

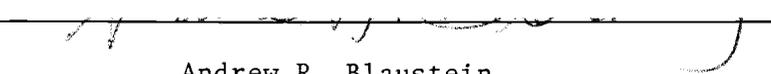
AN ABSTRACT OF THE THESIS OF

Diana Katharine Hews for the degree of Master of Science in  
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Title: An Investigation of the Alarm Response in Anuran Amphibian  
Larvae.

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Abstract approved:

  
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The results of my research suggest that tadpoles of the Western toad (Bufo boreas) and the Cascades frog (Rana cascadae) display an alarm response to chemicals from injured conspecific tadpoles. Furthermore, results suggest that the Bufo boreas alarm response can be elicited by a natural predator when it feeds on a conspecific tadpole and that this response decreases the capture efficiency of predators.

Initially, the alarm responses of B. boreas and R. cascadae were elicited by extracts containing chemical cues from injured conspecifics. B. boreas tadpoles increased their activity and avoided

areas into which conspecific extracts were added. The activity and avoidance responses were not elicited by extracts of non-conspecifics or by a control solution of water. R. cascadae tadpoles did not exhibit the avoidance response but did increase activity when a conspecific extract was added. An extract of non-conspecifics or a control solution of water did not elicit these reactions.

In the second part of my study, I tested the hypothesis that a natural predator could injure tadpoles and release the alarm substance. Results suggest that while capturing and eating a Bufo tadpole, the giant waterbug (Lethocerus americanus) caused enough damage to elicit the alarm reaction in conspecific tadpoles. Test tadpoles significantly increased their activity over control levels and avoided the side of the tank where a feeding predator was located in a visually isolated but interconnected container. When a non-conspecific tadpole (Pacific treefrog, Hyla regilla) was used as prey, B. boreas tadpoles did not avoid the side where the predator fed, although activity did increase. This increase in activity may have been due to agitation caused by the presence of predators.

I then examined two possible functions of the alarm substance: direct predator deterrence and warning of conspecifics. I found that dragonfly naiads (Aeshna umbrosa), another natural predator, neither moved away from an area into which a Bufo extract was introduced nor moved away when a control solution was introduced, suggesting that the alarm substance in extract form may not directly deter predators. If, however, the alarm substance warns conspecifics, the resulting alarm response should affect the capturing efficiency of a predator.

In fact, Aeshna naiads took significantly longer to catch B. boreas tadpoles after the introduction of a Bufo alarm substance extract than after the introduction of a control solution. For aquatic systems, this is the first evidence that suggests an alarm reaction elicited by a chemical substance may protect individuals from predation.

An Investigation of the Alarm Response  
in Anuran Amphibian Larvae

by

Diana K. Hews

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# AN INVESTIGATION OF THE ALARM RESPONSE IN ANURAN AMPHIBIAN LARVAE

## Chapter I

### INTRODUCTION

Animals defend themselves from predation in diverse ways (Edmunds, 1974). Most prey defenses, such as escape behaviors and cryptic colorations, are easy to understand evolutionarily as they obviously benefit the individual possessing the trait. Yet certain defenses pose problems for evolutionary theory. For instance, some alarm signals appear to be altruistic behaviors, performed at a risk to the signaller. Because of this, such signals are difficult to explain solely within the context of individual selection. The adaptive functions suggested for alarm signals (reviewed in Harvey and Greenwood, 1978) invoke individual, group and kin selection to explain the evolution of alarm signals. Alarm calls of ground squirrels (Sherman, 1977) and alarm signals of fish and larval anurans (Smith 1977, 1982) suggests that kin selection may be involved in their evolution or maintenance (reviewed in Harvey and Greenwood, 1978). These types of alarm signals have rarely been studied in depth. Knowledge of the relatedness of signallers and receivers is necessary to evaluate the role kin selection may play in the evolution of alarm signals. Determination of the costs and benefits, for signallers and receivers, is also critical. For example, the effectiveness of alarm signals in reducing predation must first be determined. Thus, the

study of prey defenses such as alarm signals should add greatly to our knowledge concerning kin oriented behaviors and predator prey relationships.

I investigated an alarm signal, and the response of conspecifics to the signal, in anuran amphibian larvae. The signal consists of specific chemicals released from injured animals and it elicits specific behaviors, termed collectively an alarm response, in conspecifics. Alarm substances and responses have been well studied in fish (for review see Pfeiffer, 1974; Smith 1982). Alarm responses of larval anurans have not been studied in depth and their examination is especially important in light of recent research concerning kin oriented behavior of anurans (Waldman and Adler, 1979; Blaustein and O'Hara, 1982; O'Hara and Blaustein, 1982; Waldman, 1984).

I examined alarm substances and the resulting alarm responses of tadpoles of the Cascades frog (Rana cascadae) and western toad (Bufo boreas), two anurans whose tadpoles show kin oriented behavior (Blaustein and O'Hara, 1981; O'Hara and Blaustein, 1982). I first attempted to determine whether tadpoles of these species exhibit the alarm response. Using the standard procedure of introducing an extract made from crushed conspecifics, I modified the von Frisch (1941) protocol traditionally used in alarm response studies (Pfeiffer, 1974). This protocol, recently criticized (Smith, 1979; Waldman, 1982), requires prior conditioning of test individuals to a feeding stimulus and may produce confounding effects. The methods I used allowed quantification of the behaviors involved in an alarm reaction, avoided some confounding factors associated with use of the

traditional methods, and provided experimental controls lacking in most alarm response studies.

The methods of most alarm response studies, mine included, yield little information about the amount of damage needed for an effective stimulus under natural conditions. The damage used in preparing the extract solutions is usually quite extensive. Very few studies (Vereijen and Reutter, 1969; Smith, 1979) have examined whether a natural predator can cause the type of damage needed to release an alarm substance. Therefore, I extended my research with Bufo boreas tadpoles and asked if a natural predator, while capturing and feeding on a tadpole, can release an effective amount of the alarm substance to elicit the alarm reaction in conspecific tadpoles.

I then examined possible antipredator benefits of this alarm signal, attempting to determine if the alarm substance affected predator behavior, indirectly or directly. Much circumstantial evidence suggests that the function of an alarm substance is to warn conspecifics. This warning would then protect alerted individuals from predation by eliciting an alarm reaction, and thus indirectly affect the efficiency of the predator's capturing behavior. However, there is no experimental demonstration of a decreased vulnerability to predation in prey alerted by an alarm substance. Williams (1964) has even suggested that the putative 'alarm substance' may actually be a general repellent that directly deters predators and incidentally causes agitation or avoidance in conspecifics. This hypothesis has also not been tested experimentally. Therefore, I attempted to determine if (1) the alarm response elicited by the alarm substance

affects the capturing efficiency of a predator on Bufo boreas tadpoles and (2) if the alarm substance directly affects predator behavior. With this approach, I could begin to evaluate the ecological and evolutionary implications of such a prey defense as the larval anuran alarm signal.

## Chapter II

REACTIONS OF INDIVIDUAL BUFO BOREAS AND RANA CASCADAE  
TADPOLES TO CHEMICAL CUES FROM INJURED AND UNINJURED TADPOLES

## Introduction

Chemoreception in vertebrates mediates diverse phenomena that range from navigation and orientation to species recognition. In amphibians, olfaction may be important in navigating (Dole, 1972; Grubb, 1973), in food finding (Heusser, 1958; Hemmer and Schopp, 1975; Sternthal, 1975; David and Jaeger, 1981; Dole et al., 1981) and in indicating reproductive condition (Madison, 1975). Olfaction may also play a role in individual, kin, sex, and species recognition (Twitty, 1955; Madison, 1975; Jaeger and Gergits, 1979; Blaustein and O'Hara, 1982), in intra and interspecific social interactions (Tristram, 1977; McGavin, 1978; Jaeger and Gergits, 1979), and in defense against predation (Pfeiffer, 1974). This study examined chemical cues and the predation defense they may trigger for larval anuran amphibians.

An alarm reaction to chemicals released from injured conspecifics occurs in a wide variety of aquatic animals. Invertebrates such as sea anemones (Howe and Sheikh, 1975), mud snails (Stenzler and Atema, 1977), sea slugs (Sleeper et al., 1980), and sea urchins (Snyder and

Snyder, 1970) move from areas containing injured conspecifics. This alarm reaction may be an anti-predator defense (Pfeiffer, 1974; Smith, 1977). In many fish species, the type of alarm reaction seems appropriate for the types of habitats and predators usually encountered by individuals of the prey species (Pfeiffer, 1974; Smith, 1977). Among fish, species-specific variations in these alarm reactions include resting on the bottom, remaining motionless, seeking cover in vegetation, leaping at the surface, schooling more tightly, and avoiding potentially dangerous areas. Larval amphibians also display such alarm responses, although they have not been as well studied as those in fish (Pfeiffer, 1974, 1977; Smith, 1977, 1982).

The taxonomic distribution of the larval alarm reaction in anuran amphibians is not well understood. Testing nine anuran species from a total of five families (Bufonidae, Discoglossidae, Hylidae, Pipidae, and Ranidae), Pfeiffer (1966) found larval alarm reactions in only two species of toads. Pfeiffer (1966) speculated that the alarm reaction may be common in and unique to bufonids. Nonetheless, similar alarm responses have been reported for a pelobatid (Richmond, 1947) and a ranid (Altig and Christensen, 1981). Further research is needed to clarify the taxonomic distribution of the alarm response and the aim of this study was to establish whether tadpoles of two anuran species (the Western toad, Bufo boreas and the Cascades frog, Rana cascadae) exhibit alarm responses to extracts containing chemicals from injured conspecifics.

