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An Evaluation of Immersive and Handling Methods for Collecting Salamander Skin Peptides

Kenzie E. Pereira,[1,2] Kacie A. Cava,[1] and Sarah K. Woodley[1]

[1]*Department of Biological Sciences, Duquesne University, Pittsburgh, Pennsylvania, 15282, USA*

Abstract.—**Amphibian skin secretions are a rich source of antimicrobial peptides that have long interested both the biomedical and herpetological research communities. While anuran skin peptides are well studied, little is known about skin peptides in salamanders, in part because methods for collecting them are poorly developed. In anurans, pharmacological agents are commonly used to stimulate skin peptide responses, thus increasing the amount of peptide collected. It was unclear whether similar methods would work for salamanders. We conducted a series of experiments to test the effects of immersion in pharmaceutical agents (norepinephrine hydrochloride [NE]; acetylcholine chloride [Ach]), handling, and the interaction of both on peptide collection from the skin of Red-Spotted Newts (*Notophthalmus viridescens*) and Allegheny Mountain Dusky Salamanders (*Desmognathus ochrophaeus*). Our results suggest that vigorous handling, but not immersion in NE or Ach, is a better method for collecting salamander skin peptides. Despite the widespread use of pharmaceuticals to collect anuran skin peptides, we found little evidence that NE or Ach induces peptide release in salamanders. In fact, we show that inclusion of NE in collection solutions interferes with peptide quantification in protein assays.**

Amphibian skin is highly glandular and contains diverse bioactive compounds (Duellman and Trueb, 1986; Daly et al., 2005; Conlon and Sonnevend, 2010). While the specific constituents vary widely among and within amphibian species, many species secrete peptides with broad-spectrum antimicrobial activity (Conlon, 2011). Such peptides are collectively known as antimicrobial peptides (AMPs). Ubiquitous among eukaryotes, AMPs are typically short-chained polypeptides capable of killing a wide range of bacteria, fungi, viruses, and protozoa (Zasloff, 2002). Most research of amphibian skin secretions has focused on novel AMP discovery for biomedical purposes (Conlon, 2004; Ladram and Nicolas, 2016). However, there is growing interest to also study the natural roles of skin secretions and peptides in protecting amphibians from pathogens encountered in the wild (Rollins-Smith, 2009).

Previous studies show that many amphibians produce AMPs but have primarily focused on anurans (Conlon and Sonnevend, 2010). For example, AMPs are known from at least 160 anuran species representing 14 different families (Ladram and Nicolas, 2016). In contrast, only two salamander AMPs have been isolated and sequenced (Meng et al., 2013; Pei and Jiang, 2017). Recent studies show that peptide mixtures (uncharacterized) from the skin secretions of at least five other salamander species also exhibit biological activities against various fungi and bacteria (Fredericks and Dankert, 2000; Karış et al., 2018; Pereira et al., 2018; Pereira and Woodley, 2020). Together, these data suggest that salamander skin secretions are also a rich source of AMPs and may be important for pathogen defense.

Skin peptides of most amphibians are produced and stored by dermal granular glands (Vitt and Caldwell, 2013). Each granular gland is surrounded by a myoepithelial cell layer (Wanninger et al., 2018). Contraction of this layer results in the release of gland contents into skin secretions (Toledo and Jared, 1995). Contraction is regulated by neurotransmitters and can be constitutive or episodic in response to a perceived predatory threat (Brodie, 1977; Fujikura et al., 1988; Williams et al., 2000). In anurans, it is well established that myoepithelial cells of granular glands receive adrenergic innervation and are stimulated to contract by norepinephrine (Hoffman and Dent, 1977; Fujikura et al., 1988). In salamanders, much less is known about the innervation of the myoepithelial cells. There is limited evidence suggesting that salamander gland-associated myoepithelial cells receive cholinergic innervation and are thus stimulated by acetylcholine (Hoffman and Dent, 1977; Houck et al., 2007).

