2024

Antipredator Blood-Squirting Defense in Horned Lizards (*Phrynosoma*): Chemical Isolation of Plasma Component(s), *Pogonomyrmex* Ant Dietary Origin, and Evolution

Wade C. Sherbrooke
American Museum of Natural History, Southwest Research Station, wcs@amnh.org

Bruce A. Kimball
United States Department of Agriculture, APHIS, Wildlife Sservices, National Wildlife Research Center, Fort Collins, Colorado, bkimball@monell.org

Ichthyology & Herpetology 112, No. 1, 2024, 106–116

Antipredator Blood-Squirting Defense in Horned Lizards (*Phrynosoma*): Chemical Isolation of Plasma Component(s), *Pogonomyrmex* Ant Dietary Origin, and Evolution

Wade C. Sherbrooke[1] and Bruce A. Kimball[2,3]

North American horned lizards of the genus *Phrynosoma* are largely myrmecophagous and squirt systemic blood from circumocular sinuses during encounters with certain mammalian predators. The predators react with distinct revulsion. Using bioassay-guided fractionation of blood plasma, we endeavored to identify the source and characteristics of the active chemical compound(s) responsible for this negative oral response. Our results of coyote bioassays and a series of novel mouse bioassay experiments were largely concordant. The active compound(s) was likely plasma-borne with a molecular weight (mw) of between 800 and 1,600. To identify the source of the active compound(s), we noted bioassay responses of different species of horned lizards. The active compound(s) was present in plasma fractions of species known to eat *Pogonomyrmex* and squirt blood, and it was absent in the plasma fractions of species that do not squirt blood and eat other ants or arthropods. Plasma fractions from individuals of *P. cornutum*, a blood-squirting species, fed *Pogonomyrmex* had more aversive levels of the active compound(s) compared to those given a cricket diet. In addition, extracts from *Pogonomyrmex* indicate that the active compound(s) in lizard plasma is associated with the abdomen of these ants, where venom is stored. The toxic enzymes of ant venom may be metabolized by horned lizards resulting in one or more small peptides that act as the active ingredient(s) in the lizard's circulating blood. We suggest that utilization of this widely dispersed, venomous, and abundant diet only became available to stem horned lizards with the evolution of unique prey capture techniques. This broadened diet then led to incorporation of the compound(s) from *Pogonomyrmex* spp. into their blood plasma, facilitating the evolution of antipredator blood-squirting. These transformative events apparently established an evolutionarily stable configuration that led to or expanded the unique "*Phrynosoma* suite" of characteristics from which subsequent diverse clades have evolved across arid North America.

Las lagartijas cornudas norteamericanas del género *Phrynosoma*, son en gran parte mirmecófagos, arrojan sangre de manera sistémica por los senos circumoculares durante los encuentros con ciertos mamíferos depredadores. Cuando la sangre entra en su cavidad bucal, los depredadores reaccionan con una clara repulsión. Utilizando la fracción del plasma sanguíneo en bioensayos, identificamos las características y el origen de los compuestos químicos activos responsables de esta respuesta oral negativa. Los resultados de una serie de experimentos de bioensayos con coyotes, así como una nueva serie de experimentos de bioensayos con ratones, fueron en gran medida concordantes. Es probable que los compuestos activos sean transportados en el plasma con un peso molecular de entre 800 y 1600 (mw). Para identificar el origen de los compuestos activos, se observaron las respuestas de bioensayos en ratones del plasma de diferentes especies de lagartijas cornudas. Los resultados indican que los compuestos activos están presentes en las fracciones del plasma de especies que son conocidas por comer *Pogonomyrmex* y su relación con arrojar sangre, y además, que están ausentes en las fracciones del plasma que no arrojan sangre, y que comen otras hormigas y artrópodos. Las fracciones de plasma de individuos de *P. cornutum*, una especie sanguinolenta, alimentadas con *Pogonomyrmex* presentó niveles más elevados de compuestos en su plasma en comparación con los alimentados con grillos. Además, fracciones de plasma de *Pogonomyrmex* indican que el compuesto activo(s) en la plasma está asociado con el abdomen de estas hormigas, que es donde se almacena el veneno. El rol aparente del veneno de la hormiga cosechadora en la bioactividad del plasma en las lagartijass cornudas, puede no deberse a su absorción directa. En cambio, las enzimas tóxicas del veneno de las hormigas pueden ser metabolizadas por estas lagartijas, lo que da como resultado uno a más péptidos pequeños que actúan como ingredientes activos en la sangre circundante de estas. Nuestro estudio abre un campo de investigación para comprender las complejidades de las relaciones evolutivas entre las diversas especies de lagartijas cornudas y *Pogonomyrmex* spp. Sugerimos que esta dieta abundante, venenosa y ampliamente dispersa, solo estuvo disponible para lagartijas cornudas con la evolución de técnicas únicas de captura de presas. Esta dieta condujo posteriormente a la incorporación de los compuestos de *Pogonomyrmex* spp. en el plasma sanguíneo, lo que facilitó la evolución de los chorros de sangre con funcionalidad antidepredador. Estos eventos transformadores aparentemente establecieron una configuración evolutiva del "conjunto *Phynosoma*", con características únicas, a partir de las cuales los subsiguientes diversos clados han evolucionado a lo largo de la árida América del Norte.

SUMMARIES of horned lizard biology, calling attention to their unique cranial horn morphology, unusual saurian behaviors of myrmecophagy (pronounced eating of ants), and unique ocular sinus blood-squirting as an antipredator defense, have led herpetologists to special recognition of their phylogenetic and evolutionary interest (Pianka and Parker, 1975; Baur and Montanucci, 1998; Sherbrooke, 2003; Powell and Russell, 2024). The genus *Phrynosoma* (Fig. 1), with 17–18 species (Leaché and McGuire, 2006; Leaché et al., 2021), is widespread in western North America from southwestern

[1] Southwestern Research Station, American Museum of Natural History, Portal, Arizona 85632; Email: wcs@amnh.org. Send correspondence to this address.
[2] USDA/APHIS/WS/NWRC, 4101 LaPorte Avenue, Fort Collins, Colorado 80521.
[3] Present address: Monell Chemical Senses Center, 3500 Market Street, Philadelphia, Pennsylvania 19104; Email: bkimball@monell.org.
Submitted: 20 December 2021. Accepted: 29 January 2024. Associate Editor: J. Lamb.
 DOI: 10.1643/h2021139 Published online: 22 March 2024

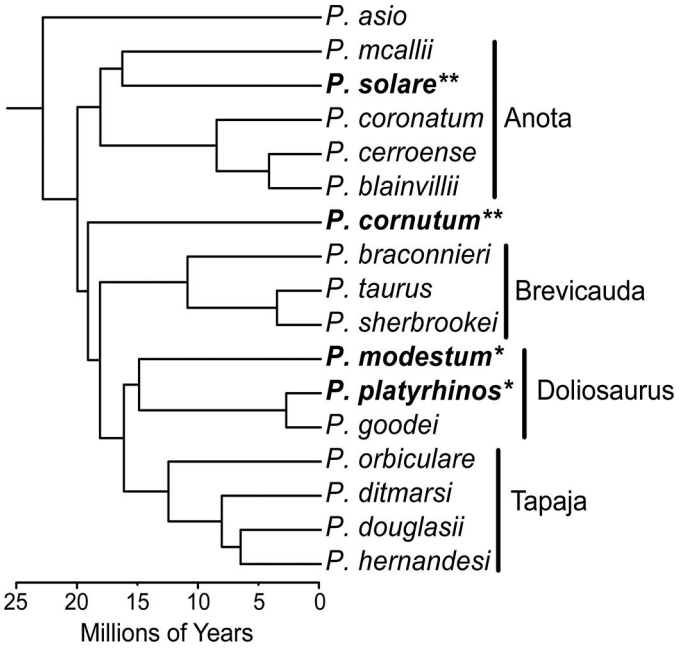

Fig. 1. Phylogeny of phrynosomatid species in the genus *Phynosoma* cladistically arranged in the four clades discussed. In bold are experimental blood-squirting and *Pogonomyrmex*-eating species *P. cornutum* and *P. solare* (with **) and non-blood-squirting and non-*Pogonomyrmex*-eating *P. modestum* and *P. platyrhinos* (with *). Modified from Leaché and Linkem (2015).

Canada to southern Mexico, occupying various arid habitats and mountain ranges. Within the family Phrynosomatidae, *Phrynosoma* is a sister taxon (Phrynosomatini) to Callisaurini (*Uma*, *Callisaurus*, *Cophosaurus*, and *Holbrookia*; Wiens et al., 2013; Leaché and Linkem, 2015).

