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Antipredator Skin Secretions of the Long-toed Salamander (*Ambystoma macrodactylum*) in Its Northern Range

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ABSTRACT.—**Distasteful, noxious, and toxic secretions from skin glands have been demonstrated to play important antipredatory roles in amphibians. Previous studies suggest that the Long-toed Salamander (*Ambystoma macrodactylum*), a widely distributed urodele found near the northern limit of its range in northern British Columbia, is distasteful to predators. The source of this distastefulness has not been exclusively identified. Our objectives were to isolate the skin secretions of the Long-toed Salamander through electrical stimulation of its granular glands and bioassay this secretion for antipredatory effects with crayfish. Previous studies suggested that proteins play an important role in these secretions; therefore, we tested for their presence and utility. The feeding response of crayfish to control food pellets was compared to pellets incorporating raw salamander skin secretions and to pellets incorporating boiled secretions. Significantly more crayfish consumed the control pellets than the raw skin secretion pellets, indicating unpalatability of these skin secretions. Thus, skin secretions appear to have an important antipredatory function. Skin secretions tested positive for presence of proteins, but the feeding response of crayfish to raw and boiled secretions was not significantly different, indicating that proteins were either only partially denatured by boiling, or are not exclusively responsible for the antipredatory nature of the secretions. A very low concentration of secretion was required to illicit a significant antifeedant response in crayfish, and we discuss the possible implications of this in relation to a proposed dual function of the Long-toed Salamander's granular glands in nutrient storage and the energetic requirements of an amphibian in its northern range.**

Amphibians have been defined as ectothermic ''vertebrates having a smooth or rough skin rich in glands'' (Noble, 1931). Chemical secretions from these cutaneous glands are used by amphibians for a variety of defensive functions, ranging from antimicrobial and antifungal activities (Clarke, 1997; Daly, 1998; Apponyi et al., 2004; Schadich, 2009) to antipredator strategies (Anderson, 1964; Clarke, 1997; Daly, 1998; Hamning et al., 2000) that are often coupled with defensive postures (Brodie and Gibson, 1969; Brodie et al., 1979) or aposematic coloration (Carpenter, 1955; Hensel and Brodie, 1976; Daly et al., 1987; Summers and Clough, 2001). The effects of these secretions on predators range considerably from simple antifeedant effects (Anderson, 1964; Brodie et al., 1979; Darst and Cummings, 2006; Grant and Evans, 2007) to serious neurological (Hamning et al., 2000) and physiological health effects often resulting in death (Brodie and Gibson, 1969; Brandon and Huheey, 1981, 1985).

Among the urodeles, glandular secretions are found principally on the skin of the tail and parotoid regions (Brodie and Gibson, 1969; Williams and Larsen, 1986; Williams and Anthony, 1994; Grant and Evans, 2007) and have been examined in varying degrees of detail in a number of genera. For example, the toxic secretions of *Taricha* newts and the role of their primary constituent, tetrodotoxin, in predator-prey relationships have been investigated for over 40 years (e.g., Brodie, 1968; Brodie et al., 1974, 2005; Hanifin et al., 1999). In comparison, the toxicity of plethodontid salamanders has only been sparsely described (Brandon and Huheey, 1981) and even less so in the Ambystomatidae (Grant and Evans, 2007). The little work done on this family has had varied, and sometimes inconsistent, results. Brodie and Gibson (1969) found skin secretions of *Ambystoma gracile* and *Ambystoma macrodactylum* to range from irritable to toxic when injected intraperitoneally into Townswend's Voles (*Microtus townsendi*) and white rats. *Ambystoma maculatum* skin secretions were among the least palatable of salamander secretions offered to Short-Tailed Shrews (*Blarina brevicauda*) (Brodie et al., 1979). Skin extracts from *Ambystoma opacum* and *Ambystoma texanum* caused strong physiological symptoms when injected intraperitoneally into white mice (Brandon and Huheey, 1985). Skin secretions from *Ambystoma*

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tigrinum were shown to inhibit neurotransmission in rat hippocampus (Hamning et al., 2000). Daly et al. (1987) found no biochemically active alkaloids, biogenic amines, peptides, or bufodienolides in ambystomatids listed by others as noxious.

