starved predators or predators that had only been fed grey tree frogs (depending on the treatment).

Behavioral observations were taken on 3 different days during the final week of the experiment. During each observation, we recorded the number of individuals visible (i.e., not hiding beneath the leaf litter) and the number of those visible animals that were moving. Dividing the number of active tadpoles by the number visible tadpoles gave us the proportion of active tadpoles in each treatment on each day, which we defined as tadpole activity (Van Buskirk 2001, Schoeppner and Relyea 2009a). All observations were conducted before the predators were fed that day. On 10 July (9 observations) and 14 July (10 observations), predators in the

Consumed 100T/100S treatment were fed snails the previous day and predators in the Consumed 100T/0S treatment were fed nothing the previous day. On 13 July (8 observations), predators in the Consumed 100T/0S and Consumed 100T/100S treatments were fed tadpoles the previous day. All observations were conducted between 900 and 1100 and 4 to 5 observers collected data on each day.

We ended the experiment on 17 July 2010 when tadpoles across treatments entered Gosner (1960) stage 43, which was 16 d after tadpoles were placed in the experimental unit. At this point in the experiment the average tadpole had increased nearly 60-fold to 884 ± 6 mg (mean ± SE). Continuing the experiment further would have resulted in the tadpoles undergoing metamorphosis, which would prevent an assessment of their larval morphology.

When we terminated the experiment, we counted and euthanized all tadpoles and then preserved them in 10% buffered formalin for subsequent morphological analysis. Photos were later taken of all preserved tadpoles from a lateral view. Using Image J, we collected landmark data on each individual as outlined by Van Buskirk and Schmidt (2000). From the landmark data, we then calculated linear distances for each individual to produce four morphological variables: body length, body depth, tail length, and tail fin depth. We selected these traits because they are the most common predator-induced morphological traits in tadpoles (Van Buskirk and Relyea 1998, Van Buskirk 2002a, Relyea 2003).

3.2.2 Statistical analysis

The data consisted of behavioral and morphological response variables. For the behavioral data, we calculated the mean number of individuals observed (i.e., out of refuge) and the mean activity for each pool on each observation day. Because the activity data were proportional, we arcsine-

transformed these data. We conducted a repeated-measures analysis of variance (rm-ANOVA) on the two behavioral responses. Our analysis of the number visible passed the test of sphericity ($W = 0.911$, $p = 0.097$) and equal variances at each time point ($F \geq 0.74$, $p \geq 0.117$). We found a few deviations from normality (Number observed, *Week 1*: Chewed 100T, Consumed 100S; *Week 2*: Consumed 50T; *Week 3*: Consumed 100T/0S; Activity, *Week 1*: Starved Predator; *Week 2*: No Predator), but analysis of variance is typically robust to such violations. The analysis of activity did not pass the test of sphericity ($W = 0.860$, $p = 0.023$), so we used the Greenhouse-Geisser correction for our results. The test of equal variances was met at each time point ($F \geq 1.19$, $p \geq 0.094$).

For the morphological data, linear dimensions of organisms increase as the mass of the organism increases, so we needed to make the morphological dimensions mass independent. The morphological data were first log transformed to improve linearity. To produce mass-independent means, we ran a multivariate analysis of covariance (MANCOVA), with the morphological measurements as dependent variables and log-transformed mass as the covariate. An important assumption of mass-independent data is that the slopes of the relationships do not differ between treatments, and we confirmed this by the lack of a significant treatment-by-mass interaction.

Using the estimated marginal means saved from the MANCOVA, we calculated size-independent measurements for each individual. We then calculated the mean morphology and mass of all tadpoles from a given mesocosm. Using these mesocosm means, we examined the effects of the treatments on mass and the four mass-independent morphological dimensions using a MANOVA. We found a few deviations from normality (Mass: Starved Predator, Crushed 100T, Consumed 50T:50S; tail length: Consumed 25T; body length: Consumed 100S), but

analysis of variance is typically robust to such violations. We also found one violation of variance homogeneity (body length), so we report Pillai's Trace from the MANOVA.

In the behavioral analyses, we conducted two types of mean comparisons. Most of our hypotheses involved comparisons between the no-predator treatment versus other treatments that had a directional response that was predicted a priori (hypotheses 1, 2, 3A, and 3B). For these hypotheses, we used Dunnett's test, which compares a control against all other treatments. Our other two hypotheses (i.e. hypotheses 3C, 4) include comparisons that between treatments that are not controls. In these cases, we compared the means using Tukey's test.

3.3 RESULTS

To more clearly address each hypothesis, we first present the rm-ANOVA and MANOVA results on the behavioral and morphological response variables. We then describe particular mean comparisons to evaluate each of the hypotheses.