Several reports, usually following isolated Human (*Homo sapiens*) encounters involving blood-squirting, led to diverse speculation on the biological use of this behavior (Middendorf and Sherbrooke, 1992) and to recordings of frequency of occurrences (4.6%: Parker, 1971; 5.9%: Lambert and Ferguson, 1985). Later experiments, utilizing a Domestic Dog (*Canis familiaris*) as a canid-predator substitute for behavioral bioassays, demonstrated a high degree of elicitation (85%) of blood-squirting by Texas Horned Lizards, *Phrynosoma cornutum* (Middendorf and Sherbrooke, 1992). Other species throughout much of the genus were later identified as exhibiting blood-squirting (Sherbrooke and Middendorf, 2001). Its role in repelling predator attacks was then addressed in trials with native canids (Kit Fox, *Vulpes macrotis*, Sherbrooke and Middendorf, 2004; Coyote, *Canis latrans*, Sherbrooke and Mason, 2005) and a felid (Bobcat, *Lynx rufus*, Sherbrooke et al., 2012). Blood of *Phrynosoma cornutum* delivered to oral tissues of all three mammalian species elicited strong revulsion responses.

Pianka and Parker (1975) linked various aspects of horned lizard morphological, physiological, and behavioral evolution to a dietary specialization focused on ants, a rare dietary specialization in lizards (Schwenk, 2000; Pianka and Vitt, 2003; Rodda, 2020). Sherbrooke and Middendorf (2004) suggested that the source of an unknown chemical defensive compound(s) employed by horned lizards in antipredator blood-squirting may be derived from their ant diet, particularly when involving harvester ants of the genus *Pogonomyrmex*.

We began a series of experiments to determine the physical and chemical characteristics, and possible identity, of the repugnant compound(s) in the blood plasma of Texas Horned Lizards. Over several decades, we developed experiments utilizing bioassay-guided fractionation to produce "fractions" of blood plasma to be presented to coyotes, and later to mice, and recorded their responses. A major breakthrough for this research was finding that we could incorporate mice in bioassays to identify fractions containing the compound(s) of interest. This allowed us to reduce blood sample size needed for bioassays and to improve on the subjectivity in scoring coyote bioassay responses (gaping, licking, head-shaking) by using quantitative measures of mouse consumption of test liquids. Utilizing the newly created mouse bioassay model, we designed experiments to further isolate and characterize the active compound(s). In these experiments, we studied blood plasma from species of horned lizards with known blood-squirting (*P. cornutum* and *P. solare*) or "non-squirting" (*P. modestum* and *P. platyrhinos*) behaviors (Sherbrooke and Middendorf, 2001). In the latter two species, *Pogonomyrmex* are normally absent or of low frequency in their diets (Shaffer and Whitford, 1981; Newbold and MacMahon, 2009). In addition, mice were offered blood-plasma test solutions from *P. cornutum* that had either been maintained on a diet of *Pogonomyrmex* or a cricket diet.

First, we ask what characteristic of the compound(s) in horned lizard blood is distasteful to some mammalian predators, and whether we can isolate it in fractionated plasma samples for further experimentation. Second, we ask if the compound(s) isolated from plasma is present in species of horned lizards that consume *Pogonomyrmex* and those that do not. Next, we ask if blood plasma from individuals of *P. cornutum* known for blood-squirting behavior will retain that plasma compound(s), when placed on diets without *Pogonomyrmex*. Given the recognized role of myrmecophagy in the evolution of unusual morphological, physiological, and behavioral features of *Phrynosoma* (Pianka and Parker, 1975; Rodda, 2020), we ask if successful inclusion of a distinct genus of ants, *Pogonomyrmex* with powerful biting and abdominal venomous defenses, into their diet might have been key in their evolutionary success as a radiation of phrynosomatid lizards. Also, we ask if the incorporation of *Pogonomyrmex* into the diet of horned lizards might have occurred within the stem clade of the genus *Phrynosoma* from whence it led to the evolution of this outstandingly distinct radiation of blood-squirting saurian species in North America.

MATERIALS AND METHODS

Collection areas.—Adult *P. cornutum* and *P. modestum* were collected during 1999–2002, 2005–2006, and 2009–2010 in Hidalgo County, New Mexico and Cochise County, Arizona (Sherbrooke, 2002). *Phrynosoma solare* were collected (2005) in Pima County, Arizona, and *P. platyrhinos* were collected (2002) in Lyon and Mineral Counties, Nevada. Seed harvester ants, *Pogonomyrmex rugosus* and *Po. barbatus* (both species with venom of significant mouse LD_{50} lethality, 0.76 and 0.39 mg/kg, respectively; Schmidt, 2016, 2019), were collected from colonies in the San Simon Valley near Rodeo, New Mexico, and Portal, Arizona.

Sample preparation.—Blood plasma was collected from *P. cornutum* maintained on either harvester ant *Pogonomyrmex*

Table 1. Fraction IDs with size exclusion column elution volume ranges and molecular weight ranges represented in the coyote and mouse bioassays. Fraction C elicited repulsive behavior in coyote bioassays, while fraction E was avoided in mouse bioassays. Together, these results suggest the compound(s) responsible for the repulsive response has a molecular weight between 800 and 1,600.

Coyote bioassay			Mouse bioassay	
Fraction	Marker	Molecular weight range	Fraction	Marker
A (39–60 mL)	Cytochrome C	>5,000	D (45–60 mL)	Cytochrome C
B (60–75 mL)	None	~1,600	E (60–140 mL)	Coenzyme B12
C (75–135 mL)	None	~1,600		
		<800	F (140–280 mL)	Fast Green FCF

spp. or cricket (*Acheta domesticus*) diets. Diet treatment lizards were maintained in two separate open/screened units, 7 m (l) x 3.8 m (w) x 2.4 m (h), of the Animal Behavior Observatory at the Southwestern Research Station (Sherbrooke, 2008) and fed daily for 30 days (2002; coyote trials) or 16 days (2010; mouse trials). Sprayed water facilitated simulated rain-harvest drinking by lizards (Sherbrooke, 1990).

Blood plasma was obtained from adults of four species of *Phrynosoma*, two with known high frequency of blood-squirting behavior (*P. cornutum* and *P. solare*) and two considered non-squirting species (*P. modestum* and *P. platyrhinos*). Heparinized 1 ml syringes were used to collect systemic blood from heart ventricles of anesthetized lizards (Ultane/Sevoflurane, Abbott Laboratories, Chicago, Illinois). Lizard blood was centrifuged for plasma, which was frozen, and shipped on dry ice to the National Wildlife Research Center, Fort Collins, Colorado. Plasma samples were lyophilized and kept frozen until used for fractionation and/or bioassay experiments. Chemicals and column media were purchased from Sigma-Aldrich Corp. (Milwaukee, Wisconsin) unless otherwise specified. Given that no single chemical in plasma was identified as aversive (and a mix of several compounds may be required to elicit negative predator responses), we use the term "compound(s)" throughout our discussions.

In order to explore the hypothesis that the source of the compound(s) being studied is in the venom-producing abdomens of ants in *Pogonomyrmex*, approximately 1,500 individual harvester ants were bisected (head/mesosoma and abdomen) with a scalpel and pooled by body component into two 50 mL glass screw-top culture tubes. Abdomens were extracted with 10.0 mL of 50 mM Tris buffer and blended with a high shear homogenizer. The homogenizer probe was rinsed with 50 mM Tris buffer and rinses were combined with the extract. The extract was filtered prior to removal of lipophilic components by liquid/liquid extraction with 20.0 mL hexane. The heads/mesosoma were similarly extracted with 15.0 mL of 50 mM Tris buffer. The final volume of extract was 20.0 mL for both samples.

Size exclusion chromatography (SEC) fractionation.—SEC is a preparative method that separates components of a mixture according to molecular weight (mw). High mw compounds pass quickly through the column and elute sooner, whereas lower mw compounds will be retained and elute later. A 30 mm (id) glass column (320 mm length) was gravity packed with 25.0 g Sephadex G-25 column media using a 50 mM Tris buffer mobile phase for each experiment. The upper mw range of Sephadex G-25 is ~5,000. Each column was calibrated with colored molecular weight markers in 50 mM Tris buffer using gravity elution.

For the coyote bioassays (Table 1), one marker (horse heart Cytochrome C; mw ~12,400) was used to guide collection of three fractions: 39–60 mL reflective of the Cytochrome C band (Fraction A), 60–75 mL (Fraction B), and 75–135 mL (Fraction C). The volume ranges for fractions B and C were estimated (not based on elution of known markers). Following calibration with Cytochrome C, the column was washed with an additional 150 mL mobile phase prior to loading samples diluted in mobile phase (typically 500 mg in 4–5 mL). Collected fractions (and 150 mL samples of Tris mobile phase) were lyophilized and maintained frozen until reconstituted for bioassay.