## Methods

Tadpoles used in these experiments were collected from lakes and ponds in the Oregon Cascade mountains in July of 1982 and 1983. Bufo boreas tadpoles were collected from Lost Lake (1219 m), Linn County. All Bufo tadpoles used in extracts and tests were at Gosner (1960) stages 27-34 ( $\bar{x} = 31$ ). Rana cascadae egg masses and newly hatched larvae were collected from a small pond near Breitenbush Lake (1290 m), Marion County. All Rana tadpoles used in extracts and tests were at Gosner stages 33-38 ( $\bar{x} = 34$ ). Tadpoles of the Pacific treefrog (Hyla regilla) at Gosner stages 27-35 ( $\bar{x} = 32$ ), were collected from ponds near Breitenbush Lake and from a pond near Fay Lake (1190 m), Linn Co. Tadpoles were maintained in 37.85 L aquaria in dechlorinated tap water with an aeration stone. Room temperature was 16-18 C, under a 16L:8D photoperiod. Tadpoles were fed Purina Rabbit Chow daily and water was changed every three days.

The protocol of von Frisch (1941) is used most often to study alarm reactions. When using this protocol, individuals are trained to feed at a station and then are presented with an extract containing the suspected alarm substance and the behavioral reaction is recorded. This method has recently be criticized by Smith (1979) and by Waldman (1982). Using this traditional assay, Smith's (1979) results were inconsistent. He observed that hunger often appeared to overcome the alarm reaction in fish. To quantify the response he used a measure of activity (number of moves). Waldman (1982) also opposed the standard

protocol because of its dependence on prior conditioning. He speculated that test individuals are presented with two opposing stimuli, one for feeding and one for the alarm response, which may confound results. Instead, Waldman (1982) compared the distribution of fish in the aquaria before and after the introduction of the suspected alarm substance.

To quantify the potential alarm reaction in Bufo boreas and Rana cascadae, I used procedures that avoided the conditioning method and its complications. Additionally, my procedures measured both the spatial distribution and activity responses of individuals to the suspected alarm substance. This allowed a more accurate description of behavioral responses than most alarm response studies.

In three experiments, I recorded responses of individual tadpoles to one of two types of stimulus solutions. In the first experiment the stimulus solution contained chemical cues from damaged conspecifics. In the second experiment, the stimulus solution contained chemical cues from undamaged conspecifics. And the stimulus solution in the third experiment contained chemical cues from damaged non-conspecific tadpoles. In the control, the stimulus solution was water. Twenty tests were run for each type of stimulus solution and for the control solution, for both tadpole species.

The stimulus solutions used in the first experiment for Bufo and for Rana will be referred to as Bufo extract and Rana extract, respectively. Both extracts were made by mutilating a known wet weight of tadpoles ( $\bar{x} = 5.10\text{g}$ ). The tadpoles were killed and the viscera removed. The remaining carcasses, primarily epidermal and

dermal tissue layers, were then mutilated. The resulting slurry was mixed with 200 ml of dechlorinated tap water and after 20 min of agitation, was filtered through a Buchner funnel and brought to a 1 L volume.

Stimulus solutions for the second experiment, which involved cues from undamaged conspecific tadpoles, will be referred to as Bufo scent and Rana scent. Fifteen minutes prior to a test, a Bufo or a Rana tadpole was placed in 200 ml of dechlorinated tap water that had been filtered through a clean Buchner funnel. These scented waters were then used after 15 min, when the next test began.

The stimulus solution for both Bufo and Rana tadpoles in the third experiment was a Hyla extract, made with the methods described above using H. regilla tadpoles. All stimulus solutions were stored on ice and used in tests within one week. Prior to use in a test, portions were removed from the stock solutions and allowed to reach room temperature (16-18°C).

Tests were conducted in an opaque plastic tub (28 x 18 x 12 cm) with a line delineating two halves (14 x 19 cm), and filled to a depth of 4 cm with dechlorinated tap water. This tub, thoroughly rinsed between each test, was placed behind an opaque black plastic observation blind. One tadpole was released in the center of the tub and allowed to acclimate, 10 min prior to each test. Through a slit in the observation blind, 5 ml of the stimulus solution (control or experimental) were introduced with a pipet, 1 cm below the surface of the water, into both corners of one end of the tub. The side of stimulus introduction was always the side in which the tadpole, at the

beginning of the test, was not located. If, after the 10 min acclimation period, the tadpole was on the side designated to be the side of stimulus solution introduction, I waited (for no longer than 2 min, otherwise the trial was discarded) for the tadpole to swim to the other side and then introduced the stimulus solution. Starting when the tadpole first swam into the stimulus side (waiting no longer than 2 min for this to occur), the time the tadpole spent (in seconds) on the stimulus and nonstimulus halves of the tub, was recorded during a 5 min observation period. One ml of stimulus solution was added to each stimulus corner every 1 min. For Rana tadpoles, the time that individual tadpoles spent swimming was recorded as a measure of activity. Pilot tests revealed, however, that individual Bufo tadpoles spent essentially the entire test period swimming. The number of times the center line was crossed over during the observation period was used as measure of activity for Bufo.

The total amount of time spent on stimulus and nonstimulus halves was used as a measure of preference (or aversion). The Wilcoxon signed-ranks test (Sokal and Rohlf, 1969) was used to test the null hypothesis that time tadpoles spent on the stimulus half did not differ from random expectation. To conduct this analysis, 150 sec (the expected time out of a possible 300 sec tadpoles would spend on each tank side if this behavior were random) was subtracted from the total time spent by each tadpole on the stimulus side. In addition, the number of individuals spending the majority of their time (>150 sec) on the stimulus side was compared to random expectation using the binomial test (Siegel, 1956) with a null hypothesis of no difference.

For both species, the activities of tadpoles in response to the three experimental stimulus solutions were compared to the activities observed in response to the control stimulus solution. Comparisons were made using the Mann-Whitney U test (Sokal and Rohlf, 1969). Statistical comparisons involving results from the first experiment were one-tailed based on the prediction that tadpoles would avoid the side containing chemical cues from damaged conspecifics and would increase their activity in response to these cues. Statistical tests of results from the second experiment were one-tailed, based on the prediction that tadpoles would be attracted to chemical cues from undamaged conspecifics. Statistical tests for the third experiment and for the control were two-tailed, because I had no a priori predictions. A significance level of  $\alpha = 0.05$  was used for all statistical tests.

## Results

Experiments testing Bufo boreas tadpoles:

Results of the Bufo extract experiments suggest that this species has an alarm response. Individual tadpoles avoided the side of the tank to which an extract solution of conspecifics was introduced (Table II.1). The time spent in the stimulus half in the first experiment differed significantly from random, and only 3 of 20 tadpoles spent the majority of their time on the stimulus half. In addition, activity was greater in experiment 1 compared to the control: the number of times test tadpoles crossed the center line was significantly higher than in the control. Thus, tadpoles became more active and avoided the extract of injured conspecifics.

Bufo tadpoles did not respond to chemical cues from damaged non-conspecific tadpoles (Table II.1). In the third experiment, which used Hyla extract, neither the amount of time spent on the stimulus side nor the number of individuals spending the majority of their time on the stimulus side differed from random expectation. Activity in the third experiment also did not differ from activity observed in the control. These results suggest the observed alarm response to bufo extract is not simply a general response to 'injured tadpole'. Tadpoles also exhibited no side preference in the control tests. The null hypothesis that the amount of time spent in the stimulus half or that the number of individuals spending the majority of their time on the stimulus half (11 of 20) were different from random could not be

rejected, indicating there were no side biases in the testing procedures.

In contrast, results from the Bufo scent experiment suggest that individuals of this species are attracted to chemical cues from uninjured conspecifics (Table II.1). Tadpoles preferred the side to which the Bufo scent solution was added, spending more time on the stimulus half than predicted by random. In addition, the number of individuals spending the majority of their time on the stimulus half significantly differed from random: 17 of 20 tadpoles preferred the stimulus half. Activity in this second experiment was not different from levels in the control.

#### Experiments testing Rana cascadae tadpoles:

Although Rana did not exhibit as distinctive a response to extracts of damaged conspecifics as Bufo, results suggest they also have an alarm response (Table II.2). Individual Rana tadpoles did not avoid the half of the tank that contained chemical cues from damaged conspecifics. Neither the time spent on the stimulus half nor the number of tadpoles spending the majority of their time on the stimulus half (10 of 20) differed from random in the first experiment. Rana activity, however, increased significantly in response to the extract of damaged conspecifics. Tadpoles spent more time swimming in the first experiment than in the control.

Results from the third experiment suggest that Rana may be attracted to chemicals from damaged non-conspecific tadpoles (Table

II.2). More time was spent on the stimulus half , although the number of individuals spending the majority of their time on the stimulus half (14 of 20) was not different from random. Although the behavior was not quantified, tadpoles frequently behaved as if feeding, opening and closing their mouths in conjunction with lateral tail undulations while remaining in a stationary position. Activity in this experiment was not different from that in the control. As for Bufo, these results suggest that the Rana alarm response is not a general reaction to 'injured tadpole'.