In the rm-ANOVAs on both behavioral response variables, we found an effect of time and treatment, but no interaction (Tables 5A, 5B). The time effect was driven by an increase in the number of tadpoles observed and their activity throughout the experiment.

In the MANOVA on tadpole mass and morphology, we found a multivariate effect of the treatment (Table 6). Subsequent univariate analyses indicated that the multivariate effect was driven by treatment effects on relative tailfin depth, body length, and body depth, but not on relative tail length or mass.

Table 5. Results of a rm-ANOVA on (A) the number of gray treefrog tadpoles observed and (B) the activity of the tadpoles when exposed to 13 predator-cue treatments. Bold fonts indicates statistical significance at α = 0.05. Asterisks indicate Greenhouse-Geisser corrected values used for analyses that violated assumptions of sphericity.

A. Number observed			
Within subjects	df	F	p
Time	2	50.44	**< 0.001**
Time x Treatment	24	1.12	0.264
Between subjects	df	F	p
Treatment	12	2.28	**0.021**
B. Activity			
Within subjects*	df	F	p
Time	1.8	7.93	**0.001**
Time x Treatment	21.0	0.78	0.759
Between subjects	df	F	p
Treatment	12	2.05	**0.038**

Table 6. Results of a MANOVA on mass and mass-independent morphological dimensions of gray treefrog tadpoles when exposed to 13 predator-cue treatments. Bold font indicates statistical significance at α = 0.05.

Multivariate tests	df	F	p
Treatment	60, 255	2.3	**< 0.001**
Univariate tests			
Tailfin depth	12	4.7	**< 0.001**
Tail length	12	1.5	0.169
Body depth	12	2.5	**0.010**
Body length	12	2.2	**0.024**
Mass	12	0.9	0.554

3.3.1 Do the prey respond to predators?

To test whether our tadpoles were inducible by predator cues, and whether they responded to starved predators or consumed snails, we compared the No-Predator, Starved Predator, Consumed 100S, and Consumed 100T treatments. We began by examining the two tadpole behaviors. Compared to the number of tadpoles observed in the No-Predator treatment, there was no effect of Starved Predator (p = 0.95), Consumed 100S (p = 0.92), but we observed fewer tadpoles with Consumed 100T (p = 0.014; Fig. 5A). Compared to the activity of the tadpoles in the No-Predator treatment, there was no effect of Starved Predator (p = 0.91) or Consumed 100S (p = 0.51), but there was an effect of Consumed 100T (p = 0.007; Fig 1B).

In terms of morphology (Fig. 6), relative to the No-Predator treatment, none of the morphological dimensions were affected by Starved Predators ($p > 0.28$) or Consumed 100S ($p > 0.54$). However, Consumed 100T induced a deeper tail fin ($p = 0.001$) but induced no change in body depth ($p = 0.98$) or body length ($p = 0.51$). Because we only detected significant effects of Consumed 100T on the two tadpole behaviors and relative tail depth, we subsequently tested our hypothesis using these three traits.

3.3.2 Do prey defenses increase as prey gain information from a predator attack sequence?

Our second hypothesis was that anti-predator responses would continually increase as tadpoles received cues from crushed conspecifics (Crushed 100T), chewed conspecifics (Chewed 100T), digested conspecifics (Digested 100T), and chewed plus digested conspecifics (Consumed 100T).

We began by examining the two behavioral responses. Compared to the number of tadpoles observed in the No-Predator treatment, there was no effect of Crushed 100T ($p = 0.52$), Chewed 100T ($p = 0.58$), or Digested 100T ($p = 0.11$) whereas there was an effect of Consumed 100T (as noted above; Fig. 5A). Compared to the activity of tadpoles in the No-Predator treatment, there was no effect of Crushed 100T ($p = 0.48$), Chewed 100T ($p = 0.70$), or Digested 100T ($p = 0.61$), but there was an effect of Consumed 100T (as noted above; Fig. 5B).

We then examined the tail depth response (Fig. 6A). Across the five treatments, we observed a general trend toward increased tail depth with each subsequent component of the attack sequence. Compared to tail depth in the No-Predator treatment (Fig. 6A), there were no

differences with Crushed 100T (p = 1.00), Chewed 100T (p = 0.58), or Digested 100T (p = 0.10), but there was an effect of Consumed 100T (as noted above).

