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Characterization of antibacterial activities of hemolymph from the desert hairy scorpion, *Hadrurus arizonensis*

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Abstract. Treatment of bacterial cultures with hemolymph collected from desert hairy scorpions (*Hadrurus arizonensis* Ewing, 1928, Hadruridae) resulted in a time- and concentration-dependent inhibition of bacterial proliferation. The hemolymph proved effective in inhibiting growth of both Gram-negative and Gram-positive bacterial species. Incubation of *E. coli* bacteria with hemolymph at different temperatures (20–40°C) showed that the antibacterial effects increased from 20–30°C, but the hemolymph was largely ineffective in killing the bacteria at 35 and 40°C. Incubation of *E. coli* with hemolymph at 25°C for different time periods revealed that the antibacterial activities were extremely rapid and resulted in killing of bacteria within 1–2 minutes of contact. Interestingly, the hemolymph exhibited no phenoloxidase enzyme activity, hemolytic activity against sheep red blood cells, or melanization activity, which is a common mechanism of immunity among many diverse arthropods. This study is the first characterization of immune function of hemolymph from any scorpion species.

Keywords: Arachnid, arthropod, immunology, innate immunity

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Multicellular organisms invoke a variety of mechanisms to prevent infiltration and colonization of potentially infectious microbes. Invertebrates display innate immune defensive strategies to respond to infection, the mechanisms of which are typically very rapid but relatively nonspecific in action. For instance, some invertebrates, in a process called melanization, produce melanin to encase and neutralize potential internal immunological threats, thus encapsulating and sealing them off from the tissues of the host organism (Gotz 1986). As part of their arsenal against invading microbes, invertebrates also utilize molecular pattern recognition to detect the presence of prokaryotic organisms. For example, many invertebrates produce lectins that recognize and bind oligosaccharides that are displayed on the surface of microbes (Yeaton 1981). Most invertebrates also produce antimicrobial peptides (AMPs) that exhibit surface activity to compromise the outer membranes of microbes to induce leakage of cellular contents and eventual lysis (Tassanakajon et al. 2015). Furthermore, invertebrates also express genes that code for toll-like receptors (TLRs). These proteins recognize common microbial molecular patterns and initiate immune responses after binding to proteins, DNA, or RNA that are specific to microbes (Hopkins & Sriskandan 2005; Coscia et al. 2011; Brennan & Gilmore 2018).

Scorpions are an ancient group of invertebrates that were present at least 437 mya during the Silurian period (Wendruff et al. 2020), and are thought to be the first animals to move from the aquatic environments of oceans to inhabit terrestrial landscapes (Dunlop &Webster 1999; Dunlop et al. 2013). There are approximately 2400 species of extant scorpions (Howard et al. 2019), all of which are members of the Order Scorpiones. They inhabit virtually all terrestrial environments and can be found on all major land masses except for New Zealand and Antarctica (Lourenço 2001).

Due to the diversity of environments in which scorpions exist, it would follow that their general basic mechanisms of immunity would act broadly to resist a wide spectrum of potential immunological threats (Lourenço 2018). In arthropods, immunological memory is achieved by employing soluble, circulating pattern recognition receptor proteins, cell adhesion proteins, and lectins that recognize molecular patterns of macromolecules that are expressed on the surface of microbes (Pal & Wu 2009). These proteins act as opsonins, binding to microbes and tagging them for phagocytosis and destruction (Bayne 1990). In addition, the proliferation of specific populations of hemocytes that express proteins that are designed to bind to distinct macromolecules on the surface of microbes can provide extended host protection (Zhang et al. 2016). Since scorpions are the oldest arachnids in the fossil record (Dunlop 2010; Dunlop & Selden 2013), the study of immune system mechanisms of these animals should provide insight into the evolution of basal forms of host defense that developed during the Silurian period (Waddington et al. 2015). In addition, immunological studies of scorpions might illuminate molecular mechanisms of immunity that developed as a result of new immunological challenges of living in a terrestrial environment.