The responses of tadpoles to Rana scent suggest that chemical cues from undamaged conspecifics did not affect the behavior of Rana cascadae tadpoles (Table II.2). Both the time spent by tadpoles on the stimulus half in the second experiment and the number of tadpoles spending the majority of their time on the stimulus half were not different from random. Also, activity was not different from that in the control. And in the control, neither the time spent on the stimulus half nor the number of individuals spending the majority of their time on the stimulus half differed from random, indicating no side biases in the testing procedures (Table II.2).

## Discussion

A chemically mediated alarm response in anuran tadpoles was first described by Eibl-Eibesfeldt (1949) and Hrbacek (1950). The introduction of juices from crushed Bufo bufo tadpoles into a feeding aggregation of tadpoles of this species caused a chaotic flight: tadpoles either changed their direction of movement and swam rapidly away or they sank to the bottom, remained motionless momentarily and then swam rapidly away. For vertebrates, an alarm substance is, by definition, located in special epidermal cells and can only be released by mechanical epidermal damage (Pfeiffer, 1963). Kulzer (1954) confirmed the epidermal location of the Bufo bufo tadpole alarm substance, testing reactions to extracts of tissues from various parts of the body. Other results (Kulzer, 1954) indicated that the alarm substance was only released by mechanical epidermal damage. Subsequent studies of larval anuran alarm responses, this one included, have assumed but not documented these two characteristics of alarm substances. In addition, no investigation of anuran alarm substances has chemically isolated a specific chemical used in the alarm response, although several studies indicate that in bufonids it may be related to a bufotoxin (Kulzer, 1954; Pfeiffer, 1966). Anuran alarm responses have been observationally reported by Richmond (1947), Eibl-Eibesfeldt (1949), and Hrbacek (1950) but few studies have experimentally examined larval anuran alarm responses (Kulzer, 1954; Pfeiffer, 1966; Altig and Christensen, 1981).

The results of this study suggest that larval B. boreas have an

alarm response. These tadpoles avoided an area containing chemicals from injured conspecifics and increased their activity but neither avoided the stimulus side nor increased their activity in response to an extract of non-conspecific tadpoles. These results suggest that the avoidance and activity changes are in responses to chemicals that occur in conspecifics and are not a general response to injured tadpoles.

Rana cascadae may also have an alarm response (Table II.2). Tadpoles did not avoid chemicals from injured conspecifics but activity did increase significantly over control levels. Tadpoles did not avoid chemicals from injured non-conspecifics: tadpoles were attracted to chemicals from injured Hyla and activity did not differ from that in the control. Rana cascadae in the field, when alarmed by quick movement of water or an approaching human, respond with an explosive burst of swimming in which individuals of a group disperse rapidly (up to 10 m away), sink to the bottom and remain motionless (personal observation; O'Hara, 1981). The alarm response to chemicals from injured conspecifics observed in this study could be a similar alarm reaction, confined by the test tank. Frequently (although this was not quantified), the increase in activity in Rana extract tests was due to chaotic spurts of activity. The test tadpole swam around the tank several times before stopping on one side or the other, apparently at random.

Larval predation defenses. Larval anuran amphibians may be more

vulnerable to predation than other phases of the anuran life cycle (Savage, 1952; Turner, 1962; Herried and Kinney, 1966; Calef, 1973; Heyer, 1976; Viertel, 1980), and predation may influence the abundance and distribution of tadpole populations (Brockelman, 1969; Licht, 1974. Heyer et al., 1975; Heyer, 1976; Heyer and Muedeking, 1976; Cecil and Just, 1979; Caldwell et al., 1980; Wilbur et al., 1983). The effects of predation, however, can be modified by a variety of morphological, physiological, and behavioral factors (Wassersug, 1971), discussed below.

Bufoiid larvae possess several traits that may reduce predation. Larval bufoiids aggregate and many of the hypothetical advantages of aggregating (Alexander, 1974; Bertram, 1978) could apply to tadpoles. In addition, larval bufoiids, like the adults, are toxic or unpalatable to many predators (Voris and Bacon, 1966; Heusser, 1971; Wassersug, 1971; Cooke, 1974; Hews, unpublished data), although this distastefulness may vary with larval stage (Formanowicz and Brodie, 1982) and may be ineffective against certain predators (Cooke, 1974; Walters, 1975; Arnold and Wassersug, 1978; Beiswenger, 1981; Morin, 1981, 1983; Hews, unpublished data). Toad tadpole aggregations may function aposematically and warn would-be predators, as suggested by the gregariousness, distastefulness and conspicuousness of these tadpoles (Wassersug, 1971, 1973). Many bufoiid tadpole aggregations are highly visible schools of hundreds to thousands of individuals (Wassersug, 1973; O'Hara, 1981), which are all the more conspicuous because of their black coloration (Wassersug, 1973). Any benefits of distastefulness are augmented by aposematic coloration or conspicuous

behaviors, both of which are bufonid traits, because predators may more readily learn to avoid such distasteful prey (Gittleman et al., 1981).

Ranid larvae also have characteristics that may lessen the impact of predation. Antipredation benefits of aggregating (Alexander, 1974; Bertram, 1978) may also apply to ranid larvae that aggregate. Large size attained by rapid growth may decrease the risk of predation by gape-limited predators (Calef, 1973; Heyer et al., 1975; Kruse and Francis, 1977; Brodie and Formanowicz, 1983; Morin, 1983; Wilbur et al., 1983; Woodward, 1983). Like bufonids, some ranid larvae are not preferred prey, compared to other anuran larvae, or are completely rejected by predators, probably because of distastefulness (Lewis et al., 1961; Liem, 1961; Walters, 1975; Kruse and Francis, 1977; Morin, 1981). R. cascadae tadpoles apparently are very sensitive to physical disturbance of water and have an explosive escape response when startled by an approaching human (personal observation ; O'Hara and Blaustein, 1981). Also like bufonids, some unpalatable ranids form conspicuous aggregations that may function aposematically (Wassersug, 1973).

The chemical cues examined in this study may trigger an alarm response that could deter predation. This alarm reaction is an example of how group-living animals such as larval Rana cascadae and Bufo boreas may increase their ability to detect and avoid predators. In these tadpoles, the area avoidance component of the alarm response may be beneficial in avoiding predation. Under laboratory conditions, I have observed three different types of tadpole predators (giant

waterbugs Lethocerus americanus, predaceous diving beetle larvae Dytiscus marginicollis, and dragonfly naiads Aeshna umbrosa) drop a tadpole they were feeding on and strike at, and sometimes catch, a nearby B. boreas. At high tadpole densities (K. Kruse, personal communication) D. verticalis larvae spend less time feeding on a B. americanus tadpole than at low prey densities. Thus, tadpoles may reduce the risk of predation by avoiding areas that contain feeding predators.

Alarm signals and reactions need to be examined more quantitatively and under more natural conditions to assess their biological significance. For example, most alarm reaction studies elicit the response using extracts obtained from mutilated entire individuals rather than using natural predators to release the alarm substance. One exception, however, was Verheijen and Reutter (1969) who found that a predatory fish, the pike Esox lucius, released enough prey alarm substance while capturing and eating an individual cyprinid, Phoxinus laevis, to elicit an alarm response in other Phoxinus. Results from a comparable study (Smith, 1979) suggest similar conclusions for pike predation on Iowa and johnny darters (Etheostoma exile and E. nigrum). Another example is my investigation of the larval B. boreas alarm response (Chapter III), which suggests that damage caused by a natural predator elicits a reaction like that reported in this study. The presumed function of alarm reactions, however, remains to be demonstrated experimentally. That is, how effective is the alarm reaction in deterring predation? Referring to fish, Smith (1982) notes that, "No one has shown that prey, in the

wild or captivity, are less vulnerable to predation when they have been warned by the alarm substance (p. 332)." With the exception of one study (Chapter III), this statement also applies to larval anuran alarm response studies. Research that examines whether natural predators can cause enough epidermal damage to elicit the alarm response and whether the alarm response affects predator efficiency are needed to fully evaluate the biological significance of alarm reactions.

Taxonomic distribution of the larval anuran alarm response. Pfeiffer (1966, 1974) suggested that the larval anuran alarm response is restricted to the family Bufonidae. Since this suggestion, alarm responses have been found in other families, including two ranid species (Rana hecksheri, Altig and Christensen, 1981; Rana cascadae, this study). Alarm reactions were also reported for a pelobatid (Scaphiopus holbrooki, Richmond, 1947), which Pfeiffer (1966, 1974) does not cite, and a discoglossid (Discoglossus pictus, Eibl-Eibesfeldt, 1961). Pfeiffer discounts the alarm response in Discoglossus for obscure reasons, questioning the existence of this alarm reaction because it is absent in two species from related genera (Bombina, and Alytes; Pfeiffer, 1966).

All larval anurans that have an alarm response aggregate in nature (Eibl-Eibesfeldt, 1961; Pfeiffer, 1966; Wassersug, 1973; Altig and Christensen, 1981; O'Hara, 1981). Benefits of alarm responses are likely only if individuals occur in social groups (Smith, 1977, 1982). The alarm response is not restricted to a taxonomic group but instead

may be restricted to species whose larvae aggregate. This generalization is helpful when attempting to understand the occurrence of alarm responses among species of a genus. For example, larvae of most ranids studied do not aggregate (Wassersug, 1973) but two species that do aggregate, Rana heckscheri (Altig and Christensen, 1981) and Rana cascadae (O'Hara, 1981), also have alarm reactions. More tests with aggregating and non-aggregating species within the same genera are needed to establish the overall accuracy of this generalization.