3.3.3 Do prey defenses increase as the biomass of consumed conspecifics increases?

We predicted that prey would induce greater defenses as the biomass of consumed tadpoles increased. The first way to address this question was to examine increases in predator consumption of tadpoles in the absence of any heterospecific prey. Compared to the number of tadpoles observed in the No-Predator treatment, there was no effect of Consumed 25T (p = 0.26) of Consumed 50T (p = 0.45) whereas there was an effect of Consumed 100T (as noted above; Fig. 5A). Compared to the activity of tadpoles in the No-Predator treatment, there was no difference in activity with Consumed 25T (p = 0.32) or Consumed 50T (p = 0.76), but there was an effect of Consumed 100T (as noted above; Fig. 5B).

When we examined relative tail depth, we found that compared to the No-Predator treatment there were deeper tails with Consumed 25T (p = 0.005), no effect of Consumed 50T (p = 0.622), and deeper tails with Consumed 100T (as noted above; Fig 2A).

Our first corollary to the above hypothesis, we also examined whether prey defenses increase as a function of increased conspecific biomass in predator's diet irrespective of other heterospecific prey that are consumed while holding the total biomass consumed by a predator constant. We first analyzed the two behavioral responses. Compared to the number of tadpoles observed in the No-Predator treatment, there was no effect of Consumed 25T:75S (p = 0.51) or Consumed 50T:50S (p = 0.20), but there was an effect of Consumed 100T (as noted above; Fig. 5A). Compared to tadpole activity in the No-Predator treatment, there was no effect of Consumed 25T:75S (p = .70), but there was lower activity with Consumed 50T:50S (p = 0.03)

and Consumed 100T (as noted above; Fig. 5B). In regard to tail depth in the No-Predator treatment, we found that tails became deeper with Consumed 25T:75S ($p = 0.05$), Consumed 50T:50S ($p = 0.01$), and Consumed 100T (as noted above; Fig. 6A)

Our second corollary to the above hypothesis was that if prey use consumed conspecific biomass as the cue to produce defenses, we should see no difference in phenotype when prey experience cues from a predator consuming conspecific prey versus predators consuming the same biomass of conspecific prey plus an additional biomass of heterospecifics that do not induce any phenotypic change. In terms of the number of tadpoles observed and tadpoles activity, we found no significant differences in behavior between Consumed 25T versus Consumed 25T:75S ($p = 1.0$) or between Consumed 50T versus Consumed 50T:50S ($p > 0.6$; Figs. 1A-B). Similarly, there were no differences in tail depth when we compared Consumed 25T versus Consumed 25T:75S ($p = 1.0$) or between Consumed 50T versus Consumed 50T:50S ($p = 0.56$; Fig. 6A).

3.3.4 Do prey respond to temporal variation in predator consumption?

Our final hypothesis was that prey would respond to temporal variation in predation cues and produce the same defenses regardless of whether the predator's temporally variable diet consisted of feeding the predator tadpoles or no tadpoles on alternate days versus feeding the predators tadpoles and snails on alternate days.

Compared to the number of tadpoles observed in the No-Predator treatment, there was no effect of Consumed 100T/0S ($p = 0.46$) but there was an effect of Consumed 100T/100S ($p = 0.01$) and Consumed 100T (as noted above; Fig. 5A). There was no difference between Consumed 100T/0S and Consumed 100T/100S ($p = 0.74$).

Compared to tadpole activity in the No-Predator treatment, there was a marginal effect of Consumed 100T/0S ($p = 0.08$) and no effect of 100T/100S ($p = 0.55$), but there was an effect of Consumed 100T (as noted above; Fig. 5B). There was no difference between Consumed 100T/0S and Consumed 100T/100S ($p = 0.98$).

Compared to relative tail depth in the No-Predator treatment, tails became deeper with Consumed 100T/0S ($p = 0.02$), Consumed 100T/100S ($p = 0.04$), and Consumed 100T (as noted above; Fig. 6A). There was no difference between Consumed 100T/0S and Consumed 100T/100S ($p = 1.00$).

3.3.5 Additional responses to the predator treatments.

It is worth noting that while body depth and body length did not show differences between No-Predator and Consumed 100T, there was a univariate effect on both dimensions (Table 6). For body length, none of the treatments induced body lengths that differed from the No-Predator treatment ($p > 0.3$). For body depth, however, the univariate effect was clearly driven by the Crushed 100T treatment, which induced shallower tadpole bodies ($p = 0.022$; Fig. 6B).

3.4 DISCUSSION

We found that predators induced grey treefrog tadpoles to exhibit reduced activity, increased hiding behavior, and deeper tails; these defenses are consistent with a multitude of previous studies across a variety of species (Laurila et al. 1997, Relyea and Werner 2000, Relyea 2001, Van

Buskirk 2001). As more types of cues became available from a predation event, there was a general trend of increased behavioral and morphological defenses. Additionally, prey defenses showed a pattern of increasing in magnitude as the biomass of consumed prey increased. Interestingly, this pattern was similar when heterospecific snails were consumed at the same time. Finally, the addition of temporal variation to changes in diet composition continued to induce strong prey defenses and the magnitude of defense was similar with and without the inclusion of snails in the predator's diet.