Additional markers were used to refine fraction collection for the mouse bioassay (Table 1). These markers included: horse heart Cytochrome C (mw ~12,400), Coenzyme B12 (mw = 1,580), and Fast green FCF (mw = 809). Elution volumes were 45–60 mL for mw greater than ~5,000 (Fraction D) as evidenced by the Cytochrome C band; 60–140 mL for mw ~1,600 (Fraction E) corresponding to the Coenzyme B12 band; and 140–280 mL for mw less than ~800 (Fraction F) as demonstrated by the Fast green FCF band. Effectively, fractions A and D represented the same high molecular weight (>5,000) fraction in the two bioassays (Table 1). Improved calibration for the mouse bioassay demonstrated that fractions B and C encompassed the range of mid-molecular weight compounds near 1,600 evident in the fraction E band (Coenzyme B12) and that the low molecular weight range (<800) had not been properly represented in the coyote bioassay (Table 1). Lyophilized plasma or extracts (500 mg) were reconstituted in five mL of 50 mM Tris buffer for loading onto the column and elution with 50 mM Tris buffer. Recovered fractions were lyophilized and maintained frozen until reconstituted for bioassay.

Hydrophobic interaction chromatography (HIC).—HIC relies on differences in hydrophobicity among the component mixture and is commonly used to separate mixtures of proteins. A gravity column (30 x 320 mm) was prepared with Toyopearl Phenyl 650M (Sigma-Aldrich, St. Louis, Missouri) using 2 M ammonium acetate. A 2.5 g sample of SEC Fraction E was loaded onto the column and eluted sequentially with 150 mL 2 M ammonium sulfate (yielding HIC Fraction A),

Table 2. Bioactivity results (none, trace, minor, or strong) for coyote bioassays (Experiments 1–3) utilizing the following samples: intact plasma (1), SEC fractions (2–12), buffer salts (13), SEC and HIC fractions (14–15), and salts (16) of *Phrynosoma cornutum* blood plasma *and P. platyrhinos* plasma (17), as well as coyote bioassays of intact plasma from *P. cornutum* on diets with or without *Pogonomyrmex* ants (18–19).

Exp	ID	Sample	Fraction or extract	Bioactivity
1	1	*P. cornutum* plasma	Intact plasma	Strong
1	2	*P. cornutum* plasma	A; >5,000 mw	Minor
1	3	*P. cornutum* plasma	B; ~1,600 mw	Trace
1	4	*P. cornutum* plasma	C; 800–1,600 mw	Strong
1	5	*Pogonomyrmex* abdomen	Intact extract	Minor
1	6	*Pogonomyrmex* abdomen	A; >5,000 mw	Trace
1	7	*Pogonomyrmex* abdomen	B; ~1,600 mw	Minor
1	8	*Pogonomyrmex* abdomen	C; 800–1,600 mw	Minor
1	9	*Pogonomyrmex* head and mesosoma	Intact extract	None
1	10	*Pogonomyrmex* head and mesosoma	A; >5,000 mw	None
1	11	*Pogonomyrmex* head and mesosoma	B; ~1,600 mw	None
1	12	*Pogonomyrmex* head and mesosoma	C; 800–1,600 mw	Trace
1	13	Tris mobile phase	Buffer salts	None
2	14	*P. cornutum* plasma	SEC fraction E	Strong
2	15	*P. cornutum* plasma	HIC fraction C	Minor
2	16	Ammonium sulfate mobile phase	Salts	Trace
3	17	*P. platyrhinos* plasma	Intact plasma	Trace
3	18	*P. cornutum* intact plasma	Diet, ants	Strong
3	19	*P. cornutum* intact plasma	Diet, no ants	Trace

150 mL 1 M ammonium sulfate (HIC Fraction B), and 150 mL water (HIC Fraction C). Owing to the very high salt concentrations of the first two fractions, an attempt was made to desalt by tangential flow filtration (diafiltration). Because desalting failed, only HIC Fraction C was lyophilized and retained for bioassay (Table 2). In addition, 150 mL of 1 M ammonium sulfate (control) was lyophilized and retained for bioassay.

Coyote bioassay testing procedures.—Test stimuli were presented orally to restrained coyotes as previously described (Sherbrooke and Mason, 2005). For each bioassay, five test subjects were selected from the captive colony of coyotes maintained at the USDA-APHIS-WS-NWRC predator ecology research facility in Millville, Utah. Briefly, 0.5 mL of a solution was loaded into a 1 mL plastic syringe (with no needle) and the full volume ejected into mouths of the test coyotes. Negative orofacial reactions noted upon release included gaping, licking, and headshaking, and were rated by the handlers as strong, minor, trace, or none (coyote experiments 1–3, Table 2).

Coyote bioassay of SEC plasma fractions obtained from* P. cornutum *and extracts from* Pogonomyrmex*. Experiment 1.—Three SEC fractions (A, B, and C) were prepared for each of three samples: *P. cornutum* plasma (3.4 mL), *Pogonomyrmex* abdomen extract (5.0 mL), and *Pogonomyrmex* head and mesosoma extract (5.0 mL). The original sample sources and resulting SEC fractions, as well as 40 mL Tris mobile phase, were lyophilized and subjected to bioassay with coyotes after reconstitution in 5.0 mL tap water (Tables 1 and 2).

Coyote bioassay with HIC plasma fractions obtained from* P. cornutum*. Experiment 2.—Samples of SEC Fraction E (56 mg), HIC Fraction C (56 mg), and ammonium sulfate control (56 mg) were each reconstituted in 2.5 mL tap water for bioassay with coyotes (Tables 1 and 2).

Coyote bioassays with plasma collected from* P. cornutum *and* P. platyrhinos*, and from* P. cornutum *on different diets, ants or no ants. Experiment 3.—Plasma samples collected from two species of *Phrynosoma*, *P. cornutum* and *P. platyrhinos*, and two dietary histories of one species (*P. cornutum*: diet of *Pogonomyrmex* or diet limited to crickets [*Acheta domesticus*] for 30 days) were presented to coyotes in bioassay. The plasma samples (Table 2), each reconstituted in 2.5 mL tap water, were as follows: 1) *P. cornutum* on ant diet (160 mg), 2) *P. cornutum* on cricket diet (160 mg), and 3) *P. platyrhinos* (160 mg).

Mouse bioassay testing procedures.—Outbred mice (*Mus musculus*; CF-1 strain) were employed for mouse bioassays. Subjects (3–5 week old) were initially purchased for the bioassays (Charles River Laboratories, Wilmington, Massachusetts). Subsequent subjects were bred in-house for additional bioassays. Eight mice were employed for each treatment group, and mice were used in only one bioassay and euthanized following completion of the test. Mice were individually housed in 29.7 cm (l) x 18.9 cm (w) x 12.8 cm (h) plastic cages with wire bar lids. When not employed in a bioassay, mice were provided *ad libitum* access to drinking water and rodent chow. During bioassays in light hours, subjects were water-restricted for seven hours prior to the test period, but water was returned immediately following the test period. Tests were conducted daily (for four consecutive days) for one hour commencing at 1500 hrs.

Test solution drinking apparatuses were fashioned from 1 mL syringe bodies and 30 mm stainless steel straight drinking tubes (no ball bearing tip). The opening of the syringe body was enlarged by cutting the tip off at the 0.0 cc indicator mark. The drinking tube was attached to the syringe body with a short (ca. 2 cm) length of Nalgene® food-grade tubing (¼″ i.d., ⅜″ o.d.).

Drinking solutions were prepared with 10.0 g sugar and 75 μL yellow food coloring in 1.0 L tap water. Fructose was used to prepare the drinking solution for pre-test training

(days 1–4) and evaluation of biases (e.g., favoring of a right- or left-side drinking tube). Sucrose was used to prepare the drinking solutions for exposure to test stimuli. Test stimuli were added to the sucrose drinking solution. The volume of solution removed from each drinking tube was recorded.

***Mouse bioassay of SEC plasma fractions obtained from* P. cornutum.**—Mouse responses to drinking tube position and three Tris buffer salt concentrations were assessed in the pre-test bioassay. Salt resulting from lyophilization of the mobile phase sample was used to prepare test solutions with the fructose drinking solution. Three salt solutions (corresponding to the mass of buffer salts arising from the three different fraction volumes) were prepared with 357 mg, 233 mg, and 65 mg salt in 100 mL fructose solution. For bioassay, positions (right, left, center) of the salt solutions were randomly assigned each day. Subjects exhibiting positional biases were removed from the study.

Following assessment of positional biases using fructose solutions, test solutions were prepared with 382.7 mg of Fraction D, 280.3 mg of Fraction E, or 161.6 mg of Fraction F (accounting for the contributions of the buffer salts) in 100 mL of the sucrose solution and delivered to mice in three-choice tests of fractions D, E, and F (Table 1).

Mouse bioassays with plasma from four lizard species.—Lyophilized plasma samples were reconstituted in sucrose drinking solution (ca. 200 mg plasma in 100 mL of sucrose solution) for testing in a series of two-choice tests. A new panel of eight mice was used for each comparison: *P. cornutum* versus *P. platyrhinos* (201.7 mg vs. 200.4 mg, respectively), *P. cornutum* versus *P. modestum* (168.6 mg vs. 169.5 mg, respectively), and *P. cornutum* versus *P. solare* (198.4 mg vs. 196.4 mg, respectively).