Table II.1 Association preferences and activities of larval Bufo boreas in response to stimulus solutions

Experiment	Stimulus Solution	N	No. spending majority of time <sup>a</sup>		seconds, out of 300 spent on stimulus half (mean ± SE)	Wilcoxon T	No. times individual crossed center line (mean ± SE)	Mann-Whitney <sup>b</sup> U
			Towards Stimulus	Towards Non-stimulus				
1	<u>Bufo</u> extract	20	3	17*	125.4 ± 5.9	15.5*	47.7 (3.3)	363*
2	<u>Bufo</u> scent	20	17	3*	161.3 ± 3.0	24*	27.3 (2.2)	251 <sup>NS</sup>
3	<u>Hyla</u> extract	20	11	9 <sup>NS</sup>	159.5 ± 11.3	99 <sup>NS</sup>	28.1 (3.1)	228 <sup>NS</sup>
Control	water	20	11	9 <sup>NS</sup>	152.8 ± 10.6	85.5 <sup>NS</sup>	26.2 (2.1)	

a Compared using Binomial Test  
 b Values compared to control  
 \* p < 0.01  
 NS p > 0.05

Table II.2 Association preferences and activities of larval Rana cascade in response to stimulus solutions

Experiment	Stimulus Solution	N	No. spending majority of time <sup>a</sup>		seconds, out of 300 spent on stimulus half (mean ± SE)	Wilcoxon T	Time in seconds spent swimming (mean ± SE)	Mann-Whitney <sup>b</sup> U
			Towards Stimulus	Towards Non-stimulus				
1	<u>Rana</u> extract	20	10	9 <sup>NS</sup>	147.4 ± 12.7	96.5 <sup>NS</sup>	54.9 ± 9.3	292 <sup>*</sup>
2	<u>Rana</u> scent	20	10	10 <sup>NS</sup>	165.6 ± 20.2	71.5 <sup>NS</sup>	42.8 ± 8.7	226 <sup>NS</sup>
3	<u>Hyla</u> extract	20	14	6 <sup>NS</sup>	182.3 ± 17.4	57 <sup>*</sup>	29.5 ± 6.2	206 <sup>NS</sup>
Control	water	20	10	10 <sup>NS</sup>	146.4 ± 16.1	94.5 <sup>NS</sup>	29.0 ± 4.8	

a Values compared using Binomial test

b Values compared to Control

\* p < 0.05

NS p > 0.05

## Chapter III

INSECT PREDATION ON LARVAL WESTERN TOADS (BUFO BOREAS):  
LARVAL ALARM SUBSTANCE RELEASE AND  
EFFECT ON PREDATOR EFFICIENCY

## Introduction

In many species of aquatic animals, chemicals released from injured individuals elicit an alarm reaction in conspecifics. Sea anemones (Howe and Sheikh, 1975), mudsnails (Stenzler and Atema, 1977), sea slugs (Sleeper et al., 1980), sea urchins (Snyder and Snyder, 1970), numerous fish species and the larvae of several anuran amphibians (reviewed in Pfeiffer, 1974) leave areas containing chemicals from injured conspecifics. In amphibian larvae and in fish, the avoidance response is frequently accompanied by an increase in activity (Pfeiffer, 1974). This alarm response is elicited by chemicals that apparently can only be released by mechanical damage (Pfeiffer, 1974). The active chemicals in alarm substances have been isolated and characterized in several invertebrate species (Howe and Sheikh, 1975; Sleeper et al., 1980) and for a number of fish species (reviewed in Pfeiffer, 1982). In these fish species, the alarm substance is epidermal. Studies of Bufo bufo tadpoles also suggests an epidermal location for the alarm substance (Kulzer, 1954).

Although alarm responses occur in many species, the adaptive significance of these responses has not been demonstrated

experimentally. Circumstantial evidence, such as the mechanism of release and the types of behavior elicited by alarm substances (reviewed in Smith, 1977, 1982), strongly suggest that alarm reactions deter predation. Few studies (Verheijen and Reuter, 1969; Smith, 1979) have examined whether a natural predator can cause the type of damage needed to release the alarm substance and elicit the alarm response. Also, no experimental evidence show a decreased vulnerability to predation in prey alerted by an alarm substance. Williams (1964) suggested that the alarm substance may actually be a general repellent that deters predators and incidentally causes agitation or avoidance in conspecific prey. Little evidence supports this hypothesis, although few studies have examined the role alarm substances may play in directly deterring predators.

This study investigated the adaptive value of the alarm response in larvae of an anuran amphibian, the Western toad (Bufo boreas). These tadpoles have an alarm reaction to an extract containing chemical cues from injured conspecifics (Chapter II); tadpoles increase their activity when exposed to this extract and avoid areas that contain it. In this study, I conducted a series of laboratory experiments to examine the Bufo alarm response in a more natural context, using two natural predators. Three questions were addressed: (1) While capturing and eating a tadpole, can a predator cause enough damage to release enough alarm substance to elicit the alarm response in conspecific tadpoles? (2) Does the alarm substance directly deter predators? (3) Does the alarm reaction in B. boreas tadpoles reduce a predator's capturing efficiency?

### Study Animals

Bufo boreas larvae occur in temporary and permanent ponds and lakes in western North America. The dark colored tadpoles form dense, conspicuous aggregations (O'Hara and Blaustein, 1982; Nussbaum et al., 1983). Most bufonid larvae are thought to be unpalatable or noxious to predators (Voris and Bacon, 1966; Heusser, 1971; Wassersug, 1971; Cooke, 1974; Hews, unpublished data), although certain predators regularly eat them (Walters, 1975; Arnold and Wassersug, 1978; Beiswenger, 1981; Morin, 1981; Formanowicz and Brodie, 1982; Hews, unpublished data). Aquatic insects such as dragonfly naiads (Aeshna umbrosa) and the giant waterbug (Lethocerus americanus) occur in many ponds with larval western toads and eat these tadpoles in the field and laboratory (personal observation). I used both these insect predators in this study.

All study animals were collected from ponds and lakes in the Oregon Cascade mountains during August and September of 1982 and 1983. Adult Lethocerus americanus (average total length 5.0 cm) were from Fay lake and an adjacent pond (Linn Co.), Aeshna umbrosa naiads (average total length 4.6 cm) were from the pond at Fay Lake and from a pond near Waldo Lake (Lane Co.), and Gosner (1960) stage 26-27 tadpoles were collected from Lost Lake (Linn Co.).

Lethocerus were individually kept in circular plastic containers (11 cm diameter) in 0.47 L of water and were fed four Pacific treefrog (Hyla regilla) tadpoles daily. Aeshna naiads were kept in groups of

ten in 20 x 30 cm tanks in 3 L of water and were fed forty small Hyla tadpoles daily. Bufo tadpoles were kept in a 38 L aquarium with an aeration stone and were fed Purina Rabbit Chow pellets daily. Dechlorinated tap water at room temperature (16-18 C) was used for all animals. Water was changed daily for the insects and every three days for the tadpoles. Animals were subjected to a 16L:8D photoperiod.

Question 1: Is an Alarm Substance Released by Lethocerus Predators?Methods

In three experiments, I examined individual tadpole responses to various sets of non-visual, waterborne stimuli, including cues from predation on a conspecific tadpole and cues from predation on tadpoles of a different species. The experiments were designed to determine if Lethocerus releases enough tadpole alarm substance to elicit an alarm reaction in B. boreas tadpoles. I used Lethocerus predators in these experiments to be conservative. As piercing and sucking predators, Lethocerus do not release large amounts of prey body fluids and damaged tissues into the water, as some chewing predators do (personal observation). Apparently, Lethocerus damage primarily prey epidermal layers with rasping actions of their raptorial forelegs when handling the struggling tadpole (personal observation).

Test Apparatus and Procedure. Tests were conducted in a large glass tank (90 x 30 cm) filled with 18.9 L (water depth 7 cm) of dechlorinated tap water at room temperature (16-18°C). The tank, divided longitudinally by a watertight opaque plastic partition, had two end-compartments. These end-compartments were created with a partition of 1.5 mm fiberglass mesh placed 15 cm from each end of the tank (Figure III.1). The central longitudinal partition divided the tank into two "test arenas", allowing for simultaneous independent testing of two tadpoles, hereafter referred to as "test tadpoles". To

aid in recording the positions of test tadpoles, both test arenas were divided in half with a line marked on the outside of the tank.

Each end-compartment contained a cylindrical (9 cm diameter) opaque plastic "stimulus chamber" (Figure III.1). Stimulus chambers each had a fiberglass mesh partition dividing the chamber in half (the purpose of this partition will be explained below). To allow diffusion of chemical cues into the tank, the back half of each stimulus chamber was a mesh window.

In all three experiments, an experimental stimulus chamber was placed in one end-compartment and a control stimulus chamber was placed in the other. Experimental and control stimulus chambers differed in the types of "stimulus tadpoles" and predators they contained (described below). These chambers were placed in the center of the end-compartments with the opaque side facing the test tadpoles in the test arenas. Thus, test tadpoles could not receive visual stimuli from the animals inside the stimulus chambers because of the end-compartment partitions.