The Long-toed Salamander (*A. macrodactylum*) is the most widely distributed salamander species in British Columbia (B.C.), and the only urodele found in the interior of northern B.C. (Matsuda et al., 2006). However, the skin secretions of this ambystomatid have not been studied in detail. Anderson (1964) reported that hungry western moles (*Scapanus latimanus*) rejected live adult *A. macrodactylum* when offered, even though these moles are generalist predators and readily eat other amphibians and meal-worms. These moles also rejected meal-worms that were rubbed over the skin of adult *A. macrodactylum*. Brodie and Gibson (1969) injected voles with macerated skin from *A. macrodactylum* tails and found they exhibited symptoms of hypersensitivity, instability, and convulsions before ultimately dying. These results suggest that the antipredator skin secretions of *A. macrodactylum* are responsible for its unpalatability and possible toxicity. Williams and Larsen (1986) analyzed the granular gland skin secretions of adult Long-toed Salamanders and found little to no fat, mucus, lipids, or polysaccharides but mostly protein. Williams and Larsen (1986) suggested that the protein might serve a dual function as both a defensive toxin and an energy source for the salamander, although this was never decisively concluded. Other studies have examined the proteinaceous nature of ambystomatid skin secretions (Williams and Anthony, 1994; Hamning et al., 2000); thus, the suggestion that *A. macrodactylum* may have strongly proteinaceous skin secretions is logical.

Although these studies suggest that Long-toed Salamanders possess antipredatory skin with secretions that are proteinaceous in nature, the secretions themselves have not been satisfactorily tested in a controlled environment. Anderson's (1964) study involved a very low sample size (one mole), and his results could have been confounded with other components of the skin. Although the testing of antipredatory effects by removing and bioassaying the whole skin of amphibians has been used in the past (Brodie and Gibson, 1969), it is difficult to pinpoint through this method what component of the skin is causing the antipredatory effect, and also, the introduction of contaminants to the sample is a concern (Grant and Land, 2002). Grant and Evans (2007) successfully isolated and bioassayed glandular skin secretions of ambystomatid salamanders through nondestructive electrical stimulation by a Transcutaneous Amphibian Stimulator (TAS) (Grant and Land, 2002). This method has been successfully used on other members of its family, but it remains untested with *A. macrodactylum*.

The primary objective of this study was to isolate the skin secretions of the Long-toed Salamander through electrical stimulation of its skin glands using the TAS device of Grant and Land (2002), and to bioassay this secretion to determine antipredator effects with crayfish (*Cambarus* spp.). Crayfish were chosen as predators for this study because they display distinct feeding behaviors (Kreider and Watts, 1998; Grant and Evans, 2007), and although most are probably not predators of adult Long-toed Salamanders, they are known predators of amphibian larvae (Holomuzki, 1989; Gamradt and Kats, 1996; Axelsson et al., 1997; Grant and Evans, 2007). Skin secretions from *Ambystoma jeffersonianum*, *Ambystoma maculatum*, and *A. tigrinum* were shown to have significant antifeedant properties against crayfish (Grant and Evans, 2007); thus, a specific examination of *A. macrodactylum* skin secretions with crayfish seemed warranted. We hypothesized that, if the skin secretions of the Long-toed Salamander were successfully isolated and bioassayed, they would have an antipredatory function on crayfish.

Another objective of this study was to confirm the presence of proteins in *A. macrodactylum* skin secretions and test the protein's role in antipredatory functions by comparing the relative effects of raw and denatured secretions. We hypothesized that proteins play an important antipredatory role and that, if denatured by boiling, the secretions would lose their antipredatory function.

Materials and Methods

Experimental Animals.—Mature, adult Long-toed Salamanders used in the study were caught through manual timed searches in randomly chosen plots along systematically placed transects in a mature mixed forest on the University of Northern British Columbia (UNBC) campus, Prince George, B.C., Canada (53°55′N, 122°49′W), between 24 July and 14 September 2008. Eleven salamanders were caught, but only individuals over 2.0 g were used in our study. Our sample size included 8 mature adults (males = 6, females = 2; mean snout–vent length = 52.85 ± 4.95 mm; mean mass = 4.11 ± 1.59 g). Salamanders were housed individually in large plastic holding containers with moist natural substrate and fed two, two-week-old crickets every two days.