Previous studies of antipredator defenses indicate that increased refuge use and decreased activity as predation risk increases is a consistent defense in treefrogs (Relyea 2001, McCollum and Van Buskirk 2006). Moving less and hiding more (i.e., reduced activity, increased refuge use) is expected to decrease the likelihood of encountering or being detected by a predator. For many organisms, anurans in particular, antipredator reduction in activity often comes at a cost of decreased growth and size at metamorphosis (Lima 1988, Skelly 1992, Anholt and Werner 1995, McCollum and Van Buskirk 1996, 2000, Laurila et al. 2004), and size at metamorphosis can have long-term effects on fitness in anurans (Altwegg and Reyer 2003, Ficetola and De Bernardi 2006). These studies that found consistent behavioral changes also commonly observe predator induction of deep tails, as we found in our study (*Hyla chrysoscelis*: McCollum and Van Buskirk 2006, Richardson 2006; *H. femoralis*: LaFiandra and Babbitt 2004; *H. versicolor*: Relyea 2001, Hoverman and Relyea 2003). Deep tails are expected to improve tadpole escape from predators by acting as a distraction and redirecting attacks away from the body and vital organs (McCollumn and Leimberger 1997).

3.4.1 Do prey defenses increase as prey gain information from a predator attack sequence?

We predicted that prey would produce stronger defenses as they gained more types of information from the predator attack sequence (i.e. crush prey, chew prey, and digest prey). Our results suggest that grey treefrogs hide more, reduce their activity, and increase their tailfin depth as more information about a predation event becomes available. Although there was a pattern of stronger responses as we moved from Crushed 100T to Consumed 100T, only the Consumed 100T induced large enough phenotypic changes in any of the traits to be significantly different from the No-Predator treatment. While few studies have examined all parts of a predation sequence, previous work suggests that cues derived from prey (Chivers and Smith 1998, Summey and Mathis 1998, Stabell et al. 2003, Laforsch et al. 2006) and cues from predator digestion (Jacobsen and Stabell 1999, 2004, LaFiandra and Babbitt 2004, Richardson 2006) can induce morphological and behavioral defenses. Moreover, organisms tend to increase morphological defenses as predation risk increases (Mirza and Chivers 2003a,b, Bordeau 2010). However, much of this same work indicates that prey should decrease activity in a continuous fashion (i.e., progressively increase) as more types of information about predation become available (Wisenden et al. 2008, Schoeppner and Relyea 2009a,b). This appears inconsistent with our results, in that only the predation sequence in its entirety (i.e., Consumed 100T) induced significant behavioral differences. Therefore, we conclude that grey treefrogs use a different antipredator strategy than other anuran species previously considered, specifically they appear to hide more with greater predator attack information, but do not reduce activity when out of refuge.

3.4.2 Do prey defenses increase as the biomass of consumed conspecifics increases?

We predicted that treefrog tadpoles should increase their defenses as predators consumed greater amounts of treefrog tadpoles. How prey decide to respond to this conspecific biomass within predator cues is less clear cut than how they responded to attack sequence cues. Prey exposed to intermediate biomasses of consumed conspecifics in their predators' diets produced intermediate hiding and activity. This response is consistent with other studies that have noted an increase in defenses as predator risk increases (i.e., predator abundance; Teplitsky et al. 2005, Fraker 2008, Schoeppner and Relyea 2009a,b). Furthermore, we saw similar responses in behavior when total diet biomass was held constant but prey biomass increased, indicating that for behavioral defenses, prey are tracking conspecifics and not total diet biomass. To our knowledge no other studies have held biomass constant while adjusting conspecific content specifically. However, work by Fraker (2009) suggests a similar pattern in that green frog tadpoles responded with increase defenses to increasing conspecific content in predators' diets, even when predators were also consuming heterospecifics (i.e., 0%, 50%, 100% green frog).

This pattern becomes complicated when we looked at changes in tailfin depth across the biomass-related treatments, or if we compared defenses between diets that included only conspecific tadpoles versus diets that included conspecific tadpoles plus snails. Grey treefrogs exposed to the lowest (25 mg) and highest (100 mg) conspecific biomass produced tailfins of approximately the same depth, but those exposed to intermediate biomass (50 mg) produced dramatically shallower tailfins. Rather than this pattern holding across all biomass treatments, we found that the 50:50 treatment produced equivalent tailfin depth to the lowest, highest, and 25:75 treatments, contrary to expectations. This odd pattern remains nearly identical in activity

measurements. However, all the aforementioned treatments showed equivalent levels of behavior except for 50:50, which showed reduced behavior.