Test tadpoles were placed in the tank 20 min prior to the beginning of a test, each confined by a clear plexiglass cylinder located in the center of the test arena. Stimulus tadpoles and predators were placed in the stimulus chambers, which were in the end compartments, 15 min prior to the start of a test. Predators caught prey stimulus tadpole within 5 min of being placed together and continued to feed for the entire test. A test began when the plexiglass cylinders were raised, releasing the two test tadpoles. I recorded the initial direction to which each test tadpole swam and the

time in seconds spent by each tadpole in the experimental and control halves of the test arenas, in two 10-min sessions with a 5-min interval between sessions. The number of times a test tadpole crossed the center line was recorded as a measure of activity. Observations were made from behind an opaque observation blind. Following each test, the tank and stimulus chambers were drained and thoroughly rinsed. The positions of the control and experimental stimulus chambers were switched to opposite sides prior to the start of the next test.

Experimental Design. Twenty replicates each of three experiments were conducted (Table III.1). Each experiment consisted of a different pair of control and experimental stimulus chambers. Experiment 1 was an apparatus control and consisted of 'experimental' and 'control' stimulus chambers that were identical: both chambers contained 5 Bufo tadpoles and no predators (Table III.1). In experiment 2, the experimental chamber contained 5 Bufo tadpoles on one side of the internal partition and a Lethocerus predator feeding on a Bufo tadpole on the other side of the partition. The control chamber for this experiment contained 5 Bufo on one side of the internal partition and a predator without a prey tadpole on the other side of the partition (Table III.1). In experiment 3, the experimental chamber had 5 Bufo tadpoles separated from a predator feeding on a Hyla tadpole. The control chamber for this experiment contained 4 Bufo and 1 Hyla separated from a non-feeding predator (Table III.1).

The third experiment was designed to determine if the alarm

response, if it was observed in experiment 2, was (1) species-specific and not due to a general reaction to injured tadpoles of any species and (2) due to chemical released from the prey tadpoles and not from the predator. Non-prey stimulus tadpoles, which were separated from the predator by the internal partitions of control and experimental stimulus chambers, were used to mask water movements from a feeding predator. Non-feeding predators in control chambers remained essentially immobile as they floated on the surface of the water in a manner typical of this predator's 'sit and wait' foraging mode.

Control and experimental stimulus tadpoles were visually matched for body size. Within a single run, test and stimulus tadpoles were of similar developmental stages, and were within 1 Gosner (1960) stage of each other, although stages used within an entire experiment ranged from Gosner stages 29-37. No tadpole was tested more than once and tadpoles used as stimulus individuals were not used as test tadpoles and vice versa. Animals used in experimental stimulus chambers were not used in control chambers nor the reverse. No stimulus tadpole was used in more than one experiment and, within an experiment, a stimulus tadpole was used in no more than 5 tests. The same individual predators were used in several tests within an experiment because of the difficulty in obtaining these predators. Predators were not fed or used in a test at least 4 hours prior to use in a test. All tests were conducted between the hours of 1300 and 1700.

The Wilcoxon signed ranks test was used to test the null hypothesis that the time tadpoles spent on the experimental half of the test arena was not different from random expectation, for each

experiment. To conduct this analysis, the expected time tadpoles would spend in each half of the test arena if choice behavior was random (600 s out of a possible 1200 s) was subtracted from the total number of seconds spent by each tadpole on the experimental side. In addition, the binomial test was used to test the null hypothesis that the number of tadpoles spending the majority of their time ( $> 600$  s) on the experimental half differed from random expectation. For experiment 2 these statistical tests were one-tailed because of the prediction that tadpoles would avoid the half of the tank in which predation on Bufo was occurring. For experiments 1 and 3, the same comparisons were made, except that statistical tests were two-tailed; for experiment 1, I predicted that there would be no side preference; for experiment 3 there was no a priori prediction as to how predation on a Hyla tadpole should affect side preference in Bufo. I also compared the levels of activity in experiments 2 and 3 to the level of experiment 1 (the apparatus control), using a Mann-Whitney U test. Comparing the number of times the center line was crossed in experiment 1 versus experiment 2, the statistical test was one-tailed because of the prediction that activity should be higher in experiment 2. The statistical test comparing experiments 1 and 3 was two-tailed because of no a priori prediction as to how predation on Hyla tadpoles should affect Bufo activity. Significance level of statistical tests was  $\alpha = 0.05$ .

## Results

Lethocerus predation appeared to release alarm substance, because tadpoles avoided the half of the tank where predation on a conspecific occurred (Table III.2). In experiment 2, more time was spent away from the side where a predator was feeding (experimental side) than predicted by random expectation. In addition, more individuals spent the majority of their time away from the experimental side. Conversely, in experiment 3 neither the time spent on the experimental side (with a predator feeding on a Hyla) nor the number of individuals spending the majority of their time on the experimental side differed from random expectation. In the apparatus control, experiment 1, tadpoles did not exhibit a side preference. Neither the number of tadpoles spending the majority of their time on the experimental side nor the total time spent on the experimental side differed significantly from random. Test tadpoles increased their activity in experiment 2, where predation on conspecifics occurred, compared to the control, experiment 1 (Table III.2). The mean number of times tadpoles crossed the center line was significantly greater ( $P < 0.05$ ) in experiment 2 than in the control, experiment 1. Test tadpoles also significantly increased their activity in experiment 3. The mean number of times the center line was crossed was significantly greater ( $P < 0.05$ ) in experiment 3 than in the control, experiment 1. These results suggest that the alarm reaction is not a general response to damaged tadpoles but may be a response to specific chemicals from

conspecific tadpoles.

Question 2: Does the Alarm Response Affect Aeshna Predators'  
Capture Efficiency?

Methods

To address the second question, I determined the capture efficiencies of individual predators on B. boreas tadpoles after the introduction of a Bufo extract, which contained the alarm substance (Chapter II) or of a control solution. I used Aeshna naiads as predators to allow accurate determination of attack rates. During a capture attempt a naiad's labial mask (a modified labium) shoots out towards the prey, a behavior easy to observe and quantify.

I conducted this experiment in a tank (44 x 22 cm) filled with 6.8 L dechlorinated tap water at room temperature (16-18°C) to a depth of 7 cm. The tank had eight small pebbles (approx. 1.5 cm diameter) placed on the bottom, equidistant from each other and from the walls in two parallel rows. Pebbles were provided because predators were more likely to attack if they had objects to sit on. Fifteen minutes before each test, an Aeshna naiad was placed in a plexiglass cylinder situated on the tank bottom in the middle. At this time, 24 Bufo tadpoles (Gosner stages 29-33) were released into the tank. Two minutes before the start of the test, an aeration stone was lowered into the water at the middle of the tank, next to the cylinder. Ten ml of either a Bufo extract or a control solution were then introduced. Diffusion tests with dyed extract indicated mixing of the

solution was complete in 1.5 min. The Bufo extract was made the day of testing by filtering an aqueous solution containing chemicals from mutilated tadpoles ( $\bar{x} = 5.2$  g tadpole/1000 ml dechlorinated water), that is, tadpole carcasses exclusive of viscera. The control solution was dechlorinated water filtered in the same manner.

Following removal of the aeration stone after 2 min, the predator was released by raising the cylinder. This marked the start of a 15-min observation period in which I recorded the times until the predator (1) first contacted a prey, (2) first attacked a prey and (3) first captured a prey. I also recorded the number of attacks, up to and including the first capture. All predators that attempted to hunt (noted by head orientations and movements and pursuits) caught a tadpole within 15 min. If no capture attempts were made during the observation period, the trial was discarded. Ten naiads were each used once in a control and once in an experimental test. Order of testing the predators in control and experimental tests was randomized. Predators were starved for 12 hr prior to use in a test to standardized hunger levels. For all 4 parameters, I tested the null hypothesis that results of the extract tests were not different from those of the control tests, using the Mann-Whitney U test. Statistical tests were one-tailed based upon the prediction that the alarm substance and subsequent alarm response would make the tadpoles more difficult to capture, interfering with predator efficiency. The time until first contact, attempt, or capture or the number of attempts needed for a successful capture was expected to increase over control levels. Level of significance for statistical tests was  $\alpha = 0.05$ .

## Results

Addition of the Bufo extract significantly increased the time until first tadpole capture by a naiad (Table III.3). In this fourth experiment, the mean time until first capture was significantly greater ( $P < 0.05$ ) with the addition of the extract solution than when the control solution was added. The effect of the extract on other parameters of capture efficiency was consistent, although not significant. These other parameters, which included the time until first contact, the time until first attempted capture, and the number of attempts required for a successful capture, were greater in extract tests than in control tests, although none of the differences were statistically significant.

Question 3: Does the Alarm Substance Directly Deter Aeshna  
Predators?

Methods

In a fifth experiment, the reactions of predators to the introduction of either a Bufo extract or a control solution were observed. To help interpret the results of experiments 4 and 5, I used Aeshna predators for both experiments.

Extract and control solutions were made as in experiment 4 (see Methods section, Question 2), using tadpoles of Gosner (1960) stages 29-33. For a test, a single naiad was placed in a small plastic container (15 x 10 cm) containing 0.6 L of dechlorinated tap water at room temperature (16- 18°C) and allowed to acclimate for 5 min, behind an observation blind. For 10 naiads, 1 ml of extract was then slowly pipetted (approx. 0.1 ml/sec) into the water 1 cm above the head of the stationary naiad. During the introduction time (approx. 10 sec) and the following 10 sec, any change in activity of the types of movements was recorded. Using Chi-Square analysis ( $\alpha = 0.05$ ), these results were compared to the reactions of 10 other naiads when the control solution was similarly introduced. If the alarm substance was aversive to predators, I expected the naiads to respond to the extract solution by moving away from the area of introduction or changing their activity in some manner. I tested the null hypothesis that the proportion of individuals exhibiting any of these behaviors should not be different between the extract and control trials.