Mouse bioassays with plasma collected from* P. cornutum *on different diets, ants or no ants.—Test solutions were prepared with plasma collected from *P. cornutum* subjects maintained in captivity (16 days) on either a cricket (*Acheta domesticus*) diet (209.8 mg plasma in 100 mL sucrose solution) or harvester-ant (*Pogonomyrmex*) diet (208.8 mg plasma in 100 mL sucrose solution) and presented to mice in a two-choice test.

Statistical analyses of mouse bioassays.—Intake data were analyzed as analyses of variance (ANOVA) with period (pre-test, test), subject, drinking tube position (left, right, or center when three tubes were used), and treatment considered as fixed effects. Day was a covariate. For all bioassays, testing biases (e.g., subjects having a drinking position bias) were examined by ANOVA using intake data from both the pre-test and testing periods. Subject, period, position, day, and all interactions were included in the model.

Intake data from only the test period were then subjected to ANOVA with subject, treatment, position, day, and all two-way interactions included in the model. An identical model was also used to determine if there was a pre-test buffer concentration bias in the bioassay using SEC fractions.

RESULTS

Coyote bioassay of SEC plasma fractions obtained from* P. cornutum *and extracts from* Pogonomyrmex. *Experiment 1.—Results of bioassays with coyotes indicate that the active compound(s) responsible for the behaviors observed is present in plasma, but does not appear to be present in intact venom of *Pogonomyrmex*. Bioassay samples #1 (intact plasma) and #4 (fraction C) of *P. cornutum* were highly active and elicited revulsion behaviors in coyotes (Table 2). This indicates that the active compound(s) found in plasma have a mw less than 5,000. Some behavioral responses occurred with each harvester ant abdomen sample (#5—licking; #6—one subject gaped; #7—some licking; #8—some licking) and head and mesosoma sample #12 (unquantified weak response).

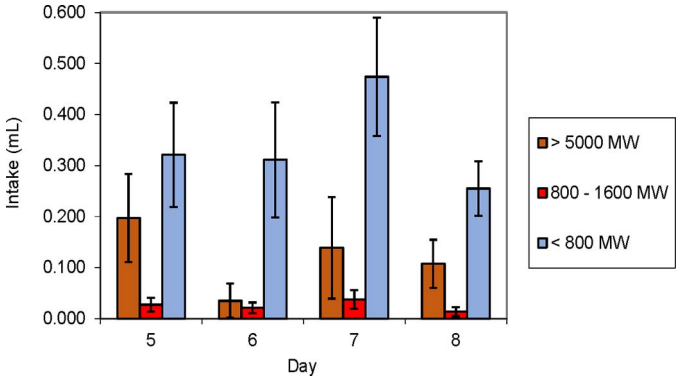

Fig. 2. Mouse bioassay (n = 24, eight per fraction) comparisons of three Texas Horned Lizards (*Phrynosoma cornutum*) plasma size-exclusion chromatography fractions. The fraction representing molecular weight between 800 and1,600 was significantly avoided ($P < 0.0001$).

Coyote bioassay with HIC plasma fractions from* P. cornutum. *Experiment 2.—Similar to experiment 1, SEC Fraction C (Table 2, #4) again elicited a strong repulsive response in coyotes. Coyote response to the HIC Fraction C (Table 2, #15) was minor, but clear. The ammonium sulfate control (Table 2, #16) only elicited a trace response. Bioactivity observed from HIC fraction C suggests that the active compound(s) is significantly hydrophobic.

Coyote bioassays with plasma collected from* P. cornutum *and* P. platyrhinos*, and from* P. cornutum *on different diets, ants or no ants. Experiment 3.—Only one subject had any response to plasma from *P. platyrhinos*, and the bioactivity ranked as trace (Table 2, #17). Plasma collected from *P. cornutum* on a diet of *Pogonomyrmex* elicited a strong repulsive response in the coyote subjects (Table 2, #18). Less activity, trace, was observed from delivery of plasma from *P. cornutum* on a cricket diet (Table 2, #19).

***Mouse bioassay of SEC plasma fractions obtained from* P. cornutum.**—Neither drinking tube position nor Tris buffer salt concentration influenced intake in the pre-test period. During the test period, molecular weight range of the plasma fraction had a significant impact on intake ($P = 0.0044$). Fraction E (representing ~1,600 mw) was avoided to the greatest extent (Fig. 2). Total fluid intake did not vary between the pre-test and test periods. These results, when compared with coyote responses to fraction C, suggest that the active compound(s) has mw greater than 800 and less than 1,600 (Table 1).

Mouse bioassays with plasma from four lizard species.—For trials with *P. platyrhinos*, evaluation of pre-test data indicated no significant position, day, or subject effects. During the test period, solutions of plasma from *P. cornutum* were strongly

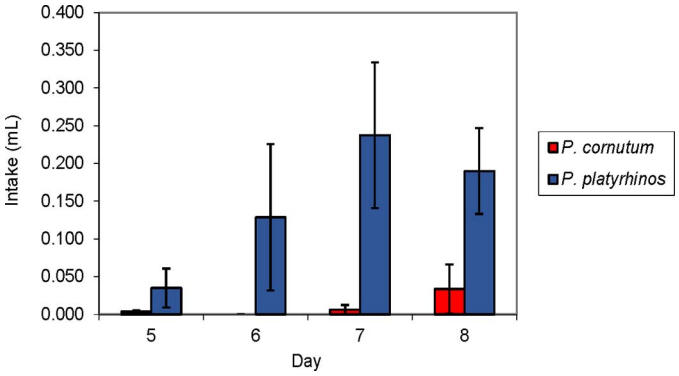

Fig. 3. Mouse bioassay ($n = 16$, eight per species) consumption for plasma solutions from two horned lizard species in pair-wise test, illustrating significant avoidance of *Phrynosoma cornutum* plasma in comparison to *P. platyrhinos* plasma ($P = 0.0004$).

avoided versus solutions from *P. platyrhinos* ($P = 0.0004$; Fig. 3). No other effects were significant.

Similar to the prior test with *P. platyrhinos*, no position, day, or subject biases were noted during trials with *P. modestum* plasma (model $P = 0.9276$). Pre-test intake (0.20 mL) and test period intake (0.17 mL) also did not differ ($P = 0.4251$). During the test period, plasma solutions from *P. modestum* were preferred versus solutions from *P. cornutum* ($P < 0.0001$; Fig. 4). Because intakes of the *P. modestum* solution varied significantly among subjects, there was also a significant subject*treatment interaction ($P < 0.0001$).

Day*position ($P = 0.0331$) and day*position*period ($P = 0.0226$) biases were noted for trials with *P. solare*. During the pre-test period, intake was greatest on day 3 and reduced on day 4. A left position bias was also evident on day 1. There were no significant effects during the test period (model $P = 0.8235$), indicating that consumption of solutions prepared with plasmas from *P. cornutum* and *P. solare* did not differ during the test period (Fig. 5). Further indicative of testing with two strongly avoided solutions, intake was significantly lower during the test period (0.016 mL) versus the pre-test period (0.19 mL; $P < 0.0001$).

Taken together, results from these pair-wise comparisons suggest that avoidance is correlated with blood-squirting behavior known for each species. Specifically, avoidance

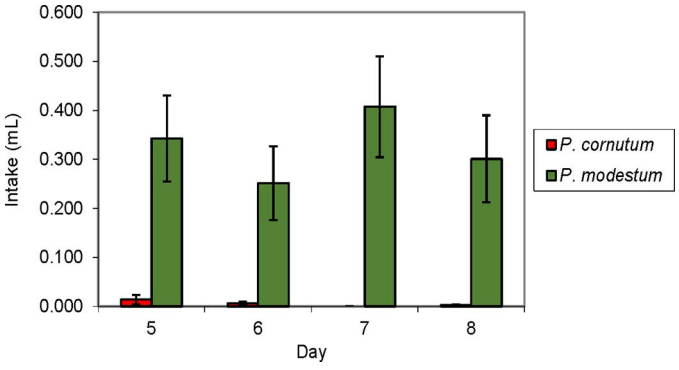

Fig. 4. Mouse bioassay ($n = 16$, eight per species) consumption for plasma solutions from two horned lizard species in pair-wise test, illustrating significant avoidance of *Phrynosoma cornutum* plasma in comparison to *P. modestum* plasma ($P < 0.0001$).