Methods for stimulating and collecting salamander skin peptides are poorly developed. Until now, mechanical stimulation has been the primary method used to collect salamander skin secretions. This method induces the release of glandular contents, in principle, by simulating a predatory attack and involves squeezing a specific body region where granular glands are abundant (e.g., tail) or by physically handling the whole body of the salamander (Williams and Larsen, 1986; Jared et al., 2009; Pereira et al., 2018; Pereira and Woodley, 2020). Skin secretions can then be scraped or rinsed off. A disadvantage of this method is that its effectiveness, indicated by the amount of peptide collected, varies greatly between individuals and species (Pereira et al., 2018; Pereira and Woodley, 2020). This may be, in part, because the amount of stimulation required to elicit a peptide response is largely unknown. Although neither squeezing nor handling techniques seem to have long-lasting adverse effects, handling, in general, induces a physiological stress response and may cause tail loss in certain species (Venesky and Anthony, 2007; Thomas et al., 2017).

Unlike salamanders, methods for collecting anuran skin peptides are well established. The most common method involves injecting frogs with the pharmacological agent norepinephrine (NE) hydrochloride. To quickly circulate the NE through the body for a rapid and systemic release of peptides, injections are given in the lymphatic system by way of the anterior lymph heart (i.e., dorsal lymph sac), which is located near the skin's surface in anurans (Hedrick et al., 2013, 2014). One advantage of this method is that copious skin peptides can be collected with a single injection and minimal damage to the skin (Gammill et al., 2012). Another advantage is that it provides a standardized stimulus for a variety of species, thereby reducing variation in the amount of peptides collected (Woodhams et al., 2006a).

[2]Corresponding Author. E-mail: bozemank@duq.edu
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It is not clear that injection with a pharmacological agent such as NE will also work for salamanders for several reasons. First, salamanders lack an easily accessible lymphatic heart or similar structure to circulate pharmaceuticals systemically (Hedrick et al., 2013). Therefore, a systemic peptide response would, hypothetically, require numerous injections and considerable skin damage. Second, salamander gland-associated myoepithelial cells are thought to receive cholinergic rather than adrenergic innervation, although the evidence for this is minimal (Hoffman and Dent, 1977). Thus, injection with a cholinergic agent like acetylcholine (Ach) chloride might work better (Hoffman and Dent, 1977).

Immersion in a pharmacological agent rather than injection may be a simple and less invasive method for collecting salamander skin peptides. Immersion involves placing an animal in a solution containing NE or Ach (Woodhams et al., 2006b; Clark, 2010; Conlon and Sonnevend, 2010). Presumably, the pharmacological agent passes through the epidermis to the dermal layer to induce gland contraction and peptide release. One advantage is that immersion would potentially avoid the stress and tail loss associated with mechanical stimulatory methods, while delivering a standardized stimulus for peptide release. A potential disadvantage is that immersion may be less effective compared to other methods (Conlon and Sonnevend, 2010). Although a few studies have attempted immersion in NE to induce peptide release in a variety of amphibians, the effectiveness of this method remains unclear (Woodhams et al., 2006a,b; Sheafor et al., 2008). To the best of our knowledge, immersive methods using Ach have not been tested in anurans or salamanders.

The goal of this work was to evaluate whether immersion in pharmacological agents can be used to collect skin peptides from salamanders. We also compared immersion with the mechanical handling. Experiments were conducted using Red-Spotted Newts (*Notophthalmus viridescens*) and Allegheny Mountain Dusky Salamanders (*Desmognathus ochrophaeus*). Both of these species are widespread and abundant (Petranka, 1998). We chose *N. viridescens* because this salamandrid species is particularly susceptible to amphibian chytrid pathogens, and a method to collect skin peptides to study the peptides' ability to inhibit chytrid growth is needed (Rothermel et al., 2008; Martel et al., 2014). We chose *D. ochrophaeus* because it represents an additional salamander family (Plethodontidae) with a very different type of skin compared to the aquatic adults of *N. viridescens*, offering an interesting contrast (Taban and Connelly, 1972; Petranka, 1998).

