

CLUTCH-LEVEL VARIATION IN PREDATOR AVOIDANCE BEHAVIOR IN WOOD FROG (*LITHOBATES SYLVATICUS*) TADPOLES

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ABSTRACT. In nature, genetics and environmental conditions contribute to the abundant variation in morphology, physiology, and behavior. Predator avoidance behavior of Wood Frog (*Lithobates sylvaticus*) tadpoles was tested from six clutches to determine if variability existed between independent clutches reared under the same environmental conditions. Exposing tadpoles to alarm cues from damaged conspecifics and kairomones from a predator (after a learning event) and the corresponding reduction in activity were recorded. While some variation between clutches existed, no significant difference was observed in this behavior. We discuss hypotheses for the lack of variability and suggest our results are due to the critical role of this behavior on the survival and fitness of individuals.

Keywords: Variation, behavioral syndromes, anti-predator, *Lithobates sylvaticus*, Wood Frog, *Rana sylvatica*, tadpole

INTRODUCTION

One of the most important characteristics of any species is the variability found within populations. This variation provides the raw material for evolution by natural selection, and has helped produce the tremendous array of diversity found today (Endler 1986; Grant 1999). Although abundantly evident among morphological characteristics (Berven 1982; Townsend & Hildrew 1994), there also can be great variability in physiology (Prosser 1955; Crespi et al. 2013) and behavior (Bendesky & Bargmann, 2011). Variation in any trait is due to the combined effect of differences in genetics through mutations and heritable variability (Brooker 2012) and environmental conditions that affect the expression of these genes (Hemmer-Henson et al. 2007). A combination of these factors (genes and environment), is primarily responsible for the additive genetic variation found within populations (Cooper & Kaplan 1982; Hemmer-Henson et al. 2007).

The effect of underlying natural variation on the ecology and fitness of organisms is well documented. Differences in environmental conditions can influence the life history of organisms within that environment, such as clutch size (Mitchell & Pague 2014), hormone levels (Harding 1981; Ketterson & Nolan 1999), and the expression of sexually selected traits (Griffith et al.

1999). The effects of environment are also seen among populations from multiple locations. For example, Wood Frog (*Lithobates sylvaticus*) populations exhibit variation in physical characteristics, reproductive characteristics, and development that correspond to differences in altitude (Berven 1982), thus leading to adaptation to local environmental conditions.

One aspect of organismal ecology that is exceedingly variable is behavior, a phenomenon often attributed to behavioral syndromes (Sih et al. 2004). Behavioral syndromes are an array of correlated behaviors that an organism exhibits through differing situations (Sih et al. 2004). These general “temperaments” tend to carry over to multiple events such as mating, parental care, and competition (Sih et al. 2004). For example, an individual that is bold or active in a situation with a mate also may have a similar tendency in situations involving a predator (Bell & Sih 2007; Pruitt et al. 2012). Within a given population, individuals possess different syndromes that occur across a broad spectrum (Huntingford 1976; Bell 2005; Johnson & Sih 2005; Bell & Sih 2007; Dingemanse et al. 2007; Dochtermann & Jenkins 2007; Duckworth & Badyaev 2007; Kortet & Hedrick 2007; Moretz et al. 2007; Reaney & Backwell 2007; Pruitt et al. 2012).

Although the variability in animal behavior is well known, there is little known about clutch-level differences in behavior. Individuals within a clutch should have (at minimum) the same

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maternal genetic background, and are typically reared under similar environmental conditions. Nevertheless, resources are often provisioned differently both within and across clutches (Reed & Vleck 2001), and these differences likely have a dramatic effect on the behavior of those organisms. For example, female birds often provision eggs with varying amounts of androgens that affect multiple life-history characteristics including behavior (e.g., parental care, aggression levels, etc.), physiology (e.g., immune function, hormone levels, etc.), morphology, growth, and even survival (Groothuis et al. 2005). In reptiles with temperature dependent sex determination, a single clutch may be exposed to different temperatures (e.g. top vs bottom of the nest), which may produce a mix of each sex. These differences in rearing conditions can have life-long effects, even affecting their mate choice decisions (Putz & Crews 2006).