By and large, the behavioral results between tadpole diets and mixed tadpole/snail diets were congruent with equal amounts of consumed tadpoles, suggesting that biomass of conspecifics is likely the most important portion of the cue. However, that the results of the equally mixed diet (i.e., Consumed 50T:50S) deviated from the equivalent tadpole biomass diet (i.e., Consumed 50T) indicates that conspecific biomass is not the sole important factor in our experiment. Our data suggest two possibilities. The first is that the addition of snails to the predators' diet created some manner of noise or interference with regard to the tadpole-derived cues. This may be due to digestive modification of the snail cues, given that digestion is required in some cases for organisms to utilize cues to make defensive decisions (Venzon et al. 2000, Brown 2003 Jacobsen and Stabell 2004). Alternatively, it could be that the chemicals from tadpole tissue and snail tissue interact with one another during digestion, since we expect chemical cues to be sensitive to the abiotic conditions in which they are embedded (Ferrari et al. 2010). The result in this case would be grey treefrogs were responding to an entirely new cue rather than two independent conspecific and heterospecific cues. That we didn't see any unusual responses in activity or morphology below 50 mg of tadpole tissue suggests that there may be a threshold to prey responses or chemical interactions, respectively (Mirza and Chivers 2003). Though prey are able to use below-threshold responses to make defensive decisions, these manifest during secondary exposure and do not induce quantifiable morphological or behavioral changes on the first pass (Mirza and Chivers 2003).