We hypothesized that immersion in pharmaceutical agents would induce skin peptide release in salamanders. First, we tested the prediction that immersion in pharmaceutical agents would induce skin peptide release at levels similar to handling. Next, we tested the prediction that immersion in Ach or NE, combined with handling, would synergize to result in greater release of skin peptides. Because we neglected to include a salamander-free control in some of our experiments, we included an experiment testing whether NE alone would be detected as 'peptides.'

Materials and Methods

Animals.—Our experiments used both free-living and captive salamanders. All animal use was conducted with an appropriate permit from the Pennsylvania Fish and Boat Commission and approved by Duquesne University's Institutional Animal Care and Use Committee.

Free-living Salamanders.—For Red-Spotted Newts (*N. viridescens*), male and female aquatic adults were caught by dip-nets in Clarion County (41°15′29′′N, 79°27′43′′W) in May 2019. For Allegheny Mountain Dusky Salamanders (*D. ochrophaeus*), male and female adults were caught by hand in Westmoreland County (40°36′28′′N, 80°7′56′′W) in June 2019. Crude skin secretion samples were immediately collected after capture. After sample collection, salamanders were rinsed and returned the same day to original capture sites.

Captive Salamanders.—Male and female adult *D. ochrophaeus* were collected by hand and brought to the lab in May 2017. Captive salamanders were individually housed in 16 × 16 × 5-cm plastic containers with moist paper towels, maintained at 16°C on a 14 : 10 light : dark cycle and fed wax worms biweekly. Crude skin secretion samples were collected in June 2017. After sample collection, salamanders were rinsed and returned to home boxes. It is worth noting that we previously found no significant difference in the amount of peptides (corrected for body mass) collected from captive and free-living salamanders (Pereira and Woodley, 2020). Therefore, we do not expect that captivity influenced our results.

Experiment 1: Effects of Immersion in Free-living N. viridescens.—To test whether immersion in pharmacological agents would cause release of skin peptides in *N. viridescens*, free-living salamanders were individually placed in a plastic bag and rocked back and forth (hereafter "swirled") in 15 mL of a vehicle (control), vehicle with 1 mM Ach chloride, or vehicle with 1 mM NE hydrochloride for 10 min. We used relatively high concentrations of NE and Ach to maximize peptide release. To compare responses to handling, a group of *N. viridescens* was gently handled for 10 min. Gentle handling consisted of massaging and lightly pressing the body of one or more salamanders (one salamander per collection bag, multiple bags) with the collector's fingers. The sample size for each treatment was five salamanders.

Experiment 2: Effects of Immersion in Captive D. ochrophaeus—To test whether immersion in pharmacological agents would cause release of skin peptides in *D. ochrophaeus*ç captive salamanders were individually placed in a glass jar and swirled in 15 mL of vehicle (control), vehicle with 1 mM Ach, or vehicle with 1 mM NE for 15 min. To compare responses to handling, a group of *D. ochrophaeus* was gently prodded for 15 min; gentle prodding consisted of lightly pressing the body with sterilized blunt metal forceps. The sample size for each treatment was four salamanders.

Experiment 3: NE Detection in Salamander-Free Solutions.—Because Experiments 1 and 2 lacked a "salamander-free" control, this experiment tested whether NE itself would be detected by the protein assay. We prepared 30 mL of vehicle solutions containing 0 mM, 0.1 mM, 0.2 mM, and 1 mM of NE. Salamanders were not placed in these solutions. Each NE concentration was divided into three 10-mL aliquots. Each aliquot was processed and assayed for peptides individually, resulting in a sample size of three per NE concentration.

Experiment 4: Synergistic Effects of Immersion and Handling in Free-living N. viridescens.—To test the synergistic effects of pharmacological agents and handling on peptide release in *N. viridescens,* free-living salamanders were individually placed in a plastic bag containing 15 mL of vehicle (control), vehicle with 0.1 mM NE, or vehicle with 1 mM Ach, and either swirled or vigorously handled for 15 min. Vigorous handling consisted of continuously massaging and firmly pressing the body of a single

salamander using the whole hand of the collector and periodically shaking the collection bag to stimulate peptide release. We chose to use 0.1 mM of NE because we wanted to avoid interference of NE in the protein assays. Salamander-free vehicle, vehicle with NE, and vehicle with Ach samples were included as no-animal controls. The sample size for each treatment was five (salamanders or no-animal controls).