Within amphibians, studies have documented variation in behavioral syndromes among individuals (Lima & Bednekoff 1998; Laurila et al. 2004; Sih et al. 2004), but an understanding of the differences in clutch-level behavior is less well known. In addition, many studies (e.g. Chivers & Mirza 2001; Mathis et al. 2008; Ferrari & Chivers 2010; Gall & Mathis 2010; Gall et al. 2013; Chapman et al. 2014) use entire egg clutches to test behavioral principles due to the large number of individuals that can be attained and their relative ease of collection. These studies can be based on relatively few clutches (i.e., 2–12). Whether these different clutches exhibit similar patterns of behavior is unknown as clutch is rarely included as a variable in analyses. In addition, it is possible that different responses by individuals from different clutches could mask trends in behavioral patterns.

Wood Frogs were chosen as a model organism to study differences between individual clutches in response to stimuli indicative of predation risk. The predator avoidance behaviors exhibited by tadpoles in response to these cues are well documented, and typically include reduction in activity and spatial avoidance (Kats et al. 1988; Petranks & Hayes 1998; Chivers & Mirza 2001; Relyea 2001). In the first experiment, the variation in predator-avoidance behavior between six clutches of Wood Frog tadpoles before and after exposure to alarm cues from damaged conspecifics was examined. In a second experiment, marbled salamander larvae (*Ambystoma opacum*) were trained via classical conditioning (e.g., a

learning event) to fear a natural predator, and the variation in behavior among these clutches in response to exposure to kairomones from that predator alone was then evaluated.

MATERIALS AND METHODS

Wood Frog (*Lithobates sylvaticus*) clutches used in this experiment were collected from three separate water-filled depressions in a flatwoods area in Jefferson County, Indiana on 16 March 2016 (air temperature 22° C). Eggs were transported to Hanover College in plastic containers using water from the collection site. Clutches were housed individually in plastic containers with approximately 8 cm of water per container. Examination of the eggs using an Olympus SZ40 dissecting microscope revealed that they were at Gosner developmental stages 13–19 (Gosner 1960). Based on rainfall patterns, all clutches were likely deposited in the previous 48 hr. To reduce density following hatching, tadpoles from a single clutch were divided into four groups. Each group was placed in a plastic container (60 × 31 × 15 cm) with approximately 6 cm of water (approximately 200 tadpoles per container). The tadpoles were fed algae powder (a mixture of *Spirulina* and *Chlorella*, Saurian Enterprises, St. Louis, MO) every two days. All clutches were housed on a 12:12 light:dark cycle at 16° C. Water was changed twice per week.

Experiment 1: exposure to alarm cue.—The experimental set up consisted of a plastic test chamber (6 × 6 × 9.5 cm) that contained 250 mL of de-ionized (DI) water. To minimize external visual stimuli, blinds were placed around the chamber. Each container had a vertical line drawn down the center (3 cm from either end) that was used to measure tadpole activity. Alarm cues were prepared by macerating tadpoles in a blender (Oster, Sunbeam Products, Inc.) and diluting the solution with DI water to a concentration of 0.003 g tadpole / 1 mL water (Mathis et al. 2008). The alarm cue was prepared at the start of each test day and stored on ice until use.

At the commencement of the experiment, a test tadpole was arbitrarily selected from a randomly chosen clutch and placed in the test chamber to acclimate for 60 min. Following the acclimation period, a four-minute pre-stimulus control period was initiated. During this time, the number of times the tadpole crossed the center line and the number of discrete movements (any movement by the animal separated by a visible period of

immobility lasting greater than 1 sec) was recorded. At the conclusion of the control period, 5 mL of alarm cue were added to the test container. The stimulus was injected with a syringe down the side of the test chamber to minimize disturbance. A 4 min post-stimulus period was then initiated immediately following the addition of the alarm cue. At the conclusion of the experiment, the tadpoles were put in separate containers and were never reused. Test containers were rinsed with hot water, then DI water, and the experimental procedure was repeated ($n = 98$ total; 15–18 from each clutch).