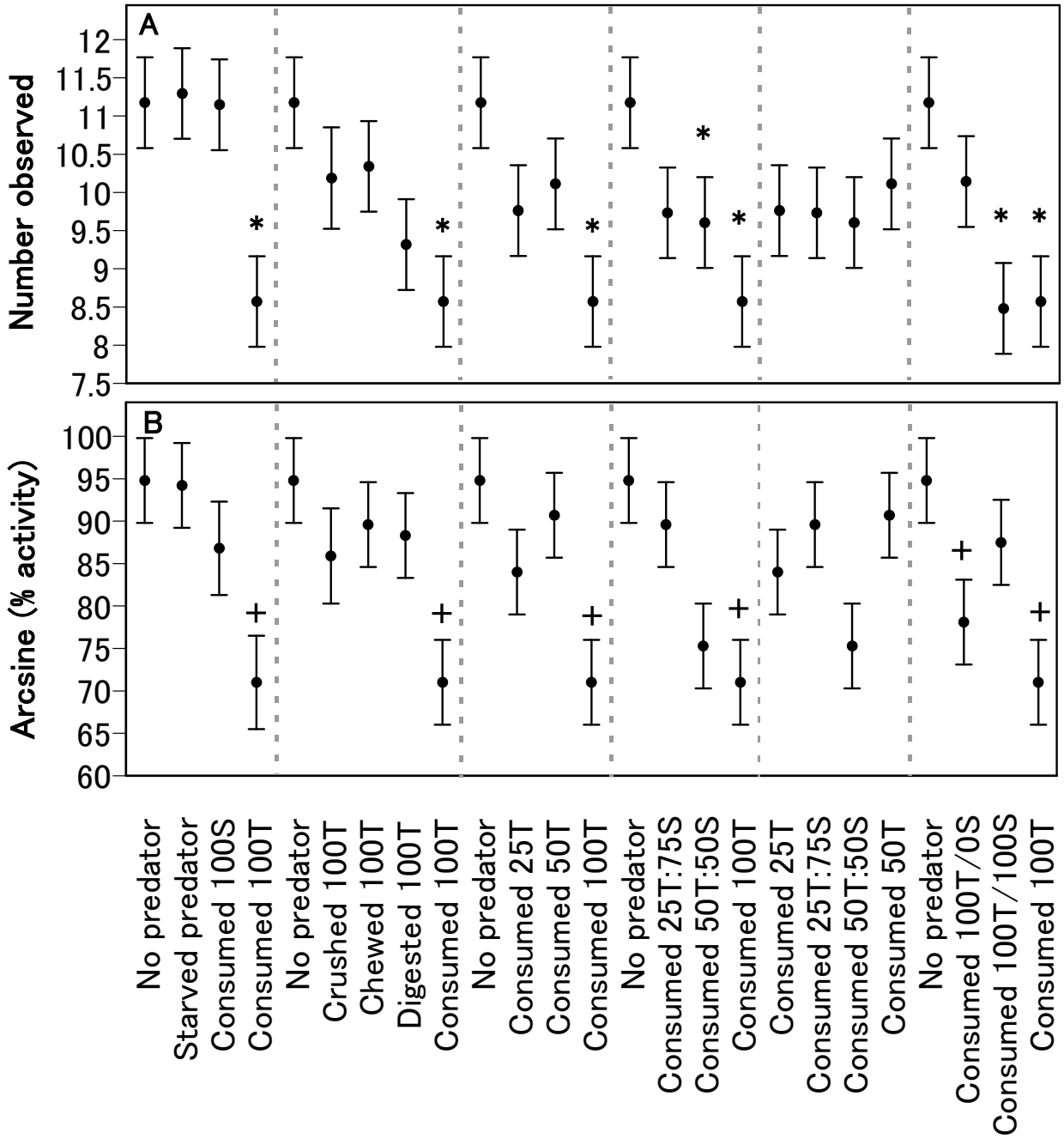

Figure 5. (A) Number of observed gray treefrog tadpoles and (B) their activity when raised in 13 predator-cue treatments. Data are the least squared means ± SE of 27 observations made over 3 d. Data are grouped by hypotheses; therefore, some treatments are repeated within panels. * indicates significance at α = 0.05; + indicates significance at α = 0.10.