Experiment 5: Synergistic Effects of Immersion and Handling in Free-living D. ochrophaeus.—To test the synergistic effects of pharmacological agents and handling on peptide release in *D. ochrophaeus*, free-living salamanders were individually placed in a plastic bag containing 15 mL of vehicle (control) or vehicle with 0.2 mM NE, and either swirled or gently handled for 10 min. A separate group of *D. ochrophaeus* was submerged in the vehicle and vigorously handled for 10 min. Salamander-free vehicle and vehicle with NE samples were included as controls. Gentle and vigorous handlings were conducted as described previously. The sample size for each treatment was five (salamanders or no-animal controls).

Preparing Pharmacological Agents.—Crystalline NE hydrochloride (Sigma A7256-1G) was dissolved in amphibian phosphate-buffered saline (APBS, 6.6 g NaCl, 1.15 g anhydrous Na_2HPO_4, 1 L nanopure water, pH = 7.4) to generate a stock NE concentration of 100 mM. An appropriate volume of the NE stock concentration was added to a collection buffer (CB) vehicle (50 mmol 1^{-1} sodium chloride, 25 mmol 1^{-1} sodium acetate) to generate final concentrations of NE (0.1 to 1 mM) for Experiments 1 through 5. Crystalline Ach chloride (Sigma A6625-256) was dissolved in APBS to generate a stock Ach concentration of 100 mM. An appropriate volume of the Ach stock concentration was added to a CB vehicle to generate final concentrations of Ach (1 mM) for Experiments 1 and 2.

Collection and Processing of Samples.—Methods utilized for the collection of crude skin secretions were adapted from Woodhams et al. (2006a) and closely followed those used by Pereira et al. (2018). Salamanders were individually submerged in 15 mL of the CB vehicle, with or without a pharmacological agent, for 10 to 15 min within a 16.5 × 8.25-cm plastic zip lock bag or a 473-mL glass jar to collect crude skin secretion samples. To control for the effects of the pharmacological agents alone on protein assays, we also set up collection containers with vehicle only (salamander-free samples).

After removal of salamanders, samples were combined with trifluoracetic acid (TFA, final combined concentration = 0.1% TFA), centrifuged at 3,220 g for 30 min, and passed through a syringe filter (0.2 μm polyethersulfone [PES] syringe filter, GE Healthcare Life Sciences, Pittsburgh, Pennsylvania, USA) to remove particulates. Each sample was passed through a C-18 Sep-Pak cartridge (cat. no. WAT 051910, Waters Corp., Milford, Massachusetts, USA) that had been primed with 100% methanol and 0.1% TFA (Conlon, 2007). Sep-Pak cartridges were then washed in 0.1% TFA and rinsed with 70% acetonitrile and 0.1% TFA to elute peptides. Wash, sample passage, and elution steps were conducted one time for Experiment 2 and repeated two times for Experiments 1, 3, 4, and 5 to maximize peptide amounts. Eluted compounds were further concentrated using a Speed-Vac concentrator (Savant Instruments, Marietta, Ohio, USA) and resuspended in nanopure water. A Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, Illinois, USA) was used to estimate the peptide content of each resuspended sample following the manufacturer's instructions, except that bradykinin (Sigma Chemical Co., St. Louis, Missouri, USA) was used as the peptide standard (Rollins-Smith et al., 2002).

Statistical Methods.—Statistical analyses were performed in RStudio version 1.2.5033 using parametric and nonparametric methods (Team, 2019). For parametric analyses, data were log transformed to meet the assumptions of normality and equal variances. Data for Experiments 1, 2, and 3 were analyzed with a one-way analysis of variance (ANOVA) with treatment as a factor. Data for Experiment 4 were analyzed with a Scheirer-Ray-Hare Test nonparametric test with handling treatment (no animal, swirled, vigorously handled) and pharmacological agent (vehicle, NE, Ach) as the factors. Because we had an unbalanced design, data for Experiment 5 were analyzed in two ways: 1) with a two-way ANOVA with handling treatment (no-animal, swirled, gently handled) and pharmacological agent (vehicle or NE) as factors, and (2) a one-way ANOVA with handling treatment as a factor. Significant parametric and nonparametric analyses were followed by Tukey and Dunn post hoc tests, respectively. Body mass (g) was not a significant covariate in initial analyses (all $P > 0.4$), thus was not included in subsequent analyses. There was no anecdotal evidence that males and females differed in responses, so sex was not included as a factor.