Ambystomatids are not believed to obtain defensive compounds from their diet (Grant and Evans, 2007); thus, this feeding prior to secretion collection should not have affected our results. All captive salamanders were housed and fed in accordance to UNBC Animal Care and Use Committee Guidelines (ACUC A2007.0509.016C).

Mature adult crayfish (*Cambarus* spp.; Decapoda: Cambaridae) were obtained from Wards Natural Science Company™ (St. Catharines, Ontario, Canada), and were kept individually in plastic housing containers with approximately 800 ml of tap water (aged for 24 h), a sand substrate, and shelter. A cardboard blind around each housing container prevented crayfish from seeing each other. This prevented stressing the animals from aggressive behavior and confounding our analysis of feeding behavior (Grant and Evans, 2007). The animals were fed freeze-dried bloodworms ad libitum. Thirty healthy, intact crayfish were used in our experiments, and a summary of their morphometrics is presented in Table 1. Crayfish were subjected to one of three feeding treatments (control, raw skin secretion, boiled skin secretion). The animals were stratified by sex and then randomly assigned to each of the three treatments, for a total of 10 crayfish in each group.

Skin Secretions.—Prior to treatment, adult Long-toed Salamanders were anesthetized (Hamning et al., 2000) by massaging approximately 0.5 ml of benzocaine-based Orajel® toothache pain relief gel (Church and Dwight Co., Inc, Mississauga, Ontario, Canada) along the ventral side of their head. Excess Orajel® was rinsed off using deionized water. Anesthetized animals were moistened with 1.0 ml of deionized water to help facilitate electrical stimulation (Grant and Evans, 2007). Skin secretions were obtained by electrical stimulation (10–15V pulsed for 2 msec at 50Hz approximate frequency) using a TAS developed by Grant and Land (2002). Because the defensive skin glands of ambystomatids are believed to be concentrated along the tail ridge (Williams and Larsen, 1986; Grant and Evans, 2007), we concentrated our stimulation efforts on this area. After approximately 30 sec of massaging the probe along the length of the tail, a sticky, milky-white secretion was produced. Salamanders were stimulated for a further 2 min, before the secretions were rinsed off into a collecting beaker using 5.0 ml of deionized water. The collected skin secretions were then centrifuged (Williams and Anthony, 1994) on high power for 50 min, at which point the top 3.0 ml of the supernatant was pipetted off and 2.0 ml of concentrated secretions were retained. To keep the salamander hydrated, it was moistened using deionized water and placed back in its holding container where it was immediately fed crickets. All salamanders recovered fully.

TABLE 1. Morphometrics of healthy, mature adult crayfish (*Cambarus* spp.) used in feeding trials (N = 30; females = 15, males = 15).

	Mass (g)	Total length (cm)[a]	Carapace length (cm)[b]
Males			
Mean	26.79	9.25	3.71
SE	0.96	0.13	0.22
Females			
Mean	21.36	8.65	3.50
SE	0.72	0.14	0.04

[a] Total length measured from end of telson to tip of rostrum.
[b] Carapace length measured from posterior end of carapace to tip of rostrum.

Skin secretions from all salamanders were pooled for the remainder of the analyses. We tested for the presence of proteins in *A. macrodactylum* skin secretions using a Bradford Protein Assay (Bradford, 1976) and the presence of a distinctive spectrophotometric absorbance peak at 280 nm (Gill and von Hippel, 1989; D. Erasmus, pers. comm.). To test for the role of proteins as feeding deterrent, 12.0 ml of secretion was boiled at 100°C for 1 h. Brodie et al. (1974) found that heating skin secretions of *Taricha* newts with this intensity decreased their toxicity by 80% (E. D. Brodie Jr., pers. comm.). Twelve milliliters of secretion was left in its raw form to test for the defensive properties of the secretion without treatment. Dry masses of skin secretions were obtained by lypholization (Grant and Evans, 2007) in a freeze-drier at −40°C for 48 h.