Experiment 2: response to kairomones after learning.—Marbled salamander (*Ambystoma opacum*) larvae were collected ($n = 20$) from an ephemeral pool in Jefferson County, Indiana. After transporting the larvae in plastic containers using the water from the collection site, the larvae were housed individually in containers ($7 \times 11 \times 11$ cm) with 250 mL of DI water for approximately 24 hr. Water from all the marbled salamander larvae was then combined to create a homogenous mixture of the kairomones from all donors and to eliminate variation among individual donor cues. Immediately following preparation of predatory kairomones, each tadpole-holding container was simultaneously exposed to 200 mL of predatory kairomones and 83 mL of freshly prepared alarm cues (0.003 g/mL). This training procedure ensured Wood Frog tadpoles learned to avoid the marbled salamander larvae through classical conditioning, as done previously in numerous studies (e.g., Brown & Smith 1998; Ferrari et al. 2010). Remaining kairomones were frozen for later testing.

The experimental procedure and set-up for the second experiment was identical to that described above, with the exception of the following change: 5 mL of kairomones from marbled salamander larvae was thawed and introduced between the control and post-stimulus observation periods ($n = 91$; $n = 15$ –16 per clutch).

Statistical Analysis.—Two Two-Way repeated-measures (RM) ANOVAs were used to examine for clutch-level differences in predator avoidance behavior in response to alarm cues from damaged conspecifics and kairomones from predatory salamander larvae. Clutch (6 levels) and treatment (RM component: 2 levels: pre-control exposure, post alarm cue/kairomone exposure) were treated as the two fixed-effect factors, while the number of lines crossed

and number of discrete movements were the two response variables. This procedure allowed us to test for interaction effects between clutch and treatment for each of the response variables (i.e., to determine whether different clutches exhibited significantly different responses to the stimuli indicative of predation risk). Assumptions for parametric statistics were assessed using Shapiro-Wilk tests for normality and Levene's test for equal variances, as well as graphical analyses of the residuals. All statistical tests were completed in SAS v9.4 (SAS Institute Inc., Cary, NC). All assumptions were adequately met for all response variables.

RESULTS

A RM ANOVA found a significant main effect of treatment on the number of lines crossed ($F_{[1,92]} = 12.09$, $P < 0.001$, Fig. 1A) and on the number of discrete movements ($F_{[1,92]} = 37.52$, $P < 0.001$, Fig. 1B) by tadpoles in response to alarm cues. These results indicate that all Wood Frog clutches responded to alarm cues with a general reduction in activity (Fig. 1). Although there was variation in the mean response of each clutch (e.g., range: change lines crossed -0.38 to -1.75; change number of moves -3.1 to -6.3), there was no significant main effect of clutch on the number of lines crossed ($F_{[5,92]} = 1.88$, $P = 0.11$, Fig. 1A) or the number of discrete movements ($F_{[5,92]} = 1.06$, $P = 0.39$, Fig. 1B) by the tadpoles after exposure to alarm cues. There was also no interaction effect between clutch and treatment for either response variable (lines crossed: $F_{[5,92]} = 0.51$, $P = 0.77$; moves: $F_{[5,92]} = 0.30$, $P = 0.91$).

After a learning event in which kairomones from marbled salamander larvae were paired with alarm cues, Wood Frog tadpoles reduced activity in response to kairomones alone from these predators [significant main effects of treatment on the number of lines crossed ($F_{[1,85]} = 3.70$, $P = 0.058$, Fig. 2A) and the number of discrete movements ($F_{[1,85]} = 7.22$, $P = 0.009$, Fig. 2B)]. Once again, there was no significant main effect of clutch on the number of lines crossed ($F_{[5,85]} = 0.34$, $P = 0.89$, Fig. 2A) or the number of discrete movements ($F_{[5,85]} = 0.18$, $P = 0.97$, Fig. 2B) by the tadpoles after exposure to predatory kairomones. In addition, there was no significant interaction between clutch and treatment for either response variable (lines crossed: $F_{[5,85]} = 0.33$, $P = 0.89$; moves: $F_{[5,85]} = 0.08$, $P = 0.99$).