Results

Experiment 1: Effects of Immersion in Free-living N. viridescens.—The amount of peptides detected in samples from free-living *N. viridescens* varied depending on treatment ($F_{3,16} = 52.05$; $P = < 0.001$; Fig. 1) and was significantly higher in the presence of NE. The amount of peptides from samples where salamanders were gently handled or swirled in 1mM Ach was not different from the control where salamanders were swirled in vehicle.

Experiment 2: Effects of Immersion in Captive D. ochrophaeus.—The amount of peptides detected from captive *D. ochrophaeus* varied depending on treatment ($F_{4,15} = 21.08$; $P = < 0.001$; Fig. 2) and was significantly higher in the presence of NE. The amount of peptides from samples where salamanders were gently handled or swirled in 1 mM Ach was not different from the control where salamanders were swirled in vehicle.

Experiment 3: NE Detection in Salamander-free Solutions.—The amount of peptides detected in samples that contained different amounts of NE, but no salamanders, varied depending on treatment ($F_{3,8} = 248.8$; $P = < 0.001$; Fig. 3). The amount of peptides detected increased with increasing concentration of NE.

Experiment 4: Synergistic Effects of Immersion and Handling in Free-living N. viridescens.—The amount of peptides detected in samples from free-living *N. viridescens* depended on the handling treatment (no-animal, swirled, or vigorously handled: $H_{2,36} = 36.4$; $P = < 0.001$; Fig. 4). Values were highest from samples where salamanders were vigorously handled followed by salamanders that were only swirled, followed by salamander-free controls. Salamanders that were vigorously handled for 15 min released the most peptides of all experiments in this study and typically secreted white frothy material (Fig. 5). In contrast, there was no effect of the pharmacological agent on the amount of peptides detected ($H_{2,36} = 1.18$; $P = 0.56$). In addition, there was no interactive effect of handling treatment and pharmacological agent ($H_{4,36} = 1.11$; $P = 0.89$).

Experiment 5: Synergistic Effects of Immersion and Handling in Free-living D. ochrophaeus.—The amount of peptides detected in samples from free-living *D. ochrophaeus* was not affected by swirling or gentle handling ($F_{2,24} = 2.56$; $P = 0.10$) or pharmacological agent ($F_{1,24} = 0.10$; $P = 0.33$). In addition, there was no interactive effect ($F_{2,24} = 1.64$; $P = 0.22$). However, salamanders that were vigorously handled secreted more peptide

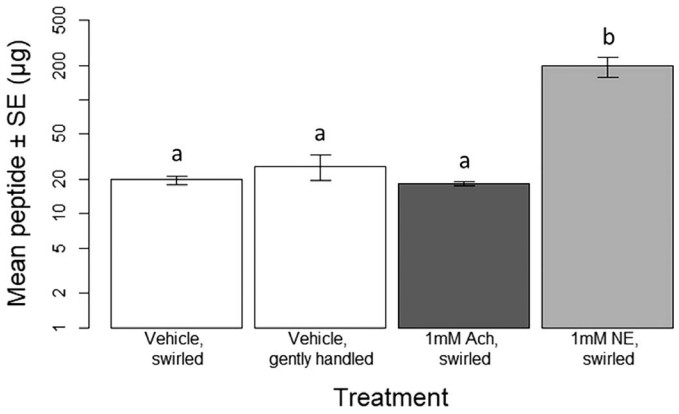

FIG. 1. Results from Experiment 1 showing the amount of peptides detected in samples from free-living *Notophthalmus viridescens* following 10 min of swirling or gentle handling in a collection buffer vehicle, with and without Ach or NE. Each bar represents the mean of five salamanders. Bars with similar letters are not statistically different. Note the logarithmic scale.

compared to the other groups in Experiment 5 ($F_{3,31} = 10.30$; $P = < 0.001$; Fig. 6).