The desert hairy scorpion *Hadrurus arizonensis* Ewing, 1928 (Hadruridae) inhabits psammophilous and lithophilus substrates and creates deep burrows (Polis & McCormick 1986). This scorpion is native to the Sonoran and Mojave Deserts of northern Mexico and the southwestern United States and is the largest scorpion species in North America, growing to total lengths of 14–15 cm. It exhibits generalist feeding habits, and routinely catches and consumes prey >20 mm (Polis & McCormick 1986). This study was conducted to investigate the antibacterial capacity and characteristics of hemolymph derived from *H. arizonensis*.

This study describes broad spectrum antibacterial activities of hemolymph derived from *H. arizonensis*. Only a few studies have described specific aspects of scorpion immunity. For instance, one study described a localized immune response to an implanted genital plug (Oviedo-Diego et al. 2019), another described the susceptibility of scorpions to bacterial toxins and scorpion venoms (Grasset et al. 1945), and several have described and characterized the cellular characteristics of different types of hemocytes (Ravindranath 1974; Patil & Shah 2012, 2013; Shah 2017). Brahmi & Cooper (1974) described an agglutinin in the hemolymph of the fat-tailed scorpion *Androctonus australis* (Linnaeus, 1758) (Buthidae). In addition, a few studies have described the isolation of AMPs in the venom of various scorpion species (Harrison et al. 2014). However, this is the first study that has characterized the antibacterial activities of hemolymph for any scorpion species. Future studies will focus on the molecular mechanisms of these immunological activities.

METHODS

Treatment of scorpions.—We collected twelve wild adult *H. arizonensis* near Red Rock, Arizona at night using a UV flashlight (Pavan 1958; Stahnke 1972) during July and August 2019. We maintained the scorpions individually in 43 cm wide x 23 cm deep x 25 cm high plastic aquaria with screened lids for adequate ventilation (Brenes & Gomez 2016). Each aquarium contained approximately 7.5 cm of substrate composed of 70% fine sand and 30% clay, which was saturated with water, mixed thoroughly, and allowed to dry to produce a substrate adequate for subterranean tunneling. Each aquarium was also supplemented with several pieces of cork bark to provide overhead cover. Ambient temperature was a constant 25°C and we provided a thermal gradient of 25–36°C in the substrate using under-tank heating pads (Zoo Med ReptiTherm® UTH). We fed each scorpion two to three large crickets once or twice per week.

Collection of hemolymph.—We collected hemolymph directly from the tubular heart (Moghadam et al. 2013) by puncture of the opisthosoma between the tergites using a 1.0 mL syringe and a 1.27 cm 26 gauge needle. Immediately upon collection, we transferred the hemolymph to ice-cold 500 µL microcentrifuge tubes, pooled the collected material from all individuals and stored it in 400 µL aliquots at -20°C until ready for use. The hemolymph was typically used within a few days of collection.

Concentration-dependent antibacterial activity.—We diluted confluent bacterial cultures 10^7-fold with nutrient broth. We inoculated various concentrations of hemolymph diluted in nutrient broth with approximately 10^6 bacteria (*Escherichia coli*, *Klebsiella oxytoca*, *Shigella flexneri*, *Salmonella enterica*, *Streptococcus pyrogenes*, *Bacillus subtilis*, *Enterobacter cloacae*, and *Staphylococcus epidermitis*) from a log phase culture as previously described (Merchant et al. 2003). These bacterial species provided a broad spectrum of diverse taxa, some of which might have been encountered by scorpions in their natural environment, and some of which should not (Merchant et al. 2003). We transferred 40 µL of each culture to a well of a sterile 384-well microtiter plater. We used a BioRad Benchmark Plus™ microtiter plate reader to measure the optical density of each well at 0, 3, 6, 9, 12 hr for Gram-negative bacterial species and at 0, 3, 6, 9, 12, and 24 hr for Gram-positive species after inoculation. *Salmonella enterica* and *Streptococcus pyrogenes* cultures were allowed to grow longer due to slower growth rates. To calculate the changes in optical density, the absorbance changes for each individual replicate at each time were subtracted to that of the same sample at time zero. We conducted these assays using five replicates per sample.