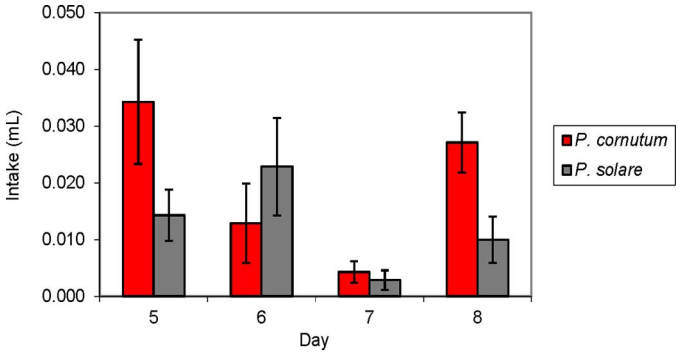

Fig. 5. Mouse bioassay ($n = 16$, eight per species) consumption for plasma solutions from two horned lizard species in pair-wise test, illustrating no difference in intakes of *Phrynosoma cornutum* plasma and *P. solare* plasma ($P = 0.8235$). However, the significant decrease in intake of both solutions versus the pre-test period (0.19 mL) indicates that both were avoided by mice ($P < 0.0001$).

behavior was observed for plasma from the two species known to squirt blood (*P. cornutum* and *P. solare*), while no avoidance was observed to plasma from non-blood-squirting species (*P. modestum* and *P. platyrhinos*).

Mouse bioassays with plasma collected from* P. cornutum *on different diets, ants or no ants.—During the pre-test period, intake differed among subjects ($P = 0.0106$). Pre-test (0.21 mL) intake was greater than test (0.013 mL) intake ($P < 0.0001$). Although consumption of the cricket-diet derived solution (0.023) was higher than the ant-diet derived solution (0.0034 mL), the test period ANOVA was not significant (model $P = 0.303$). Though there was no treatment effect, one subject consumed significant volumes of the cricket-diet solution on days 7 and 8 (Fig. 6).

DISCUSSION

Coyote bioassays summary.—Coyote bioassays (Table 2) show that plasma of *Phrynosoma cornutum* elicits the same repulsive oral responses previously demonstrated in canids and felids with whole blood (Sherbrooke and Middendorf, 2004;

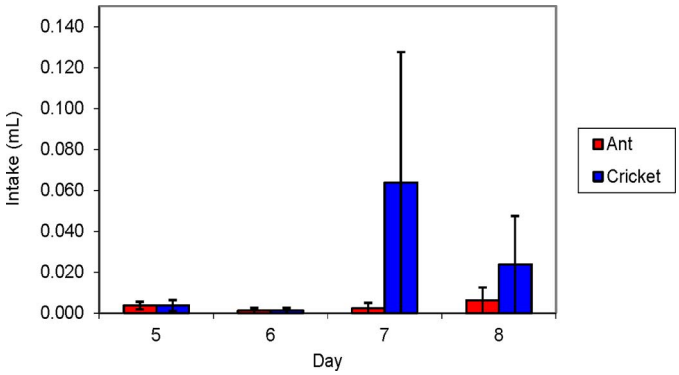

Fig. 6. Mouse bioassay ($n = 16$, eight per diet) consumption for plasma solutions from Texas Horned Lizards (*Phrynosoma cornutum*) maintained on diet of crickets only or diet of *Pogonomyrmex* spp. ants in pair-wise test, illustrating no difference in intake of the two plasma solutions ($P = 0.303$). However, the significant decrease in intake of both solutions versus the pre-test period (0.21 mL) indicates that both were avoided by mice ($P < 0.0001$).

Sherbrooke and Mason, 2005; Sherbrooke et al., 2012). This established that the active compound(s) is present in their plasma, supporting further plasma studies. We found that the active compound(s) has a mw of less than 5,000 (Table 2). The second finding indicated a somewhat stronger response by coyotes to the ant abdominal fraction than to the ant head + mesosoma fraction (Table 2). If activity in samples #1 (plasma) and #4 (plasma fraction C) resulted from sequestration of ant venom by *P. cornutum*, an analogous positive response would be expected in samples #5 (abdomen extract) and #8 (abdomen fraction C), which were minimal. This suggests either a concentration effect (the active ingredient in circulating blood is at a greater concentration than in the ant abdomen extract) or that intact venom present in the abdomen is not itself responsible for the repulsive response in coyotes. This suggests that some source of the active compound(s) might be harbored in the ant abdomen, perhaps at a much lower concentration than in the plasma of *P. cornutum*.

Further coyote bioassay results from plasma of *P. cornutum* placed on an ant-less diet illustrate that a diet including *Pogonomyrmex* is important to the presence of the compound(s) (Table 2). Also, they establish that the activity of the compound(s) is diminished when lizards were denied dietary access to these ants over a period of 30 days while on a cricket diet. In contrast (Table 2), the very low response by coyotes to blood plasma of *Phrynosoma platyrhinos*, a "non-squirting" species not usually feeding on *Pogonomyrmex*, suggests that this species' plasma contains little or no active compound(s).

Mouse bioassays summary.—In comparative mouse bioassays, mice displayed strong avoidance for the solution from *P. cornutum* but not from *P. platyrhinos* (Fig. 3), supporting the results of similar coyote bioassays. In both coyote and mouse bioassays, plasma from *P. cornutum* is repulsive (coyotes) or avoided (mice). This could be a result of dietary differences between the two species of horned lizards. *Phrynosoma platyrhinos* is reported to not be a harvester ant specialist (*Pogonomyrmex*), and non-ant prey may dominate its diet (Meyers and Herrel, 2005; Newbold and MacMahon, 2009), although certain populations may feed on *Pogonomyrmex* (Rissing, 1981; Hilsinger et al., 2011). In contrast, in many populations of *P. cornutum*, including those in these trials, diets are high in *Pogonomyrmex* spp. (Whitford and Bryant, 1979; Blackshear and Richerson, 1999; Eifler et al., 2012).

Similar to trials with *P. cornutum* and *P. platyrhinos*, the solution containing plasma of *P. modestum* is significantly more acceptable to mice than the solution containing plasma of *P. cornutum* (Fig. 4). The plasma solution from *P. modestum* may lack or contain only minor amounts of the active compound(s). The diet of *P. modestum* is very different from that of *P. cornutum* (Shaffer and Whitford, 1981), usually lacking *Pogonomyrmex* (Munger, 1984). *Phrynosoma modestum* is considered a "non-blood-squirting" species (Sherbrooke and Middendorf, 2001; Leaché and McGuire, 2006).

In sharp contrast to the *P. modestum* and *P. platyrhinos* trials, *P. solare* plasma solutions are highly avoided by mice. There is no significant difference in consumption compared to *P. cornutum* (Fig. 5). Similar rejection of samples from both suggests that blood plasma from *P. solare* contains the same active compound(s) as *P. cornutum*. Both species feed heavily on *Pogonomyrmex* (Eifler et al., 2012; Sullivan et al., 2014).

In mouse trials utilizing plasma from *P. cornutum* on either harvester ant or cricket diets there was no significant difference in the quantity of solution consumed by mice (Fig. 6). These results are not consistent with the coyote bioassay results for somewhat similar plasma samples, where coyotes appeared to react more negatively to plasma from lizards on an ant diet than on a cricket diet (Table 2, #19). The difference may reflect the potential for mice to develop a learned taste aversion to lower quantities of the active compound(s) during the 1 hr test. Conversely, learning was unlikely to occur from a single exposure in the brief access trials employed with coyotes. Learned taste aversions arise from a sequence of events that begin with the ingestion of a food item that causes malaise or nausea (Provenza, 1995). Though not required, aversions occur more readily with ingestion of unfamiliar (novel) food items. The flavor of the food serves as a cue, which facilitates avoidance of that specific food item when encountered on future occasions. Alternatively, the duration of ant removal from the diets of lizards in the coyote trials (30 days) versus in the mouse trials (16 days) may have resulted in the different responses in the two bioassays. If so, these differing results suggest that the circulating compound(s) in plasma of *P. cornutum* may require periodic replacement from dietary *Pogonomyrmex*, although the aversive compound(s) may remain in circulation for several days to weeks before losing potency. This suggests that in the shorter mouse trials, the compound(s) may have still been present in cricket-eating lizards. In support of that interpretation, we note that the consumption levels of solutions by mice on plasma from ant-eating or cricket-eating lizard diets was low (statistically similar to levels in *P. cornutum/P. solare*; Figs. 4, 5). These issues deserve further investigation as they may reflect the need for local availability of ants in the genus *Pogonomyrmex* for continuous or timely acquisition of the compound(s) utilized in blood-squirting defensive actions.

Mice proved to be excellent models for bioassays and yielded quantitative results suitable for statistical analyses, whereas behavioral measurements from test coyotes were subjective. Furthermore, care and use of mice is far easier and less costly as compared to a canid model such as coyotes. Importantly, results obtained from coyote and mouse bioassays were largely concordant, suggesting that the mouse bioassay can be an important tool for conducting bioassay-guided fractionation of *P. cornutum* plasma in future studies.