Experimental Food Pellets.—Food pellets used in our crayfish feeding trials were made using a modified technique of Grant and Evans (2007). Each pellet was made by crushing 0.25 g of freeze-dried bloodworms with a mortar and pestle, mixing them with 5.0 ml of deionized water and then filtering the suspension through cheese-cloth (Grant and Evans, 2007). This bloodworm solution, which is a known crayfish feeding stimulant (Grant and Evans, 2007), was added to a boiled solution of 0.15 g of agar and 5.0 ml of water and pipetted into prepared plastic molds to cool and harden. Each mold was approximately 0.235 ml; thus, a single batch of solution created several pellets. Pellets did not disintegrate when placed in water.

A pilot study indicated that crayfish quickly consumed agar pellets incorporating the bloodworm solution. Ten pellets consisting of the bloodworm solution served as a positive con-

trol. Ten other pellets incorporated the raw skin secretions. These were made by combining 5.0 ml of a 0.2504 mg/ml aqueous skin secretion solution with 5.0 ml of prepared bloodworm and agar solution. The 10 remaining pellets were created as above but using the boiled skin secretions. All pellets were refrigerated at 2°C until they were used in the feeding trials.

Feeding Trials.—Before the feeding trials, each crayfish was starved for 24 h to enhance feeding response (Kreider and Watts, 1998; Meakin et al., 2008; Volpe et al., 2008). After starvation, each crayfish was placed in a 2.1-L Rubbermaid® container with 1.0 L of 24 h aged tap water (Kreider and Watts, 1998). A large cardboard box was placed over the container to serve as a blind. Each animal was given 3 min to acclimatize to its new environment. A food pellet was presented directly in front of the crayfish's mouthparts, using a ''feeding rod'' constructed of a long wire with the food pellet attached to the end. The crayfish was given 3 min to respond to the pellet (Kreider and Watts, 1998), and their feeding behavior was closely observed during this time. As crayfish will either ingest or immediately reject a food item presented to them (Kreider and Watts, 1998), we used a binomial score of either ''consumed (1)'' or ''rejected (0)'' to assess feeding behavior. Consumption was defined as the complete ingestion of a pellet, whereas, rejection was defined based on two sequential behaviors: (1) bringing the pellet to the mouthparts (''tasting'' it); and (2) either dropping the pellet or extracting and discarding strands of skin secretion from the pellet while consuming the rest of it. This behavior of manipulating the pellet to extract and discard the skin secretion was noted in several individuals fed pellets with raw skin secretions. Those individuals who did not respond at all to the pellet were listed as ''ignored'' and were not included in subsequent statistical analyses. Time to completely consume a pellet was also recorded as a metric of preference. If a pellet was not consumed within a 3-min time limit, then a time of ''3 min+'' was recorded. No individual crayfish was used more than once for each treatment (Kreider and Watts, 1998; Volpe et al., 2008).

Statistical Analysis.—Behavioral response to food pellet was examined using logistic regression, where ''consumed'' was given the binomial score of ''1'' and ''rejected'' was given a score of ''0.'' As we were primarily interested in the possible antipredatory nature of the salamander skin secretions, the response of crayfish to food pellets with raw skin secretions was first compared to the response to control food pellets. To determine whether the defensive nature of the secretion was caused by proteins, the response of crayfish to food pellets with boiled skin secretions was compared to the response to raw skin secretions, as well as to the control pellets. The effect of mean time to consume food pellets was analyzed using ANOVA. All statistical analyses were completed using R v.2.5.1 (R Development Core Team, Vienna, Austria, available from www.R-project.org, 2007), and $\alpha = 0.05$ statistical significance.

Results

We confirmed the presence of proteins in *A. macrodactylum* skin secretions through a Bradford Protein Assay (Bradford, 1976) and detection of the presence of a distinctive spectrophotometric absorbance peak found at 278 nm (Gill and von Hippel, 1989; D. Erasmus, pers. comm.).