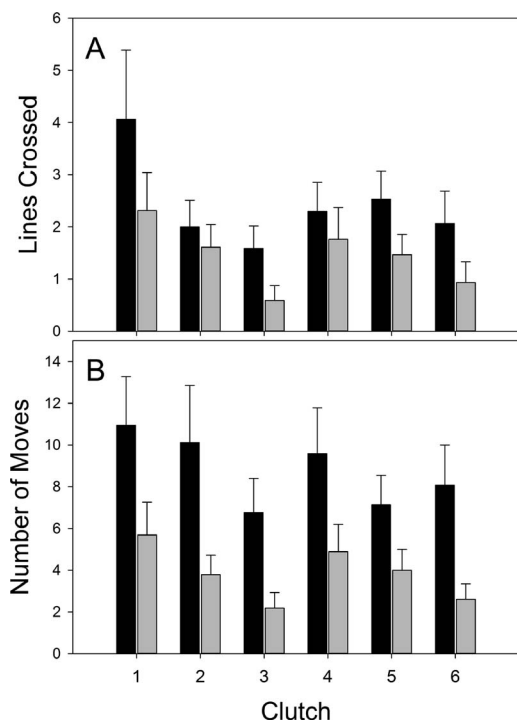


Figure 1.—Mean ($\pm$ SE) change in lines crossed (A) and number of moves (B) by Wood Frog (*Lithobates sylvaticus*) tadpoles both before (black bars) and after (gray bars) exposure to alarm cues from macerated conspecifics. Wood Frog tadpoles reduced activity in response to alarm cues (lines crossed: $F = 12.1$, $P < 0.001$; moves: $F = 37.5$, $P < 0.001$), but there was no effect of clutch (lines crossed: $F = 1.88$, $df = 5$, $P = 0.11$; moves: $F = 1.06$, $df = 5$, $P = 0.39$) and no interaction between clutch and treatment (lines crossed: $F = 0.51$, $df = 5$, $P = 0.77$; moves: $F = 0.30$, $df = 5$, $P = 0.91$), indicating each clutch exhibited similar responses to the alarm cues.

DISCUSSION

Amphibians possess numerous predator avoidance and antipredator behaviors including physical displays (e.g., unken), toxic and distasteful secretions, and numerous behavioral changes including hiding, fleeing, and immobility (Brodie 1977; Brodie et al. 1984; Kats et al. 1988; Williams et al. 2000). Not surprisingly, Wood Frog tadpoles from different clutches were found to significantly decreased the number of lines crossed and the number of discrete movements in response to alarm cues and kairomones from a predator. Behavioral changes by Wood Frog tadpoles in response to these stimuli have been

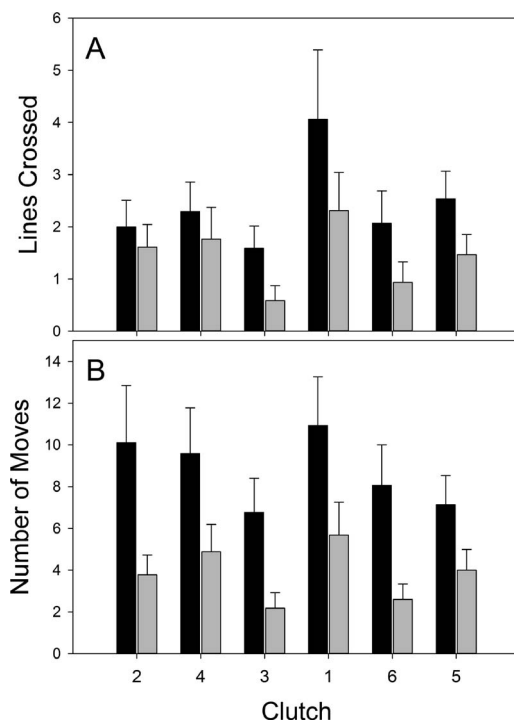


Figure 2.—Mean ($\pm$ SE) change in lines crossed (A) and number of moves (B) by Wood Frog (*Lithobates sylvaticus*) tadpoles both before (black bars) and after (gray bars) exposure to kairomones from a natural predator, the marbled salamander larvae (*Ambystoma opacum*). Wood Frog tadpoles reduced activity in response to kairomones (lines crossed: $F = 3.7$, $P = 0.058$; moves: $F = 7.2$, $P = 0.009$), but there was no effect of clutch (lines crossed: $F = 0.34$, $P = 0.89$; moves: $F = 0.18$, $P = 0.97$) and no interaction between clutch and treatment (lines crossed: $F = 0.33$, $P = 0.89$; moves: $F = 0.08$, $P = 0.99$), indicating each clutch exhibited similar responses to predator kairomones.

well documented and the reduction in activity observed here is a common predator avoidance behavior in this species (Kats et al. 1988; Petranksa & Hayes, 1998; Chivers & Mirza, 2001; Relyea 2001; Ferrari et al. 2010).