Chemical nature of* Phrynosoma *blood-plasma compound(s) eliciting negative responses.—Of the three molecular weight size exclusion plasma fractions of *P. cornutum* tested with mice, one (800 to 1,600 mw) was significantly avoided (Fig. 2). From our initial coyote and mouse trial bioassays and given the apparent molecular weight and hydrophobicity of the active compound(s), we suspect an alkaloid is not the active compound(s). If an alkaloid bound to a blood protein were responsible, the molecular weight of this complex would be exceedingly higher than 5,000 (Leeman et al., 2018) owing to the high mw of blood proteins e.g., myoglobin (17,000) and albumen (67,000). Likewise, unbound alkaloids (as free bases) have molecular weights ranging from less than 1,000 (Svatos, 2010).

Though it appears that harvester ant venom plays a role in bioactivity, it is unlikely to be a consequence of direct sequestration due to the high mw of venom. Harvester ant venom is composed of a mixture of six classes of macromolecular enzymes and low levels of free amino acids (Schmidt and Blum, 1978). These enzymes have been qualitatively identified in ant venoms but have not been well characterized. Much more is known about venom proteins in bee venoms (Schmidt, 1982). Phospholipases, present in large quantities in venom of *Pogonomyrmex badius*, have molecular weights of approximately 10,000 in bee venom (Schmidt, 1982). Hyaluronidases may range in molecular weight from 35,000 to 53,000 in bee venom. Acid phosphatase has a molecular weight of 49,000 in bee venom and is present in great quantities in *Po. badius* venom (Schmidt, 1982). The venom of some species of *Pogonomyrmex* also contains peptides with molecular weights less than 10,000, such as barbatolysin (ca. 3,300) and an 8,000 mw peptide that are present in *Po. barbatus* venom (Bernheimer et al., 1980). Rather than a *Pogonomyrmex* venom being sequestered directly into horned lizard blood, it is likely that the toxic enzymes and/or peptides from ant venom are metabolized by the lizards and the resulting active antipredator ingredient in circulating blood is a small peptide with a mw of approximately 800 to 1,600. The genus *Pogonomyrmex*, in the subfamily Myrmicinae, is implicated in having peptide venoms containing vertebrate-selective pain-causing sodium channel toxins that have contributed to their successful defenses against vertebrate predators (Robinson et al., 2023).

Contrasts with alkaloid dietary-sequestered poison-frog chemical defenses.—In contrast to *Phrynosoma* acquiring a defensive compound(s) that is not an alkaloid, various diet-acquired alkaloids are used by several groups of ant-eating anurans in their chemical defenses. Ants, millipedes, and other arthropods are the source of defensive alkaloids for aposematically colored frogs in the Neotropics, Dendrobatidae, and Madagascar, *Mantella* (Clark et al., 2005; Saporito et al., 2012). Several other frog groups also employ prey-acquired alkaloids against predators. These well-studied and independently evolved frog assemblages exemplify several aspects of convergent evolution of their prey-acquired defensive compounds. In contrast, horned lizards, despite also obtaining a defensive compound(s) from ants, differ in that their acquired compound(s) is not a directly ingested intact defensive chemical, is circulated in their blood which is defensively squirted, and is derived from ants of a single genus, *Pogonomyrmex*. Also strikingly distinct are their nearly opposite color and pattern features for avoiding predation, aposematic frogs versus cryptic lizards.

Incorporation of dietary ants, genus* Pogonomyrmex*, by* Phrynosoma *and its significance for the evolution of a blood-squirting defense.—The close evolutionary relationships between species of horned lizards and their ant prey have long been recognized. Pianka and Parker (1975) characterized these as a unique integrated constellation of anatomical, behavioral, physiological, and ecological adaptations that facilitate efficient exploitation of ants (termed the "*Phrynosoma* suite" by Powell, 2016), setting them distinctly apart from other phrynosomatids, and other lizards (Rodda, 2020). The Pianka and Parker (1975) integrated view features adaptations for enhancing an ant diet while feeding at sites of concentrated resources, such as colony surface entries and dispersal trails. Such adaptive features include modified teeth, a stomach for large prey capacity, broad and flattened body form, and wide temporal and thermal feeding-activity periods. Additional features that reduce predation while being exposed at feeding sites include color/pattern camouflage, immobility responses, and cranial horns.

An important early insight into the physiological linkage between species of *Phrynosoma* and *Pogonomyrmex* was the discovery of a blood plasma factor in *P. cornutum* that detoxifies the venom of *Pogonomyrmex* (Schmidt et al., 1989; Schmidt 2016, 2019). A similar resistance to venom of *Pogonomyrmex* is found in the blood-squirting species *P. hernandesi* and the "non-blood-squirting" *P. modestum* (Schmidt, 2019). Other species remain unstudied. *Sceloporus jarrovii*, in the sceloporine clade of the Phrynosomatidae, has very little resistance against venom of *Pogonomyrmex* (Schmidt and Schmidt, 1989; Schmidt et al., 1989). This suggests that the ability to detoxify venom of P*ogonomyrmex* spp. may have evolved in response to, or occurred with, incorporation of a diet of *Pogonomyrmex* in the stem clade of *Phrynosoma*. Perhaps the compound(s) responsible for predator aversion are metabolites of venom detoxification to protect the lizards from *Pogonomyrmex* venom, which then became incorporated in blood plasma and led to the evolution of a blood-squirting defense.

Not mentioned by Pianka and Parker (1975) was blood-squirting, which had not been suggested as a possible consequence of diet until recently (Sherbrooke and Middendorf, 2004). Nor was there mention of the lizard's resistance to the venom of *Pogonomyrmex*, of their blood containing a predator-repellant compound(s) derived from *Pogonomyrmex*, nor of adaptations for safely consuming *Pogonomyrmex*. These defensive and prey capture revelations clearly further relate to and significantly expand the integrated view of the ecology of *Phrynosoma* by tying it to a focus springing from its apparent natural selection acquisition of a diet incorporating ants of a very specific venomous genus, *Pogonomyrmex*. We suggest that the stem clade of *Phrynosoma* evolved and shifted its selective dietary regime with myrmecophagy of *Pogonomyrmex*, and this shift was followed by selection that helped enhance and expand this focused myrmecophagy. Few other vertebrate predators utilize *Pogonomyrmex* as prey (Schmidt and Schmidt, 1989), despite their frequent clumped abundance and diurnally exposed above-ground foraging in arid North American ecosystems (MacKay and MacKay, 1989; Uhey and Hofstetter, 2021). This was then coupled with an antipredator defensive compound(s) and a blood-squirt delivery system as a new broader defensive optimum.

Several apparent specializations of the genus *Phrynosoma* for a myrmecophagous diet that focus on *Pogonomyrmex* involved their abilities to overcome the ants' diverse defenses. During prey approach, lizards have defensive response behaviors for outmaneuvering socially organized *Pogonomyrmex* attacks (Rissing, 1981; Fertschai et al., 2021). Other defenses include hindering venomous stings and thwarting penetrating mandibular bites, both utilized during prey capture and ingestion. Prey capture is by tongue protrusion (typical of Iguania; Schwenk, 2000). But in contrast to many other lizards, ingestion proceeds without mastication (Schwenk, 2000; Meyers and Herrel, 2005; Fertschai et al., 2021). This rapid ant-prey capture is highly coordinated with millisecond directional adjustments of head and tongue while employing visual lens accommodation to judge and track prey distance and movements (Ott et al., 2004). Additionally, horned lizards combine

prey capture, transport, and swallowing into a single rapid feeding stage that keeps ant prey from contacting oral tissues (Schwenk, 2000, 2021; Meyers and Herrel, 2005; Fertschai et al., 2021). These derived feeding-kinematics are associated with unique mucus-secreting lingual, pharyngeal, and esophageal papillae that bind prey with copious mucus and thus immobilize potentially dangerous *Pogonomyrmex* (Sherbrooke and Schwenk, 2008; Fertschai et al., 2021; Schwenk, 2021). Feeding strikes at ants are very precise, their prehensile tongue's papillary cushion avoiding the ant's jaws and stinger (Fertschai et al., 2021). This facilitates rapid entry of inverted ants into mucus-enveloping coatings continuously applied during swallowing (Sherbrooke and Schwenk, 2008; Fertschai et al., 2021; Schwenk, 2021). This combination of unique prey capture characteristics, behavioral, morphological, and physiological, is a major evolutionary advancement that has allowed the integrated evolutionary relationships of *Phrynosoma* to evolve with a primary prey, *Pogonomyrmex*, exhibiting outstanding defenses that led to its own blood-squirting defense, as we have shown.