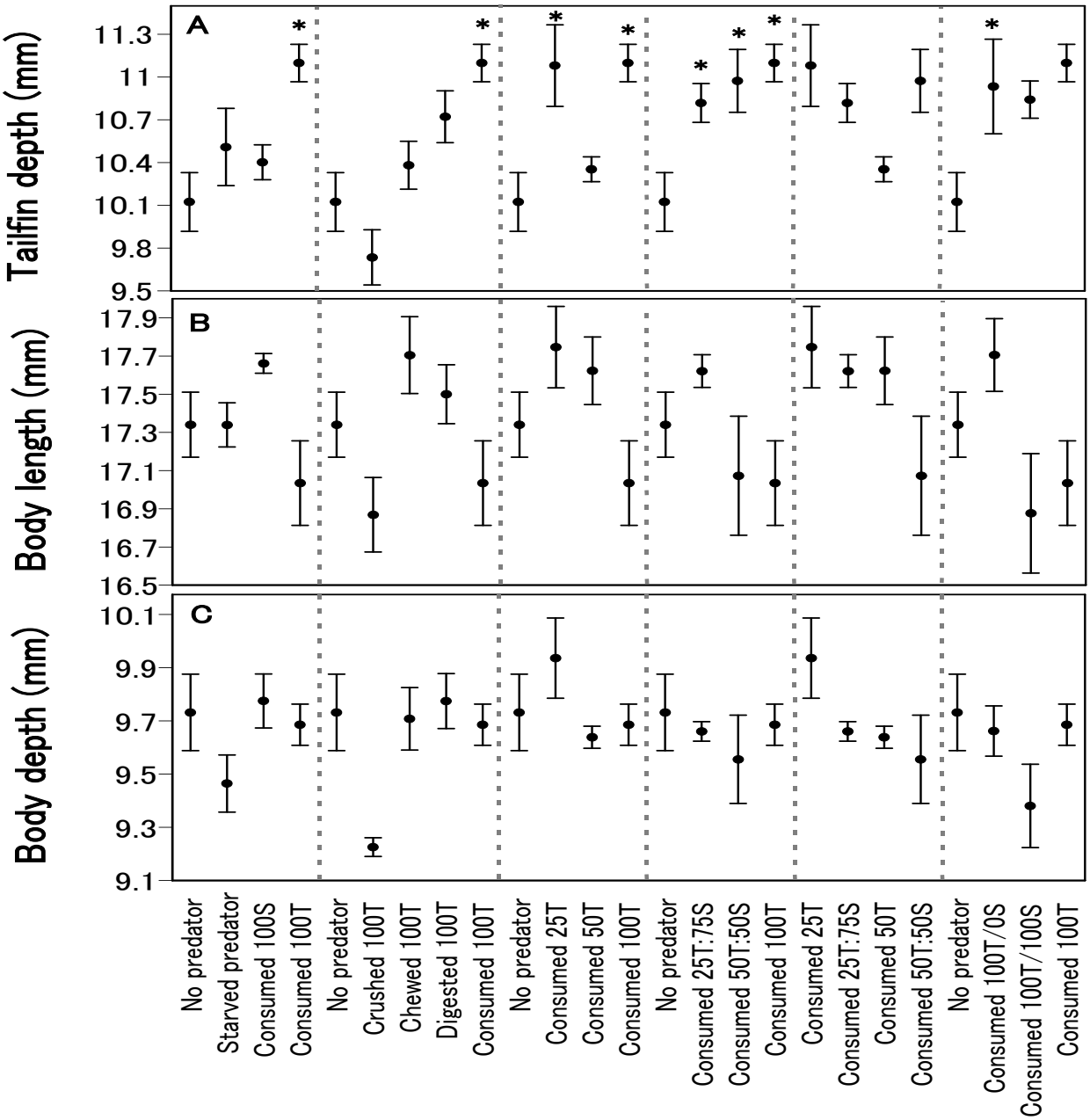

Figure 6. Mass-independent morphological dimensions for gray treefrog tadpoles when raised in 13 predator-cue treatments: (A) tail depth, (B) body length, and C) body depth. Data are means ± SE of animals measured at the end of the experiment on day 16. Data are grouped by hypotheses; therefore, some treatments are repeated within panels. * indicates significance at α = 0.05.

3.4.3 Do prey respond to temporal variation in predator consumption?

We predicted that tadpoles should continue to respond only to treefrog biomass in predator diets regardless of the temporal pattern of predation regardless of whether heterospecifics were included in the predator's diet. However, our results are only partially consistent with this prediction. We found that this was only the case for tailfin depth. Regardless of timing of consumption or whether predators also consumed heterospecifics, tadpoles increased their tailfin depth approximately the same amount. We found that behavioral responses were more complicated. Consumption of tadpoles alone, regardless of temporal variation (i.e., Consumed 100T/0S, Consumed 100T), decreased activity significantly. The number of visible tadpoles showed an opposing pattern in that the addition of heterospecifics (i.e., Consumed 100T/100S) decreased the number of tadpoles visible while temporal variation alone (i.e., Consumed 100T/0S) did not.

There is a significant body of literature about how prey address the complexity of predator identity in their environment, but limited data on how they deal with the any complexity of predator dietary cues, let alone temporal complexity of these cues. Studies looking at temporal variation typically have found animals faced with fluctuating periods of risk tend to show greater activity levels than those in constant risk environments (Sih and McCarthy 2002, Laurila et al. 2004, Foam et al. 2005, Mirza et al. 2006, Creel et al. 2008). This is consistent with our results if we consider Consumed 100T/0S a constant risk environment, in the sense that only tadpoles are ever being consumed. In terms of diet, most of our information relates to how prey respond primarily to consumption of their conspecifics by predators, which is to induce greater antipredator defenses in the presence of those predator diets (Mirza and Chivers 2003b, Wilson and Lefcort 1993, Laurila et al. 1997, 1998). Comparatively, the rare study of predators

consuming both conspecifics and heterospecifics in their diets seem to indicate an intermediate defensive response similar to what we observed in the number of visible tadpoles in the Consumed 100T/0S treatment (Smee and Weissburg 2006).

We challenged prey with conflicting information about predator timing and diet by temporally varying tadpole exposure to predators consuming prey that produce strong defenses (i.e., conspecifics) and those that produce weak defenses (i.e., snails). In the face of predators who consumed both tadpoles and snails over time, grey treefrogs produced a defensive compromise: developing a short body and hiding as if the predator was only eating conspecifics, but as active as with predators only eating snails. They also increased tailfin depth linearly with both timing and proportion of tadpole consumption. When faced with only temporal variation in feeding, all defenses seemed to track average conspecific biomass. Tadpoles appear to have allocated different defensive strategies simultaneously and we suggest that this compromise is a strategy for fine-tuning their defenses to address a complex predator environment (Ferrari and Chivers 2009).

When one does not observe a response to heterospecific prey being consumed, one cannot determine whether this is caused by an inability of prey to detect heterospecific cues or if heterospecific cues are detected but subsequently interpreted as indicating no need for a phenotypic response. In previous work, tadpoles tend to be unresponsive to snail-based cues (Schoeppner and Relyea 2005, 2009a,c). Our results lend support to the notion that tadpoles can detect snail cues in their predators' diets, but the importance of these cues depends on context. Specifically, we see this in the differential response in activity and tailfin depth between Consumed 50T and Consumed 50T:50S (Fig. 5B, 2A) and the behavioral divergence between Consumed 100T/0S and Consumed 100T/100S (Fig. 5). However, we remain unable to

determine whether this is because snail cues are unimportant unless they co-occur with grey treefrog tadpoles, or if the ability to detect them is due to digestive modification of snail cues within the predator. Regardless of the mechanism, we demonstrate that the additional cues of snails in the predator diet cause a non-additive defensive response by prey.