Two males out of the 10 crayfish total in each feeding treatment ignored the pellets, whereas all females reacted by either consuming or rejecting the pellets. The males that did not react were not included in subsequent analysis; thus, each treatment sample size was reduced to $N = 8$ (males = 3, females = 5) animals. Mean ($\pm$ SE) time taken by crayfish to completely consume pellets did not differ significantly across feeding treatments ($F = 3.31$, df = 2,21, $P = 0.06$), because crayfish quickly either consumed or rejected the pellet when offered. The crayfish took 129.38 $\pm$ 14.83 sec, 153.75 $\pm$ 23.74 sec, and 82.25 $\pm$ 20.38 sec to consume the control, raw skin secretion, and boiled skin secretion pellets, respectively. There was no significant difference between male and female crayfish in any of the feeding responses (all $P > 0.05$).

Eighty-seven and a half percent of crayfish feeding on control pellets consumed the pellets completely, with only one animal exhibiting a rejection response. In contrast, only 37.5% of crayfish given pellets with raw salamander skin secretions consumed these pellets, with five animals rejecting the pellets. Control pellets were consumed in a significantly greater number than pellets incorporating the raw salamander skin secretions ($\chi = 4.56$, df = 1, $P = 0.0328$). Seventy-five percent of crayfish consumed the pellets with boiled salamander skin secretions, with only two animals exhibiting a rejection response (Fig. 1). There was no significant difference in the number of pellets consumed by animals feeding on control pellets and those feeding on pellets incorporating boiled skin secretions ($\chi = 0.42$, df = 1, $P = 0.52$). No statistically significant difference was detected between pellets consumed by crayfish feeding on pellets with raw and boiled salamander skin secretions ($\chi = 2.35$, df = 1, $P = 0.13$).

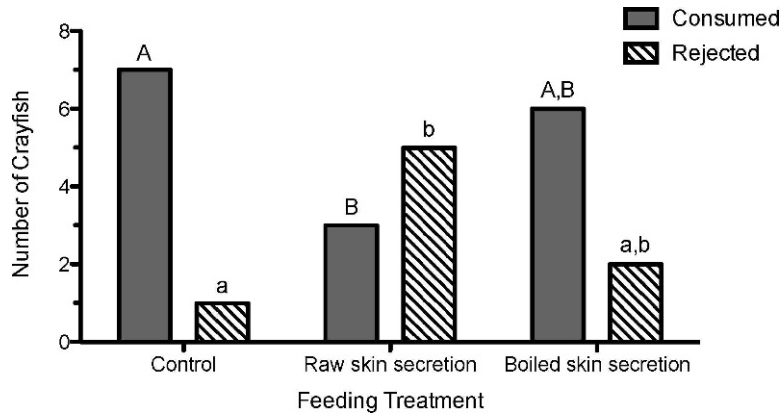

FIG. 1. Behavioral responses of crayfish ($N = 8$ for each treatment) to the presentation of food pellets incorporating different feeding treatments. (Different upper- [consumed] and lower-case [rejected] letters indicate significant differences between treatments [$P < 0.05$]).

DISCUSSION

Our results contribute to the small body of literature on chemical defenses in *A. macrodactylum* (Anderson, 1964; Brodie and Gibson, 1969) by isolating a source of these chemical defenses to glandular skin secretions. In addition, our successful use of the TAS device (Grant and Land, 2002) to extract Long-toed Salamander skin secretions in a nonlethal, noninvasive manner provides further justification for the use of this underused method of examining amphibian skin secretions.

Because crayfish consumed significantly more control pellets than those treated with raw skin secretions, our data support the hypothesis that skin secretions of the Long-toed Salamander play an important antipredatory role for this amphibian. Thus, we confirm the antipredatory role of skin glands as proposed by Williams and Larsen (1986). The antipredator function of ambystomatid skin has been previously suggested (Brodie and Gibson, 1969; Brodie et al., 1979; Brandon and Huheey, 1985; Hamning et al., 2000; Grant and Evans, 2007), and our results confirm that the glandular skin secretions are responsible for this antipredatory function.

Antipredator skin secretions allow ambystomatid salamanders to successfully defend themselves against both microbial and fungal pathogens (Clarke, 1997; Daly, 1998; Apponyi et al., 2004; Schadich, 2009), and vertebrate and invertebrate predators (Anderson, 1964; Clarke, 1997; Daly, 1998; Hamning et al., 2000). Reducing risk of injury, and surviving encounters with predators mean that producing these secretions is a highly successful evolutionary strategy. In addition, having the majority of granular glands positioned at the rear of the salamander (Williams and Larsen, 1986; Grant and Evans, 2007) allows these animals to maximize their fitness, where the tail, being a nonessential body part, can shield vital anatomy (Brodie and Gibson, 1969), and autotomize if necessary.