DISCUSSION

We conducted multiple experiments to evaluate whether immersion in pharmacological agents can be used to collect salamander skin peptides. While there was some overlap in design between Experiments 1 and 4, and Experiments 2 and 6, collectively our results show that vigorous handling, but not immersion in pharmacological agents, may be the best way to collect skin peptides from salamanders. Specifically, we found that 10 to 15 min of vigorous handling significantly increased peptide recovery from Red-Spotted Newts (*N. viridescens*) and Allegheny Mountain Dusky Salamanders (*D. ochrophaeus*) compared to swirling, gentle handling, or immersion in the pharmacological agents NE or Ach chloride. Contrary to our prediction, neither NE nor Ach had an effect on peptide recovery from either species, either alone or in combination with handling. In fact, our results suggest that because NE is concentrated during the processing of skin secretions and subsequently quantified by protein assays, including NE in collection solutions may confound the quantification of skin peptides.

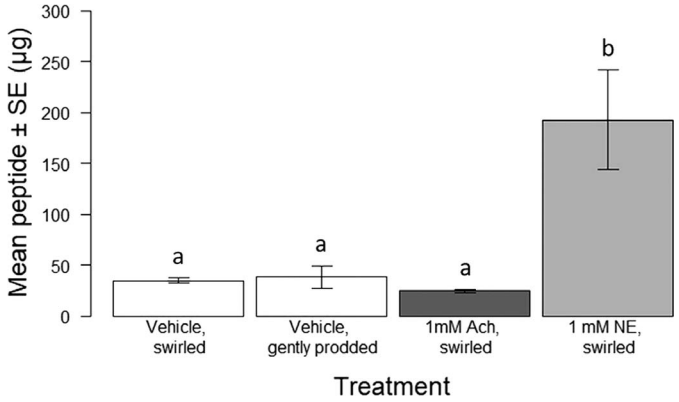

FIG. 2. Results from Experiment 2 showing the amount of peptides detected in samples from captive *Desmognathus ochrophaeus* following 10 min of swirling or gentle prodding with forceps in a collection buffer vehicle with and without Ach or NE. Each bar represents the mean of five salamanders. Bars with similar letters are not statistically different.

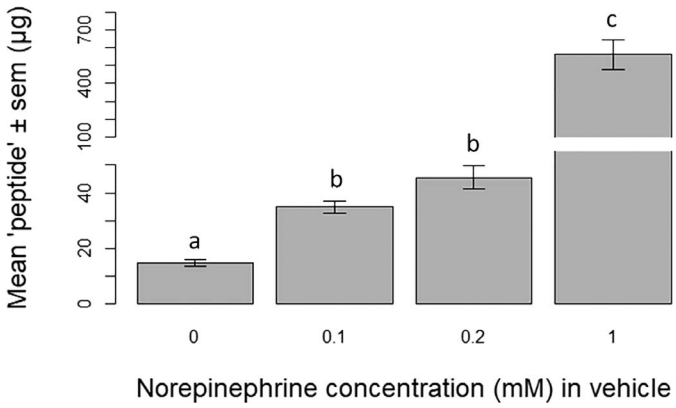

FIG. 3. Results from Experiment 3 showing the amount of peptides detected in no-animal (salamander-free) controls containing NE. Each bar represents the mean of three individually processed samples. Bars with similar letters are not statistically different.