The close biological relationship between these two genera has been recognized and exploited by the unique two-host nematode life-cycle of *Skrjabinoptera phrynosoma* that is closely dependent on *Phrynosoma* eating *Pogonomyrmex*, and on *Pogonomyrmex* collecting expelled (with horned lizard fecal pellets) female nematodes containing many encysted capsules of eggs. These soon desiccated females are grasped by foraging *Pogonomyrmex* to be taken to their subterrain nests and fed to developing larval ants that become living carriers for cyst-emerged larval nematodes. Metamorphosed callow ants grow and later forage for seeds where they become potential meals for horned lizards, thus carrying their nematode load into the lizard's stomach where they reside, mature, and mate (Sherbrooke, 2003; Hilsinger et al., 2011; Flann, 2020). The evolution of this unique parasite life cycle has clearly arisen as a confirmatory biological evolutionary response to the close specific *Phrynosoma/Pogonomyrmex* evolved relationship that we report.

The presence of blood-flow restricting muscles encircling veins that carry blood from the head and capillary beds forming ocular sinuses adjacent to eyes are apparently a synapomorphy in Lepidosauria (Bruner, 1907). Bruner (1907) reported these muscular actions regulating cranial blood flow, when employed, serve as the physical and physiological base for blood-squirting in *Phrynosoma*. Within the family Phrynosomatidae, two closely related clades, Phrynosomatini and Callisaurini, are sister to the Sceloporinae which includes the genera *Urosaurus* and *Sceloporus* (Wiens et al., 2010). In that context, it is worth noting that *Sceloporus jarrovii* and *Urosaurus ornatus* have been reported to ooze blood during lassoing captures. This was attributed to ocular-sinus autohemorrhaging (Mahrt, 1996; Sherbrooke, 2000). Sherbrooke (2000) suggested that ocular-sinus bleeding during such cranially associated tactile trauma during predator attacks may have been an event(s) that led to the evolution of a blood-squirting defense in *Phrynosoma* carrying blood chemicals distasteful to some predators.

Within the genus *Phrynosoma*, the most parsimonious reconstruction of the evolutionary history of blood-squirting suggests that this character evolved once in the stem clade. Leaché and Linkem (2015) estimated divergence time for the stem group of *Phrynosoma* of up to 30 million years (mean = 24.4 Mya). They noted that *P. asio* is the sister clade of all other species, and that *P. cornutum* is the sister clade to three of four recognized clades. Weins et al. (2013) suggested that both *P. asio* and *P. cornutum* may be sister clades of the other species of *Phrynosoma*, having a stem of 36.3 Mya (Wiens et al., 2013; Powell, 2016). Both *P. asio* and *P. cornutum* are well recognized as blood-squirting species (Sherbrooke and Middendorf, 2001, 2004; Hodges, 2004). All known lineages of *Phrynosoma* are North American, whereas the origins of the genus *Pogonomyrmex* was in northwestern South America 60 million years ago, with a later expansion into North America, perhaps 30 million years ago (Taber, 1998; Ward et al., 2015). Subsequent widespread species radiations include ranges in Canada, the United States (particularly western) and Mexico. Broadly, that arrival timing would have placed them historically and geographically in places and habitats at a time frequented by the stem lineage of phrynosomatid lizards that led to *Phrynosoma* (Wiens et al., 2013; Leaché and Linkem, 2015).

We believe that our data from extant horned lizards suggest that the incorporation of a chemical compound(s) from the venom of *Pogonomyrmex* into the blood plasma of stem *Phrynosoma* was critical to their evolution of a unique antipredator blood-squirting defense. The inadvertent acquisition of this blood-borne compound(s) was probably an exaptation event (Gould and Vrba, 1982) that occurred during periods of adaptation for feeding on *Pogonomyrmex*. Acquisition of this compound(s) was further built upon by selective forces for it to be utilized with pre-existing, otherwise selectively adapted structures and behaviors. These included vascular sphincter muscles regulating cranial blood pressure for thermoregulation and ecdysis and ocular sinuses for lens cleaning (Bruner, 1907; Heath, 1964). New modifications of this system manifested as blood-squirting, an effective predator defense in *Phrynosoma*. Thus, our work explicitly expands and further contextualizes the "*Phrynosoma* suite" hypothesis (Pianka and Parker, 1975; Powell, 2016) so as to now include the incorporation of a widespread, abundant, and species-rich dietary taxon of well-defended ants (*Pogonomyrmex*) that also provide a compound(s) of defensive value to the lizards.

DATA ACCESSIBILITY

ACKNOWLEDGMENTS

For assistance with bio-assaying with coyotes and mice we thank R. Mason, D. Zemlicka, R. Mauldin, and J. Figueroa. Bioassays were conducted between July 1999 and November 2005 at USDA-APHIS-WS-National Wildlife Research Center facilities as approved by the NWRC Institutional Animal Care and Use Committee (protocols QA-724 and QA-1239). We thank volunteers, researchers, and staff at the Southwestern Research Station for assistance with collection of horned lizards. The abstract translation to Spanish was graciously provided by T. V. Gutiérrez-Santillán. A. D. Leaché provided the cladogram for Figure 1. Helpful suggestions on an early version

of the manuscript from Gary Beauchamp were appreciated. Scientific collecting permits for lizards were obtained from the New Mexico Department of Game and Fish (No. 1149) and the Arizona Game and Fish Department (ID A01124495). S. Ferrand provided *P. platyrhinos* (Nevada Permit 521673). Lizard care and blood collection procedures adhered to protocols, American Museum of Natural History, Institutional Animal Care and Use Committee.

LITERATURE CITED

Baur, B., and R. R. Montanucci. 1998. Krötenechsen: Lebensweise, Pflege, Zucht. Herpeton, Offenback, Germany.

Bernheimer, A. W., M. S. Avigad, and J. O. Schmidt. 1980. A hemolytic polypeptide from the venom of the red harvester ant, *Pogonomyrmex barbatus*. Toxicon 18:271–278.

Blackshear, S. D., and J. V. Richerson. 1999. Ant diet of the Texas horned lizard (*Phrynosoma cornutum*) from the Chihuahuan Desert. Texas Journal of Science 51:147–152.

Bruner, H. L. 1907. On the cephalic veins and sinuses of reptiles, with description of a mechanism for raising the venous blood pressure in the head. American Journal of Anatomy 7:1–117.

Clark, V. C., C. J. Raxworthy, V. Rakotomalala, P. Sierwald, and B. L. Fisher. 2005. Convergent evolution of chemical defense in poison frogs and arthropod prey between Madagascar and the Neotropics. Proceedings of the National Academy of Sciences of the United States of America 102:11617–11622.

Eifler, D. A., M. A. Eifler, and T. K. Brown. 2012. Habitat selection by foraging Texas horned lizards, *Phrynosoma cornutum*. Southwestern Naturalist 57:39–43.

Fertschai, I., W. C. Sherbrooke, M. Ott, and B. P. Chagnaud. 2021. Avoiding being stung or bitten—prey capture behavior of the Texas horned lizard, *Phrynosoma cornutum*. Biology Open 10:bio058453.

Flann, H. 2020. Effect of a coevolved parasitic nematode on fitness of the desert horned lizard (*Phrynosoma platyrhinos*). Unpubl. M.S. thesis, Western Washington University, Cedar, Washington.

Gould, S. J., and E. S. Vrba. 1982. Exaptation—a missing term in the science of form. Paleobiology 8:4–15.

Heath, J. E. 1964. Head–body temperature differences in horned lizards. Physiological Zoology 37:273–279.

Hilsinger, K. C., R. A. Anderson, and D. Nayduch. 2011. Seasonal dynamics of *Skrjabinoptera phrynosoma* (Nematoda) infection in horned lizards from the Alvord Basin: temporal components of a unique life cycle. Journal of Parasitology 97:559–564.

Hodges, W. L. 2004. Defensive blood squirting in *Phrynosoma ditmarsi* and a high rate of human-induced blood squirting in *Phrynosoma asio*. Southwestern Naturalist 48:267–270.

Lambert, S., and G. M. Ferguson. 1985. Blood ejection frequency by *Phrynosoma cornutum* (Iguanidae). Southwestern Naturalist 30:616–617.

Leaché, A. D., H. R. Davis, S. Singhal, M. K. Fujita, M. E. Lahti, and K. R. Zamudio. 2021. Phylogenomic assessment of biodiversity using a reference-based taxonomy: an example with horned lizards (*Phrynosoma*). Frontiers in Ecology and Evolution 9:678110.

Leaché, A. D., and C. W. Linkem. 2015. Phylogenomics of horned lizards (genus *Phrynosoma*) using targeted sequence capture data. Copeia 103:586–594.

Leaché, A. D., and J. McGuire. 2006. Phylogenetic relationships of horned lizards (*Phrynosoma*) based on nuclear and mitochondrial data: evidence for a misleading mitochondrial gene tree. Molecular Phylogenetics and Evolution 39:628–644.

Leeman, M., J. Choi, S. Hansson, M. U. Storm, and L. Nilsson. 2018. Proteins and antibodies in serum, plasma, and whole blood—size characterization using asymmetrical flow field-flow fractionation (AF4). Analytical and Bioanalytical Chemistry 410:4867–4873.