Although variation existed in each clutches' mean response to these stimuli, there was no main effect of clutch (and no interaction effect) indicating all clutches exhibited similar predator avoidance responses. The lack of significant variation across clutches in predator avoidance behavior was surprising given studies that have documented highly variable behavioral syndromes among individuals. These correlated behaviors occupy a spectrum from bold/aggres-

sive/active to reclusive/submissive/inactive and may be responsible for the maintenance of the high levels of variability within animal behavior (Sih et al. 2004). These behaviors are presumed to have a genetic basis (Bendesky & Bargmann 2011), and therefore related individuals (i.e., individuals within the same clutch) should exhibit similar patterns along this spectrum, whereas unrelated individuals may not. One possible explanation for a consistent response across clutches as shown in our study may be the relative "importance" of predator avoidance behaviors relative to others. Organisms perform a wide range of activities, and the role in enhancing fitness varies both intrinsically and depending on the general state of the animal. For example, many animals forgo foraging during reproductive periods, likely due to limited life-time reproductive opportunities and the relative need to focus time and energy toward this critical activity (Fleischer et al. 2003). Some behaviors, such as foraging frequency, timing of mating and courtship, and the window of oviposition may not have consistent dramatic fitness consequences from year to year and therefore have more intrinsic variability (Lima & Dill 1990). However, avoiding predators is critical to survival and fitness, and the consistent responses of Wood Frog tadpoles from different clutches in this study may be due to the overwhelming fitness advantage that responding to these cues (as opposed to ignoring them) entails. In this case, the lack of significant variation in the tadpoles' responses to these cues is critical because these stimuli signal the presence of imminent danger (Fraker et al. 2009; Ferrari et al. 2010).

Within predator-prey systems, organisms are exposed to situations and stimuli that are indicative of differing levels of risk (Lima & Bednekoff 1998; Ferrari & Chivers 2010). For example, an organism that detects kairomones from a predator may recognize a risky situation and react accordingly, yet may assign less risk to this situation than if a predator is visually detected in the vicinity. Predation-risk allocation hypothesis. Leopard Frog (*Lithobates pipiens*) tadpoles exposed to a diverse set of predator kairomones (starved, digesting conspecifics) and alarm cues (alone and combined with kairomones) were capable of discriminating among these cues and only responded with a full suite of defenses (both antipredator behavior and morphological changes) in response to the most risky cue (predators chewing and digesting prey) (Schoeppner &

Relyea, 2009). While the intensity of predator avoidance behavior varies depending on the situation and level of risk (Lima & Bednekoff 1998; Schoeppner & Relyea, 2009; Ferrari & Chivers 2010), variability in response to a single type of danger (i.e., within a level of risk) may be minimal and may account for the results of our study.

Although there was no significant difference between clutches in response to the chemical threat of predation, the presence of minor variability between clutches is evident (Fig. 1). Some variation was expected, and can be attributed to genetic or environmental factors. To reduce the role of environmental effects in our study, clutches were reared under identical conditions, indicating that any variability present is primarily the result of genetic differences between the clutches (Bendesky & Bargmann 2011).

Variation can arise through genetic differences or the effect of environmental factors on the expression of those genes. By eliminating the possibility of learned experiences by rearing individuals under the same conditions, it allowed this study to focus on the natural variation between clutches in response to predation risk. Rearing these tadpoles under uniform conditions resulted in similar predator avoidance behaviors in response to the same stimuli. Each individual holds genetic variability that results in slight differences in its particular characteristics, including behavior. However, Wood Frog tadpoles do not show significant variation in predator avoidance behaviors across clutches that were reared under the same environment. This suggests tadpoles from different clutches respond to predation risk with similar behavior, therefore studies focusing on behavioral responses toward predators may not need to incorporate clutch as a variable in their analysis.

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