Our results indicate that immersion in NE is not a useful method for collecting salamander skin peptides because the NE itself is detected by the protein assays, and any evidence that NE is inducing release is minimal. We found that NE alone, even the lowest concentration of 0.1 mM NE, was extracted and detected with our methods. This ability to detect NE confounded interpretation of results, such as those in Experiments 1 and 2. Despite this confounding, our findings provide scant evidence that immersion in NE is a viable method for inducing peptide release in these salamander species. First, at 1 mM NE, the mean amount of 'peptide' detected was actually less in samples containing salamanders (~250 µg in samples from both *N. viridescens* and *D. ochrophaeus* compared to ~600 µg in salamander-free samples). These differences could be because of experimental differences, or perhaps the presence of salamanders degraded the amount of NE. Regardless, our results suggest that NE does not induce release of skin peptides. Furthermore, our experiments that tested for synergistic effects of NE and handling also failed to provide strong evidence that

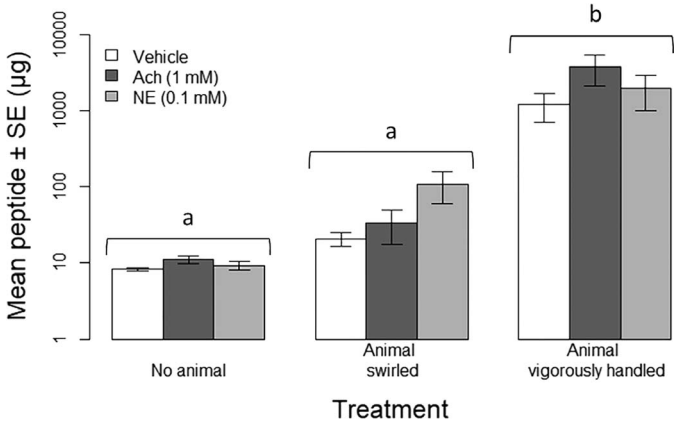

FIG. 4. Results from Experiment 4 (using free-living *Notophthalmus viridescens*) showing the amount of peptides detected in salamander-free samples (no-animal), samples from animals that were swirled, or animals that were vigorously handled. Within each handling treatment, the collection solution was vehicle, vehicle with NE, or vehicle with Ach. Each bar represents the mean of five salamanders or no-animal controls. Bars under brackets sharing the same letter are not statistically different. Note the logarithmic scale.

FIG. 5. Accumulation of skin secretions from an aquatic adult male *Notophthalmus viridescens* (body mass: 5.4 g) following 15 min of vigorous handling in a collection buffer vehicle (without pharmacological agents).

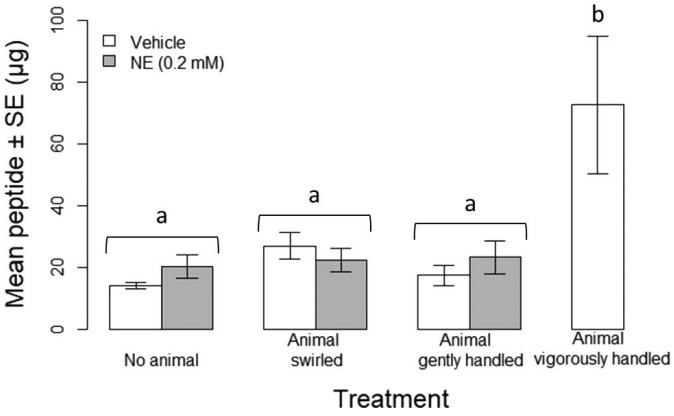

FIG. 6. Results from Experiment 5 (using free-living *Desmognathus ochrophaeus*) showing the amount of peptides detected in salamander-free samples (no animal) or samples from animals that were swirled, animals that were gently handled, or animals that were vigorously handled. Within each treatment, the collection solution was either vehicle or vehicle with NE. Each bar represents the mean of five salamanders or no animal controls. Bars under brackets sharing the same letter are not statistically different.

NE was inducing skin peptide release. These experiments used lower concentrations of NE to minimize the interference of NE in peptide detection. Specifically, in *D. ochrophaeus*, the same amount of peptides was detected in the presence of NE regardless of whether salamanders were present (see gray bars, Fig. 6). In *N. viridescens*, there is a hint that NE increased skin peptide release, but the amount detected was an order of magnitude less than with vigorous handling (light gray bars, Fig. 4).

There are several potential reasons why NE failed to induce skin peptide release. First, it is possible that NE was not able to transverse the epidermal skin layer when administered via immersion, and thus was unable to interact with underlying dermal granular glands. Alternatively, granular gland contraction may not be under adrenergic control in salamanders. Hoffman and Dent (1977) provided evidence that salamander granular glands receive cholinergic and not adrenergic innervation.