## Results

The alarm substance does not appear to directly affect Aeshna predators. In experiment 5, the reactions of Aeshna naiads to the Bufo extract and the control solution did not differ. The proportions of naiads responding to the two introduced solutions were not statistically different ( $P > 0.05$ ), so the null hypothesis could not be rejected. In both sets of tests, only 3 of 10 naiads responded in any manner, moving away from their previous positions. No other changes in behaviors were observed.

## Discussion

This study reinforces and adds to an earlier study (Chapter II) that documented an alarm reaction in Bufo boreas tadpoles to a filtered aqueous solution containing mutilated conspecifics. Individual B. boreas tadpoles avoided the side of a tank containing the Bufo extract and increased their activity over control levels when exposed to this extract (Chapter II). The results of this earlier study suggested that the B. boreas alarm response was a specific reaction to chemicals released from injured conspecifics and not a general response to damaged tadpoles; there was neither an activity increase nor an avoidance response when a control solution of plain water or of a Hyla tadpole extract was added.

In the present study, Bufo increased their activity and avoided the side of a tank containing cues released while a predator, in a visually isolated but interconnected container, preyed upon a conspecific tadpole. Bufo tadpoles did not avoid the side of the tank in which predation on a Hyla tadpole occurred. Activity, however, did increase over the control level in tests where predation on Bufo or on Hyla occurred. These results differ somewhat from those of a previous study (Chapter II), where Bufo activity did not increase in response to a Hyla extract. In the present study, tadpoles may have been more active in the experiment with Hyla predation than in the control because of the presence of predators. Cues from the predators themselves, which were absent in the control experiment, may have stimulated tadpole activity.

My results suggest that chemicals released by predation caused the alarm response. In the first three experiments, test tadpoles were unable to receive visual stimuli from individuals within the stimulus chambers. This suggests that waterborne chemical cues produce the alarm reaction. Auditory cues probably did not produce these results as tadpoles may lack a mechanism for sound generation other than tail vibrations, which can produce near subsonic (ca. 10 hz) sounds (Katz et al., 1981; Blaustein and O'Hara, 1982). In addition, any sounds from the predator feeding on Bufo are assumed to be similar to those from feeding on Hyla. Since auditory cues probably are not involved in the alarm reaction chemical cues, as suggested by the results of an earlier study (Chapter II), are the logical alternative. Furthermore, Bufo did not avoid the side where predation on Hyla occurred, but did avoid the side where Bufo predation occurred. These results, which are consistent with previous results (Chapter II), suggest that the chemical cues eliciting the response occur in Bufo and not in Hyla tadpoles.

The methods of most alarm response studies yield little information about the amount of damage needed for an effective stimulus under natural conditions. Investigators usually damage animals substantially when preparing the stimulus solution (for example see Reed, 1969; Smith, 1979; Waldman, 1982; Chapter II). Few studies have examined whether predators cause damage sufficient to release an alarm substance and elicit the alarm reaction in prey conspecifics. The northern pike (Esox lucius) releases sufficient chemical stimuli when eating a small roach (Leuciscus rutilus) to

cause an alarm reaction in conspecific prey that are located in a visually isolated but interconnected tank (Verheijen and Reuter, 1969). Other evidence (Smith, 1979) suggests similar conclusions for pike predation on Iowa and johnny darters (Etheostoma exile and E. nigrum), although some controls were lacking. The first three experiments of this study suggest that even a piercing predator such as Lethocerus, which does not chew and cause major epidermal damage to the prey, causes damage sufficient to release effective amounts of the Bufo alarm substance.

Although many studies have demonstrated alarm responses (reviewed in Pfeiffer, 1974), few have experimentally examined the suggested selective advantages for maintaining the alarm substance system. This study examined two possible roles of the Bufo alarm substance: direct deterrence of predators and warning of conspecifics. If the direct deterrence suggested by Williams (1964) is related to noxiousness or unpalatability, predators might be expected to reject prey that possess an alarm substance. Aquatic insects can acquire an avoidance response to prey with toxic epidermal secretions. Predaceous diving beetle larvae (Dytiscus verticalis) acquired an avoidance response to newt (Notophthalmus viridescens) skin secretions (Brodie and Formanowicz, 1981). Brodie et al. (1978) found that these insect predators also acquired an avoidance response to B. americanus tadpoles after repeatedly eating this prey. An alarm response in this species has not been studied but these tadpoles may possess such a reaction, as larval alarm responses to injured conspecifics have been observed in all reported bufonid studies, which now total four (reviewed in

Pfeiffer, 1974; Chapter II). In the present study, an avoidance reaction arising from the introduction of the Bufo extract was not observed in predators that were experienced in feeding on Bufo tadpoles. If olfaction is the primary mechanism used by predators to sense the alarm substance, these results suggest that this substance does not directly deter Aeshna predators. If, however, gustation is more important than olfaction in sensing the alarm substance, then this experiment may not have been an adequate test of the possible deterrent nature of an alarm substance.

The findings of Bernstein (in Smith, 1982) suggest that the alarm substance of fathead minnows (Pimephales promelas) is not a direct predator deterrent. Bernstein examined the palatability of fathead minnows to their predators, rainbow trout (Salmo gairdneri). These minnows seasonally lose specialized epidermal alarm substance cells (ASC's) that contain an alarm substance. Bernstein found no Salmo taste preference between fathead minnows with ASC's and fatheads that had been androgen-treated to eliminate ASC's. Studies that examine if alarm substances directly deter predators are few. Such studies, however, would be difficult to conduct as, for most organisms, either ASC's have not yet been identified or the presence of ASC's cannot be manipulated. In addition, studies that compare the palatability of species that possess an alarm response (and are assumed to possess an alarm substance) to those that do not would be difficult to interpret, unless the predators are actually observed to capture and reject prey. Interspecific differences in prey behavior, such as activity, response to predator, and escape behaviors could result in differential

susceptibilities to predation, independent of palatability.

I also investigated another possible role of the alarm substance by examining whether alarmed prey are less vulnerable to predation. The results suggest that predators are less efficient at capturing prey that have been alerted by the alarm substance; predators took significantly more time to capture tadpoles after the introduction of the alarm substance. Several mechanisms may account for this decrease in capture efficiency, including a heightened prey alertness, a confusion effect, or a change in predator behavior directly in response to the alarm substance. The minnow (Phoxinus phoxinus) alarm substance causes bradycardia (Pfeiffer and Lamour, 1976). For black tetra (Gymnocarymbus ternetzi), results indicate that the tetra alarm substance induces a specific central nervous system activation, increasing optical alertness (Pfeiffer and Riegelbauer, 1978). Similar physiological responses to the alarm substance could occur in Bufo tadpoles. The increased activity that accompanied an alarm response in B. boreas tadpoles (Chapter II; this study) could reflect a general state of central nervous system excitation. The resulting increased tadpole activity could, for example, have reflected increased tadpole alertness, making capture more difficult. The decreased capture efficiency could also have been due to a confusion effect from increased prey activity (Humphries and Driver, 1970; Neill and Cullen, 1974). The predator's capturing success could also have been directly affected by the introduction of the alarm substance extract. Results from experiment 5 however, which indicate that predators did not overtly change behavior when Bufo extract was

introduced, suggest that there was no direct effect of the alarm substance on predator behavior.

Williams (1964) suggested that an alarm substance may actually be a compound that deters predators and secondarily causes distress, or an alarm reaction, in conspecifics. Although speculative, several lines of reasoning suggest that both direct predator deterrence and warning of conspecifics could function in the B. boreas alarm substance system. Larval and adult bufonids are unpalatable to many predators (Voris and Bacon, 1966; Heusser, 1971; Wassersug, 1971; Cooke, 1974; Hews, unpublished data) and the substance responsible for this distastefulness could also be the substance that elicits the larval alarm reaction. In a histological examination of tadpole epidermis (of 2 bufonid and 7 non-bufonid species), Pfeiffer (1966) identified an epidermal cell type, which he termed the giant-cell, unique to bufonids. Giant-cells extend to the surface of the skin but have no external opening, suggesting that their contents may be released only by mechanical damage. These and other characteristics indicate that giant-cells may contain the larval alarm substance. In addition, Wassersug (1973) suggests that these giant-cells may develop into the poison-containing granular glands, which first appear during metamorphosis. If so, the alarm substance may actually be a bufotoxin, a substance that directly deters predation, through distastefulness. The secretions of the epidermal granular glands contain bufotoxins (bufodienolides, Flier et al., 1980), a class of noxious compounds responsible for the distastefulness of many bufonids. Pfeiffer (1966) found that skin secretions from B. bufo

tadpoles elicited the alarm response in larval natterjack toads (B. calamita) and vice versa. Non-bufoinids, however, did not respond to the bufoinid skin extracts and the bufoinids did not respond to the non-bufoinid skin extracts. Kulzer (1954) tested a number of substances for their effectiveness in eliciting the alarm reaction in B. bufo and found that tests of bufotoxin and gama-bufotoxin were indistinguishable from those of skin extracts. In addition, paper chromatographs and absorption spectra of skin extracts and bufotoxin were similar. These results, however, only suggest that bufotoxin and the alarm substance are the same compound; isolation and identification of the active compound(s) in Bufo alarm substances are needed to conclusively demonstrate that the alarm substance is identical to or contains a bufotoxin.