Our results are potentially doubly significant because they demonstrate not only that skin secretions of the Long-toed Salamander are antipredatory in nature but also may be required in low concentrations to achieve this defensive effect, in *A. macrodactylum*'s northern range. Work by Grant and Evans (2007) on three different southern ambystomatid salamander species showed that crayfish were repelled by pellets incorporating skin secretions concentrated to 4.80 mg/ml. Although Grant and Evans (2007) do not report a minimum antipredatory concentration of their salamanders' secretions, it is still interesting to note that our pellets, from a salamander in the same genus, repelled the same type of predator at a far lower concentration of skin secretion (0.25 mg/ml).

Williams and Larsen (1986) proposed a double function hypothesis for the granular skin glands of Long-toed Salamanders as both an antipredatory secretion production center and protein nutrient storage depository. Further to this hypothesis, we speculate that Long-toed Salamanders, in their northern range, may produce skin secretions potentially potent at low concentrations (0.25 mg/ml). This may be an adaptive strategy to survive the short summers and harsh, prolonged winters in their northern habitat. The ability to conserve protein reserves could enhance a salamander's winter survival and spring reproduction and, therefore, increase the overall individual fitness of the animal, despite the physiological costs of

producing and storing chemical defenses (Longson and Joss, 2006). Further work, involving the examination of geographical variations in skin secretion potency and determining the effective antipredatory concentration of secretions with more natural predators should be conducted to help advance our knowledge of the double function hypothesis.

Boiling the skin secretions of the Long-toed Salamander before incorporating them into food pellets resulted in 75% of crayfish consuming these pellets (Fig. 1), and these results were not significantly different from those consuming control pellets (87.5%). Although our results indicate that boiling increased the consumption of pellets, compared to the raw skin secretions, these results were not significant and may indicate only a partial denaturation of the protein. However, because of the intensity of the boiling treatment, a perhaps more plausible explanation is that other chemical components work in conjunction with proteins to have antifeedant effects; thus, denaturing the proteins alone does not completely reduce the secretion's antipredator nature (D. Erasmus, pers. comm.). The proteinaceous nature of Long-toed Salamander skin secretions has been reported (Williams and Larsen, 1986; Williams and Anthony, 1994), and our Bradford Protein Assay (Bradford, 1976) and spectrophotometric analysis (Gill and von Hippel, 1989; D. Erasmus, pers. comm.) confirmed these findings. However, the degree of utility of these proteins in providing antipredator functions remains uncertain in *A. macrodactylum*.

Across the amphibia, the four major categories of skin secretion constituents are amines, alkaloids, steroids, and peptides/proteins (Daly, 1998; Clarke, 1997). Within the urodeles, the distribution and functional significance of each of these major components varies widely (Toledo and Jared, 1995). Some salamanders principally use compounds such as tetrodotoxin (Brodie, 1968; Brodie et al., 1974; Hanifin et al., 2008), whereas others enable proteins as their primary antipredatory constituent (Jaussi and Kunz, 1978; Hamning et al, 2000). Although most work on ambystomatid salamanders seems to indicate proteins as their major antipredatory constituent (Williams and Larsen, 1986; Williams and Anderson, 1994; Hamning et al., 2000; Grant and Evans, 2007), the vast diversity of chemical constituents found in amphibian skin glands (Toledo and Jared, 1995) strongly suggest the need for further, more detailed biochemical and structural analysis of the constituents of *A. macrodactylum* glandular skin secretions.

In conclusion, we have here, for the first time, shown that the skin secretions of the Long-toed Salamander appear to be antipredatory in nature. Although proteins were identified as a component of these secretions, and their utility in providing antipredator defense is suggested from our results, the degree of their utility compared to other biochemical components requires further analysis. Long-toed Salamanders in northern British Columbia have chemical defenses potentially effective at low concentrations, and this knowledge provides important ecological and energetic insights into the life of an amphibian in its northern range.

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