Determination of colony forming units (CFUs).—We diluted hemolymph samples to 50% with nutrient broth and incubated them with *E. coli* for different amounts of time (2, 5, 10, 20, 30, and 60 min) at ambient temperature (approximately 25°C). After incubation, we plated the samples onto the surface of 2.0% low EEO agarose containing nutrient broth in 150 mm Petri dishes. We plated six to eight replicates of each sample to obtain a reasonable estimate of bacterial density (20–80 CFUs/plate). We incubated the plates overnight at 37°C and colonies were counted manually.

To determine the thermal profile of the antibacterial activity of hemolymph from *H. arizonensis*, we incubated 30 µL of hemolymph with 30 µL of a bacterial culture containing 10^6 *E. coli* CFU/mL at different temperatures (20, 25, 30, 35, or 40°C) for 30 min. We plated 20 µL of each culture on nutrient broth containing 2% low EEO agarose. The samples were analyzed using eight replicates to allow us to determine the statistical validity of results.

Statistics and controls.—We subjected the results obtained from kinetic and temperature-dependent studies to one-tailed t-tests to determine the level of significance relative to untreated control bacterial cultures.

RESULTS

Inoculation of nutrient broth with approximately 10^5–10^6 CFUs/mL resulted time-dependent bacterial proliferation as measured by spectrophotometry at 610 nm (Figs. 1, 2). Hemolymph derived from *H. arizonensis* exhibited antibacterial activity against gram negative (Fig. 1) and gram positive (Fig. 2) bacteria in a concentration-dependent fashion. The activity against *E. coli* was the strongest with respect to the Gram-negative bacteria, with 50 and 75% hemolymph exhibiting approximately 65 and 100% growth inhibition at 24 hrs, respectively. Comparatively, these same dilutions produced growth inhibition values of 27 and 81% for *S. flexneri*, 37 and 63% for *K. oxytoca*, and only 0 and 45.1% for *S. enterica*, respectively (Fig. 1). In addition, 25% hemolymph produced a 28.4% inhibition of growth in *E. coli* cultures compared to 9.1% for *S. flexneri*, 13.7% for *K. oxytoca*, and 0% for *S. enterica* (Fig. 1).

Challenge of 10 and 25% hemolymph derived from *H. arizonensis* with Gram-positive bacteria *S. epidermidis*, *E. cloacae*, *B. subtilis* and *S. pyrogenes* produced no antibacterial effects. However, antibacterial activities against the Gram-positive species were most potent toward *E. cloacae* (53.0 and 100% activity), followed by *S. epidermidis* (44.4 and 100% activity), *B. subtilis* (21.8 and 67.8% activity), and *S. pyrogenes* (0 and 42.5% activity).

The antibacterial action of hemolymph from *H. arizonensis* was rapid, producing strong activities within a minute or two of exposure (Fig. 3). Incubation of 50% hemolymph with liquid *E. coli* bacterial cultures at ambient laboratory