Several functions for alarm substances have been proposed. For fish (Cameron and Endean, 1973) and amphibians ( Bachmayer et al., 1967; Preusser et al., 1975), the deterrence of pathogenic, parasitic or fouling organisms is a role frequently suggested but seldom studied. Although it has never been examined, such a protective mechanism could operate in the B. boreas alarm system. Two other anti-predator functions of the alarm substance, direct predator deterrence and warning of conspecifics, were examined in this study. Results provide the first direct evidence in an aquatic system that an alarm reaction elicited by an alarm substance may protect the performers from predation. This is one of the few experimental demonstrations of decreased vulnerability to predation in prey alerted by an alarm substance.

Table III.1 Experimental design of alarm response experiments 1, 2, and 3, which use a predator to release the alarm substance

Experiment	Experimental Chamber	Control Chamber	Purpose
1	Five <u>Bufo</u> and no <u>Lethocerus</u>	Five <u>Bufo</u> and no <u>Lethocerus</u>	To test if either side of the tank biases tadpole side preference: an apparatus control.
2	Five <u>Bufo</u> separated* from a <u>Lethocerus</u> preying on a <u>Bufo</u>	Five <u>Bufo</u> separated* from a non-feeding <u>Lethocerus</u>	To test if a predator, while capturing and feeding on a <u>Bufo</u> tadpole, can release enough alarm substance to cause an alarm reaction in <u>Bufo</u> tadpoles.
3	Five <u>Bufo</u> separated* from a <u>Lethocerus</u> preying on a <u>Hyla</u>	Four <u>Bufo</u> and one <u>Hyla</u> separated from a non-feeding <u>Lethocerus</u>	To test if the alarm response is (a) species-specific or (b) caused by chemicals released from the predator.

\* separation achieved by a fiberglass mesh partition.

Table III.2

Changes in larval Bufo boreas side preferences and activities in response to chemicals released from predation on a conspecific, on a non-conspecific, and to an apparatus control

Experiment	No. test tadpoles spending majority of time towards		Mean ( $\pm$ SE) time spent (out of 1200 sec) in experimental half <sup>b</sup>	Mean ( $\pm$ SE) No. times crossed over centerline <sup>c</sup>	
	N	Experimental Half			Control Half
Bufo Predation	20	3	17**	449.5 ( $\pm$ 29.7) <sup>a</sup>	44.5 ( $\pm$ 3.7) <sup>a</sup>
Hyla Predation	20	8	12 <sup>NS</sup>	634.2 ( $\pm$ 11.9) <sup>NS</sup>	45.7 ( $\pm$ 1.2) <sup>a</sup>
No Predation	20	9	11 <sup>NS</sup>	587.4 ( $\pm$ 11.3) <sup>NS</sup>	32.2 ( $\pm$ 4.4)

\*  $P < 0.05$ , \*\*  $P < 0.01$ , NS = not significant  $p > 0.05$ .

<sup>a</sup> Binomial Test

<sup>b</sup> Wilcoxon signed rank test

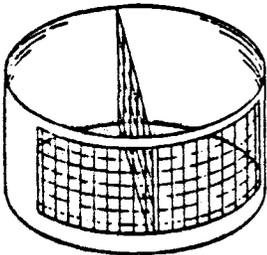
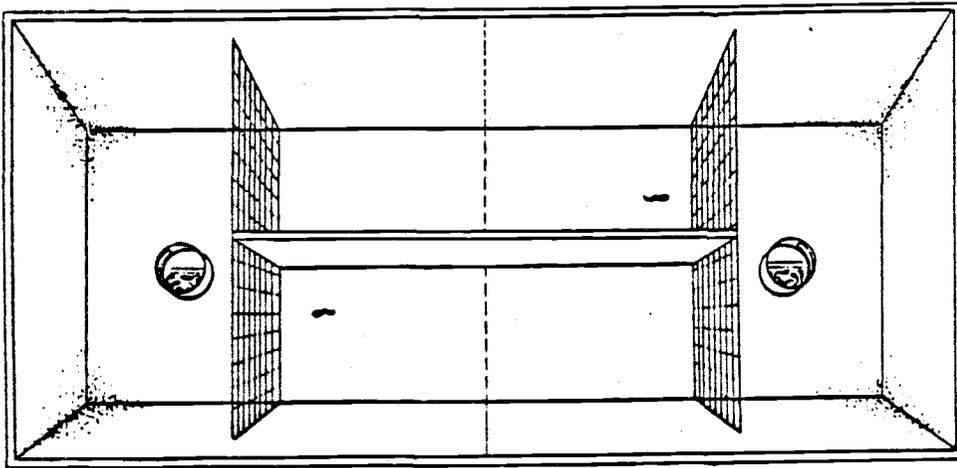
<sup>c</sup> Mann-Whitney U test, experiments 1 and 2 compared to experiment 3.

Table III.3 Parameters of Aeshna naiad capture efficiency after the introduction of a Bufo alarm substance solution or a control solution

Stimulus Solution	N	Time (sec) until predator first contacts a tadpole (mean $\pm$ SE)	Time (sec) until first attempted capture (mean $\pm$ SE)	Time (sec) until first capture (mean $\pm$ SE)	No. of attempts up to and including the first capture (mean $\pm$ SE)
Experimental ( <u>Bufo</u> extract)	10	69.3 $\pm$ 17.4	178.6 $\pm$ 76.4	324.5 $\pm$ 64.1*	2.2 $\pm$ 0.39
Control (Water)	10	42.2 $\pm$ 12.6	117.1 $\pm$ 38.3	159.5 $\pm$ 57.6	1.5 $\pm$ 0.17

\* Significantly different from control,  $P < 0.05$ .

Figure III.1 Test apparatus for alarm response experiments 1, 2, and 3



## Chapter IV

## GENERAL DISCUSSION

Animals defend themselves from predation in diverse ways (Edmunds, 1974). The anuran larvae I studied may defend themselves from predation in several manners, as discussed in detail in Chapters II and III. The defenses of Bufo boreas appear to include aggregating and its possible associated benefits, unpalatability, and an alarm response to chemical cues from injured conspecifics. The defenses of Rana cascadae are less obvious but may include aggregating and its associated anti-predator advantages and alarm reactions to mechanical disturbance of the water and to chemical cues. Most prey defenses, such as cryptic coloration and escape behaviors are easy to understand evolutionarily, as they obviously benefit the individual possessing the trait. Yet certain predation defenses, such as alarm signals, pose some evolutionary problems. Kin selection may have been involved in the evolution and/or maintenance of the distastefulness, aggregating behavior, and alarm signalling of larval anurans (Wassersug, 1973; Waldman and Adler, 1979; O'Hara and Blaustein, 1981; Blaustein and O'Hara, 1982; Smith, 1982; Waldman, 1984). In the following sections, I discuss the evolution of alarm signals, with special reference to anuran larvae. I then briefly consider how distastefulness and aggregating in anuran larvae may also have been influenced by kin selection.

### Alarm signals

In response to the presence or attack of a predator, many animals perform specific alarm behaviors (Edmunds, 1974; Curio, 1976). A variety of adaptive functions have been proposed for these alarm behaviors, which may be signals to the predator, to conspecifics, or to both (for review see Harvey and Greenwood, 1978). Most hypotheses about the evolution and adaptive value of alarm signals fall into four basic categories, not necessarily mutually exclusive. These proposed functions involve mechanisms that invoke group selection, individual selection, kinselection, and reciprocal altruism.

The suggestion that group selection (Wynne-Edwards, 1962) can account for the widespread occurrence of alarm signals is unlikely, as most vertebrate populations do not have the restricted types of group structures and group dynamics that would permit this type of selection to occur (Maynard Smith, 1976). More plausible roles for alarm reactions and signals are based upon mechanisms involving either individual or kin selection (Harvey and Greenwood, 1978). Many scenarios increase a signaller's individual fitness by reducing the risk from predation, either immediately or in the future. For instance, alarm calls in birds may be ventriloquial and draw the predator away from the caller (Perrins, 1968). Prey may also startle the predator and escape, if their signals are unpredictable (Humphries and Driver, 1970). Rump patch signalling of many cursorial mammals (Walther, 1969) may announce to predators that the signalling individual is alert to their presence and thus difficult to catch. Alarm behaviors of many group-living animals may act in several ways

to reduce a predator's success at capturing members of an alerted group. Signallers may reduce their own risk of predation if their alarm signal elicits a chaotic response from conspecifics, confusing the predator (Neill and Cullen, 1974; Milinski, 1977; Gillett et al., 1979; Treherne and Foster, 1981). Callers may also reduce their own chances of being killed by alerting other group members to the presence but not the location of a predator, thus increasing the susceptibilities of these group members to predation. Such a behavior has been termed selfish manipulation (Charnov and Krebs, 1975; Owens and Goss-Custard, 1976). Warning unrelated individuals that reciprocate in the future with similar aid (reciprocal altruism) could also be favored (Trivers, 1971). Warned prey could also be more difficult to catch. This lowered hunting success may reduce the probability that the predator forms a search image of the signalling individual or continues hunting in the immediate area (Trivers, 1971). In addition to increases in the signaller's individual fitness, the latter scenario suggested by Trivers (1971) could also increase the sender's inclusive fitness. Finally, some alarm signals, which may endanger the sender and decrease its individual fitness but save nearby kin, could increase the sender's inclusive fitness (Maynard Smith, 1965; West-Eberhard, 1975). Alarm pheromones of aphids (Nault and Montgomery, 1979) and social insects (for example see Boch et al., 1962; Regnier and Wilson, 1964; Moore, 1968) and alarm calls of colonial-living ground squirrels (Sherman, 1977) may be alarm signals that are maintained primarily by kin selection.