It is also interesting that the Crushed 100T treatment induced shallower bodies in gray treefrogs. This response to crushed tadpoles was also observed a study of leopard frog tadpoles by Schoeppner and Relyea (2009a). However, we remain unable to explain why such a change might occur, and future studies are needed to determine whether the changes are adaptive.

3.5 CONCLUSIONS

This study demonstrated that grey treefrogs respond to increases in information gained from predator attack sequences by increasing their hiding behavior but not reducing their activity. Furthermore, grey treefrogs use biomass of consumed conspecifics as a primary cue for determining strength of induced defenses in the absence of other dietary cues. However, with the addition of additional dietary components, particularly an otherwise neutral heterospecific, defenses no longer appear to directly track biomass, and prey induce non-additive responses for some defensive traits. Future work should seek to determine what other information in the cue is being used by the prey, what precisely changed in the cue due to the consumption of multiple prey types, and whether the response is specific to conspecifics combined with a neutral heterospecific. Conspecific biomass alone doesn’t appear to be the information within predator diets that prey are utilizing. We also determined that the interaction between temporal variation and diet variation produces behavioral compromises wherein multiple defenses were used

simultaneously. This is in contrast to other studies of variation in predator environment that suggest prey should respond to the most dangerous predator context, particularly in the presence of conflicting information. Future studies should consider addressing the generality of these results among taxa, and to determine how they differ over other combinations of diet and temporal variation.

4.0 OVERARCHING CONCLUSIONS

In order to gain a full understanding of how predator-induced plasticity operates, we must understand it from an evolutionary perspective and we must understand the immediate cues and mechanisms that prey use to make defensive choices. Using larval amphibians, we addressed both ends of this spectrum. By using a genus of salamander that has not been well studied previously, we were able to fill a hole in our general understanding of amphibians while carrying out a comparative study to give us a better picture of large-scale patterns of induced defenses. We then selected a species of tadpole for which we already understand the general patterns of antipredator plasticity relatively well, which gave us a baseline from which to investigate induced defenses based on fine-scale variation in predator cues.

Our results suggest that *Ambystoma* salamanders are less plastic than previous studies would suggest. When defenses exist, morphological responses to predators vary among species in both magnitude and direction, and we are unable to generalize any defense across all species examined. Furthermore, not all species respond to predators behaviorally. Though behavior was more consistent than morphology across the five species, contrary to our predictions, neither reduced behavior nor increased refuge use were predictable responses. Most of the salamanders that induced behavioral defenses appeared to rely on them less the longer the experimental predator exposure lasted. To make reliable conclusions about the patterns of specific *Ambystoma* inducible defenses or interspecific variation requires more data. Doing so may allow us to begin

taking a phylogenetic approach to the study of salamander inducible defenses.

Theory and evidence suggest that plasticity itself can evolve (Bradshaw 1965, Pigliucci et al. 1999, Agrawal 2001, Pollard et al. 2001, Pigliucci 2005), and that it may play a critical role in evolution (Agrawal 2001, West-Eberhard 2003, Pigliucci et al. 2006, Ghalambor 2007, Crispo 2008). We suggest that a phylogenetic signal in plastic traits may explain the variation within the literature. Future studies of salamander plasticity should aim to examine species with an eye to phylogenetic analysis so we can better understand the evolution of these defenses.

At the opposite end of the spectrum, our study of grey treefrog responses to predator cues revealed some unique insights into the ability of prey to use cue information. Our data suggest that as grey treefrog tadpoles gain more information from an attack, they increase the strength of their hiding behavior but do not reduce activity. Furthermore, treefrog tadpoles are able to use the biomass of consumed conspecifics, in the absence of other dietary cues, to determine the strength of their induced defenses. However, the non-additive defenses developed with the addition of an otherwise neutral heterospecific to predator diets indicates to us that conspecific biomass is not the specific information within predator diets that prey are utilizing. Contrary to our predictions and previous work, we found that the interaction between temporal variation in attack and diet variation of the predator induced multiple defenses simultaneously in treefrogs. Previous work suggests that prey should either average their defensive response or prioritize the most dangerous predator. That the treatment we observed this defensive compromise in was one meant to incorporate more complex feeding regimes than are typically used in simplified experimental studies, suggests that prey defenses in nature may be more complex than we have estimated or measured previously.

The results of our work with grey treefrog indicate a more nuanced use of indirect cues than previously reported and value to studying more complex interactions among cues, diet, and the timing of predation. Future studies should consider assessing the generality of our results, determining the identity of cue components being utilized by prey to fine-tune their defenses, and incorporating more complex interactions into experiments than simple single-species diet regimes.

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