MacKay, W. P., and E. E. MacKay. 1989. Diurnal foraging patterns of *Pogonomyrmex* harvester ants (Hymenoptera: Formicidae). Southwestern Naturalist 34:213–218.

Mahrt, L. A. 1996. *Urosaurus ornatus* (Tree Lizard). Autohemorrhaging. Herpetological Review 27:21–22.

Meyers, J. J., and A. Herrel. 2005. Prey capture kinematics of ant-eating lizards. Journal of Experimental Biology 208:113–127.

Middendorf, G. A., III, and W. C. Sherbrooke. 1992. Canid elicitation of blood-squirting in a horned lizard (*Phrynosoma cornutum*). Copeia 1992:519–527.

Munger, J. C. 1984. Optimal foraging? Patch use by horned lizards (Iguanidae: *Phrynosoma*). American Naturalist 123:654–680.

Newbold, T. A. S., and J. A. MacMahon. 2009. Spatial and seasonal dietary patterns of the desert horned lizard (*Phrynosoma platyrhinos*): harvester ant specialist or generalist ant feeder? Canadian Journal of Science 87:112–123.

Ott, M., J. Ostheim, and W. C. Sherbrooke. 2004. Prey snapping and visual distance estimation in Texas horned lizards, *Phrynosoma cornutum*. Journal of Experimental Biology 207:3067–3072.

Parker, W. S. 1971. Ecological observations on the regal horned lizard (*Phrynosoma solare*) in Arizona. Herpetologica 27:333–338.

Pianka, E. R., and W. S. Parker. 1975. Ecology of horned lizards: a review with special reference to *Phrynosoma platyrhinos*. Copeia 1975:141–162.

Pianka, E. R., and L. J. Vitt. 2003. Lizards: Windows to the Evolution of Diversity. University of California Press, Berkeley, California.

Powell, G. L. 2016. Evolution of dermatocranial shape in horned lizards (*Phrynosoma*). Unpubl. Ph.D. diss., University of Calgary, Calgary, Alberta, Canada.

Powell, G. L., and A. P. Russell. 2024. The veritable horns of a dilemma: assessing homology of the parietal and squamosal asperities of *Phrynosoma* (Squamata: Iguania: Phrynosomatidae). Acta Zoologica 105:57–80.

Provenza, F. D. 1995. Tracking variable environments—there is more than one kind of memory. Journal of Chemical Ecology 21:911–923.

Rissing, S. W. 1981. Prey preferences in the desert horned lizard: influence of prey foraging method and aggressive behavior. Ecology 62:1031–1040.

Robinson, S. D., J. R. Deuis, A. Touchard, A. Keramidas, A. Mueller, C. I. Schroeder, V. Barassé, A. A. Walker, N. Brinkwirth, S. Jami, E. Bonnafé, M. Treilhou, E. A. B. Undheim, J. O. Schmidt . . . **I. Vetter.** 2023. Ant venoms contain vertebrate-selective pain-causing sodium channel toxins. Nature Communications 14:2977.

Rodda, G. H. 2020. Lizards of the World: Natural History and Taxon Accounts. Johns Hopkins University Press, Baltimore, Maryland.

Saporito, R. A., M. A. Donnelly, T. F. Spand, and H. M. Garraffo. 2012. A review of chemical ecology in poison frogs. Chemoecology 22:159–168.

Schmidt, J. O. 1982. Biochemistry of insect venoms. Annual Review of Entomology 27:339–368.

Schmidt, J. O. 2016. The Sting of the Wild. John Hopkins University Press, Baltimore, Maryland.

Schmidt, J. O. 2019. Predator–prey battle of ecological icons: horned lizards (*Phrynosoma* spp.) and harvester ants (*Pogonomyrmex* spp.). Copeia 107:404–410.

Schmidt, J. O., and M. S. Blum. 1978. A harvester ant venom: chemistry and pharmacology. Science 200:1064–1068.

Schmidt, P. J., and J. O. Schmidt. 1989. Harvester ants and horned lizards predator–prey interactions, p. 25–51. *In*: Special Biotic Relationships in the Arid Southwest. J. O. Schmidt (ed.). University of New Mexico Press, Albuquerque, New Mexico.

Schmidt, P. J., W. C. Sherbrooke, and J. O. Schmidt. 1989. The detoxification of ant (*Pogonomyrmex*) venom by a blood factor in horned lizards (*Phrynosoma*). Copeia 1989:603–607.

Schwenk, K. 2000. Feeding in lepidosaurs, p. 175–291. *In*: Feeding: Form, Function, and Evolution in Tetrapod Vertebrates. K. Schwenk (ed.). Academic Press, San Diego, California.

Schwenk, K. 2021. Tongue morphology in horned lizards (Phrynosomatidae: *Phrynosoma*) and its relationship to specialized feeding and diet. Journal of Russian Herpetology 28:309–317.

Shaffer, D. T., and W. G. Whitford. 1981. Behavioral responses of a predator, the round-tailed horned lizard, *Phrynosoma modestum* and its prey, honey pot ants, *Myrmecocystus* spp. American Midland Naturalist 105:209–216.

Sherbrooke, W. C. 1990. Rain-harvesting in a lizard, *Phrynosoma cornutum*: behavior and integumental morphology. Journal of Herpetology 24:302–308.

Sherbrooke, W. C. 2000. *Sceloporus jarrovii* (Yarrow's Spiny Lizard). Ocular sinus bleeding. Herpetological Review 31:243.

Sherbrooke, W. C. 2002. Seasonally skewed sex-ratios of road-collected Texas horned lizards (*Phrynosoma cornutum*). Herpetological Review 33:21–24.

Sherbrooke, W. C. 2003. Introduction to Horned Lizards of North America. University of California Press, Berkeley, California.

Sherbrooke, W. C. 2008. Antipredator responses by Texas horned lizards to two snake taxa with different foraging and subjugation strategies. Journal of Herpetology 42:145–152.

Sherbrooke, W. C., and J. R. Mason. 2005. Sensory modality used by coyotes in response to antipredator compounds in the blood of Texas horned lizards. Southwestern Naturalist 50:216–222.

Sherbrooke, W. C., and G. A. Middendorf III. 2001. Blood-squirting variability in horned lizards (*Phrynosoma*). Copeia 2001:1114–1122.

Sherbrooke, W. C., and G. A. Middendorf III. 2004. Responses of kit foxes (*Vulpes macrotis*) to antipredator blood-squirting and blood of Texas horned lizards (*Phrynosoma cornutum*). Copeia 2004:652–658.

Sherbrooke, W. C., A. Mitchell, K. Sweet, L. Searles, and D. Braastad. 2012. Negative oral responses of a non-canid mammalian predator (bobcat, *Lynx rufus*; Felidae) to ocular-sinus blood-squirting of Texas and regal horned Lizards, *Phrynosoma cornutum* and *Phrynosoma solare*. Herpetological Review 43:386–391.

Sherbrooke, W. C., and K. Schwenk. 2008. Horned lizards (*Phrynosoma*) incapacitate dangerous ant prey with mucus. Journal of Experimental Zoology 309A:447–459.

Sullivan, B. K., K. O. Sullivan, D. Vardukyan, and T. Suminski. 2014. Persistence of horned lizards (*Phrynosoma* spp.) in urban preserves of central Arizona. Urban Ecosystems 17:707–717.

Svatos, A. 2010. Mass spectrometric imaging of small molecules. Trends in Biotechnology 28:425–434.

Taber, S. W. 1998. The World of the Harvester Ants. Texas A&M University Press, College Station, Texas.

Uhey, D. A., and R. W. Hofstetter. 2021. From pests to keystone species: ecosystem influences and human perceptions of harvester ants (*Pogonomyrmex*, *Veromessor*, and *Messor* spp.). Annals of the Entomological Society of America 115:127–140.

Ward, P. S., S. G. Brady, B. L. Fisher, and T. R. Schultz. 2015. The evolution of myrmicine ants: phylogeny and biogeography of a hyperdiverse ant clade (Hymenoptera: Formicidae). Systematic Entomology 40:61–81.

Whitford, W. G., and M. Bryant. 1979. Behavior of a predator and its prey: the horned lizard (*Phrynosoma cornutum*) and harvester ants (*Pogonomyrmex* spp.). Ecology 60:686–694.

Wiens, J. J., K. H. Kozak, and N. Silva. 2013. Diversity and niche evolution along aridity gradients in North American lizards (Phrynosomatidae). Evolution 67:1715–1728.

Wiens, J. J., C. A. Kruzynski, S. Arif, and T. W. Reeder. 2010. Phylogenetic relationships of phrynosomatid lizards based on nuclear and mitochondrial data, and a revised phylogeny for *Sceloporus*. Molecular Phylogenetics and Evolution 54:150–161.