We also tested whether immersion in Ach would increase peptide recovery from salamander skin. Because Hoffman and Dent (1977) demonstrated that an Ach dose of 0.001 mM stimulated peptide release in *N. viridescens* when administered via subcutaneous injection, we presumed that a concentration of 1 mM Ach would effectively induce peptide release when passively delivered using an immersive method. However, immersion in a 1-mM Ach concentration had no effect on peptide recovery from *N. viridescens* or *D. ochrophaeus*.

The lack of peptide response to Ach was unlikely because of an inefficient exposure time or degradation of Ach in collection solutions. Hoffman and Dent (1977) showed that peptide discharge in *N. viridescens* occurred within 1 min following direct exposure of excised skin to Ach. Likewise, physiological responses to Ach passively delivered to human subjects via transdermal patches were evident within 2 min of patch application (Schonberger et al., 2006). While we expected that peptide responses to passive Ach delivery to granular glands via immersive methods would be delayed compared to more-direct delivery methods, we believed that a period of 10 to 15 min would be sufficient for eliciting peptide responses. Further, because we used Ach solutions within 24 h of being prepared, and Ach solutions are stable for up to 28 days when stored at 25°C, it was unlikely that degradation of Ach explained our negative results (Sletten et al., 2005).

As with NE, the lack of peptide response to Ach in salamanders may have resulted from inefficient delivery of Ach to dermal granular glands. In mammalian models, including humans, transdermal delivery methods are commonly used to administer Ach, but often utilize an anodal electrical current to drive positively charged Ach ions across the skin, indicating that Ach does not easily transverse the epidermis (Singh and Singh, 1993; Tesselaar and Sjöberg, 2011). Also, transdermal Ach delivery in mammalian models are dose-dependent, so perhaps Ach delivery would be enhanced by increasing the concentration of Ach in collection solutions above 1 mM, as used in the present study (Christen et al., 2004).

Handling has previously been used to collect skin secretions in other species of salamander with varying effectiveness (Largen and Woodley, 2008; von Byern et al., 2017a,b; Pereira and Woodley, 2020). We expand on these earlier studies by showing that vigorous, rather than gentle, handling was necessary to cause release of skin peptides. Neither gentle handling nor swirling produced peptide levels above background (salamander-free controls). These findings suggest that, in general, *N. viridescens* and *D. ochrophaeus* do not secrete skin peptides without a strong external stimulus. Consistent with a previous study, the release of skin secretions (inferred by the presence of frothy accumulations in the collection bags, Fig. 5) from *N. viridescens* and *D. ochrophaeus* were often not observed until at least 10 min into the handling period (von Byern et al., 2015). It is not clear why such vigorous handling was necessary to induce noticeable release of skin secretions. Presumably, animals would need a rapid release of skin secretions for predator defense. However, *N. viridescens* is toxic, thus, may not rely on bulk release of skin secretions for predator defense (Marion and Hay, 2011). Likewise, *D. ochrophaeus* may utilize other strategies such as biting or tail autotomy for predator defense even though neither of these behaviors was observed in the current study (Brodie et al., 1979, 1989; Labanick, 1984).

We also tested whether handling and pharmacological agents might synergize, such that the presence of NE or Ach would

result in additional peptide release with handling. However, there was no difference in the amount of peptides detected between salamanders that were handled without pharmacological agents vs. with pharmacological agents (*N. viridescens*: Fig. 4, right-most cluster of bars; *D. ochrophaeus*: Fig. 6, third cluster of bars).

In conclusion, our study is one of the first to investigate the effects of the pharmacological agents NE and Ach on the recovery of skin peptides from salamanders using a minimally invasive immersive method. Although the use of NE or Ach in collection solutions failed to increase peptide collection in a salamandrid (*N. viridescens*) and plethodontid (*D. ochrophaeus*) salamander species, vigorous handling greatly increased the amount of skin peptides collected from both species. Our results should be useful to other investigators attempting to collect skin secretions from salamander species for study of their potential antimicrobial effects.

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