Alarm signalling systems in some anuran larvae and fish

(Pfeiffer, 1974) could have evolved or been maintained through kin selection. Arguments based on individual selection and reciprocal altruism do not seem applicable (Smith, 1977, 1982). Mechanical damage is required to release the alarm substance, frequently implying the signaller's death. Therefore, the scenarios invoking increases in the signaller's individual fitness because of future beneficial events are not likely. Williams (1966) suggests that alarm signals are for warning mates and that their retention in the non-breeding season is non-adaptive or neutral. These arguments do not apply to fish or anuran larvae. Those fish that have an alarm signal do not have parental care, and the alarm signals of anuran larvae are only received by other larvae. Therefore, for anuran larvae the mechanisms involving individual selection and group selection are not likely to account for the occurrence of alarm signals. Kin selection, however, could play a role in the evolution or maintenance of alarm signals.

Kin selection could play a role in the evolution or maintenance of the alarm substance, if signallers and nearby individuals are more closely related to each other than the average degree of relatedness of all individuals in the population. Rana cascadae tadpoles preferentially associate with siblings in the lab (Blaustein and O'Hara, 1981, 1982; O'Hara and Blaustein, 1981) and field (R. O'Hara and A. Blaustein, unpublished data). Thus, alarm signalling in R. cascadae may have been selected for by kin selection. In contrast, although larval Bufo boreas also associate preferentially with siblings in the laboratory (O'Hara and Blaustein, 1981), short-term exposure to non-siblings results in loss of sibling preferences.

Field observations indicate that from an early larval stage, tadpoles may mix with non-siblings (O'Hara and Blaustein, 1982). In the first analysis, then, the kin selection interpretation of the B. boreas alarm signal may not be parsimonious. Other factors, however, suggest that both the B. boreas and R. cascadae alarm signals could be maintained by kin selection. West-Eberhard (1975) argues that proximity can be associated with an above-average degree of relatedness and that weak kinship may be adequate to maintain low-cost or high-benefit signals. She suggests that low-cost altruistic acts will raise the value of  $K$  (the ratio of gain to loss in fitness), making the value of  $r$  (the coefficient of relatedness) comparatively unimportant in the evolution of alarm signals. Smith (1982) believes that the cost to the sender is not injury or death, but simply the cost of developing and maintaining the alarm substance cells and their contents. These alarm substances, then, could be relatively low-cost signals. Signals could also be low-cost if they are multifunctional, which may be the case for some anuran alarm substances (see below). Additionally, if more than one somewhat related individual was aided simultaneously, any increase in the signaller's inclusive fitness would be multiplied (West-Eberhard, 1975). Such a situation would be possible in larval anuran aggregations. Thus, for a variety of reasons, kin selection could account for alarm signals in anuran amphibian larvae.

West-Eberhard (1975) also notes that kin selection could help maintain behaviors such as alarm signals, "whether or not they have originated or been maintained also, or even primarily, by selection in

other contexts." Indeed, alarm substances in fish and tadpoles could have evolved and been maintained primarily by direct selection on the sender, based on a function unrelated to their use by conspecifics as an alarm signal (Williams, 1964; Smith, 1977,1982; Chapter III). I have suggested in Chapter III that the primary function of the bufonid 'alarm substance' may actually be to confer noxiousness or unpalatability. The response of conspecifics to the release of this noxious chemical could be a secondary adaptation, maintained primarily by individual selection. In these anurans, kin selection may not have been responsible for the evolution of the alarm substance but once present, the substance could be maintained by kin selection in addition to individual selection. The alarm response could also have evolved through individual and/or kin selection. The response could decrease the responder's risk of predation through a number of previously mentioned mechanisms (for example, by startling or distracting the predator). The response could also decrease the susceptibility of other group members (for example, by decreasing the predator's capturing efficiency) and if these individuals were kin, then the original responder's inclusive fitness would increase.

#### Aggregating and Distastefulness

Of the theoretical advantages to aggregating (Alexander, 1974; Bertram, 1978), several may benefit tadpoles. The ability of tadpoles to locate and obtain food may be enhanced by group living (Beiswenger, 1975; Wilbur, 1977). Tadpoles in groups may also be more able to

detect and avoid predators. Species of anuran larvae that aggregate, such as Bufo boreas and Rana cascade do, could accrue other anti-predator benefits, besides the aposematic advantages already mentioned. Although anti-predator advantages of group-living have not been well documented experimentally, suggestions are numerous. Aggregating could reduce the risk of a particular individual being attacked by a predator (Hamilton, 1971; Treisman, 1975; Milinski, 1977a,b; Calvert et al., 1979; Duncan and Vigne, 1979; Foster and Treherne, 1981), by a numerical 'dilution' effect. A similar numerical effect could lower the probability an individual would be eaten because of predator satiation (Harvey and Greenwood, 1978). Aggregation could also increase the preys' ability to confuse predators with sudden or erratic behaviors (Humphries and Driver, 1970; Neill and Cullen, 1974; Milinski, 1977a,b; Gillet et al., 1979; Lim, 1981; Treherne and Foster, 1981, 1982). Group-living could also increase an individual's ability to detect and evade predators (Powell, 1974; Siegfried and Underhill, 1975; Kenward, 1978; Treherne and Foster, 1980, 1981, 1982; Seghers, 1981). Aggregating in tadpoles, therefore, could have evolved by individual selection alone. Tadpoles that preferentially associate with kin in nature, however, could gain additional advantages of aggregating, through increases in their inclusive fitness (Alexander, 1974; West-Eberhard, 1975). For example, individuals in groups of many siblings that warn others of a predator may increase their inclusive fitness compared with similarly signalling individuals in a group with few or no sibs. Thus, kin selection could contribute to the maintenance of aggregating behavior

in tadpoles.

Fisher (1930) suggested that gregariousness and distastefulness in insects could be the result of kin selection. With a similar argument, Wassersug (1973) proposed that the social behavior of Bufo tadpoles may be due to kin selection. Although it is not necessary to invoke kin selection to explain the evolution of aggregating (see discussion above), it may be required for evolutionary explanations of distastefulness. If a predator must eat one or several distasteful individuals before learning to avoid such prey, individual selection could not account for the establishment of distastefulness. The loss of these few individuals, however, could benefit other members of the group if they and the sacrificed individual were more related than average. In this situation enhanced inclusive fitness could account for the spread of an allele for distastefulness.

Recent research has revealed that tadpoles of several anuran species can distinguish sibs from non-siblings (Waldman and Adler, 1979; Blaustein and O'Hara, 1981; O'Hara and Blaustein, 1982; Waldman, 1984). The kin recognition abilities of these species, however, differ in the degree to which they are modifiable and to which they are influenced by familiarity. Demographic and life-history characteristics may determine whether traits for preferential treatment of kin can evolve (Blaustein and O'Hara, 1982; O'Hara and Blaustein, 1982). Field and laboratory observations of R. cascadae larvae are consistent with a kin selection model. Prior exposure to kin is not necessary for recognition of relatives in R. cascadae, and tadpoles can also distinguish familiar sibs from familiar non-sibs.

This species lays clutches communally with conspecifics and simultaneously with other anurans (B. boreas and H. regilla) resulting in early larval development that occurs in the presence of kin, non-kin, and non-conspecifics (Blaustein and O'Hara , 1982). Under such conditions, a recognition system that is not modifiable or based solely on familiarity would be necessary. That R. cascadae has such a recognition system suggests that there may be an advantage to associating with kin, although a recognition system is not necessary for kin selection to operate.

Field and laboratory observations of B. boreas suggest that it is not necessary to invoke kin selection to explain the larval social behavior of this species. In this species, short-term exposure to non-sibs results in the loss of the preference to associate with sibs (O'Hara and Blaustein, 1982). This probably happens in nature as clutches are laid in large communal masses and larval aggregations contain thousands of individuals (O'Hara and Blaustein, 1982; personal observation). Although kin selection is not necessary to explain the evolution of aggregating in Bufo, O'Hara and Blaustein (1982) recognize that kin selection could have influenced the evolution of social behavior in Bufo sometime in the past, perhaps under different environmental and social conditions. They speculate that some components of a recognition system still may exist in B. boreas larvae, enabling sibling preference to be exhibited under certain environmental conditions. For example, small ponds, isolated portions of lakes, or small breeding populations could allow for clutches to remain segregated and the development and maintenance of sibling

preferences could then occur.

### Concluding Remarks

This research centered on a prey alarm behavior that larval anurans exhibit to chemicals from injured conspecifics. In Chapter II I presented data suggesting that both Bufo boreas and Rana cascadae have such an alarm reaction. My experiments used controls that are usually absent from studies of larval anuran studies. In addition, finding an alarm response in the larvae of not only a bufonid but also a ranid supports the conclusion that Pfeiffer's (1966) generalization, which restricts the alarm response to the Bufonidae, is not tenable. In Chapter III, I examined the alarm response of B. boreas from a more natural perspective. I found that a natural predator, while feeding, could release a sufficient amount of alarm substance to cause the alarm response in conspecific tadpoles. This question had only twice before been examined in alarm response studies (Verheijen and Reuter, 1969; Smith, 1979). I also found that a predator's capturing efficiency was lowered in the presence of an alarm substance extract. This has never before been demonstrated in an aquatic system. Finally, I discussed the evolutionary implications of alarm signals, with specific reference to anuran larvae.

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