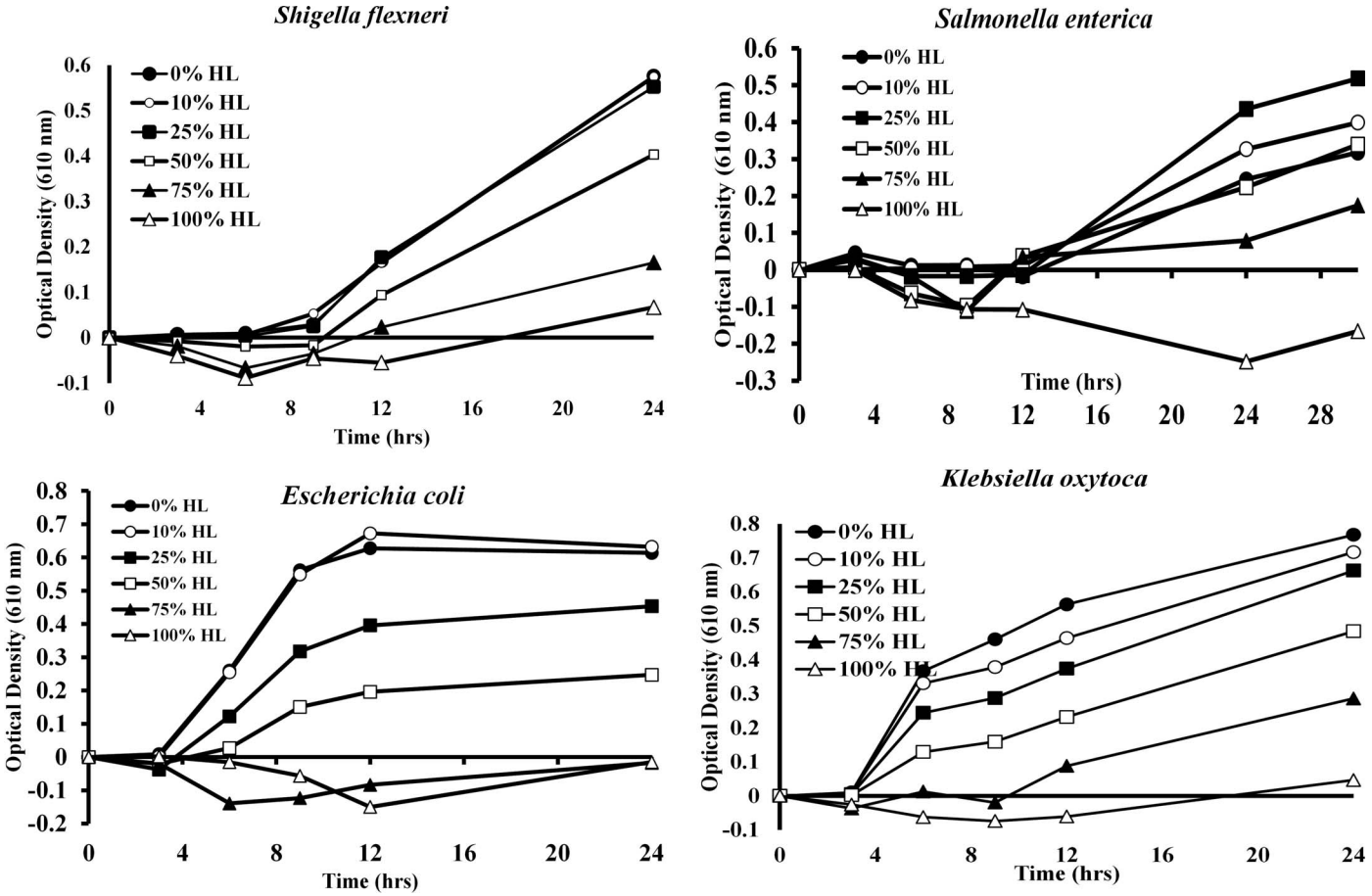

Figure 1.—Antibacterial activities against Gram-negative bacteria. Liquid cultures were maintained in 384-well microtiter plates at 37°C and the OD_{610} was measured in a microtiter plate reader at various times. The results represent the means of five replicates for each hemolymph dilution. (HL = hemolymph)

temperature for only two minutes produced a 59.6 ± 24.4% reduction ($P < 0.005$) in growth compared to bacterial cultures treated with the same volume of sterile saline. The inhibition of growth increased to 79.0 ± 13.9% after 5 minutes ($P < 0.004$) and reached maximum activity at 20 minutes (86.2 ± 4.5% inhibition).

Incubation of hemolymph from *H. arizonensis* with liquid cultures of *E. coli* bacteria at different temperatures resulted in a temperature-dependent antibacterial activity (Fig. 4). The antibacterial activity was relatively low at 20°C (14.9 ± 13.6% bacteria killed) but rose stepwise to 31.9 ± 9.5% ($P = 0.02$) at 25°C and 40.4 ± 28.7% ($P = 0.02$) at 30°C. The antibacterial activities at 25–30°C were all statistically higher than untreated E. coli cultures $P < 0.009$). However, the activity plummeted to 2.1% ± 15.3 ($P = 0.003$) and 0 ± 19% ($P = 0.0003$) antibacterial activity at 35 and 40°C, respectively.

DISCUSSION

The hemolymph collected from *H. arizonensis* exhibited similar antibacterial activities toward Gram-negative (Fig. 1) and Gram-positive (Fig. 2) bacteria. The hemolymph was only moderately effective in killing the various bacteria, only one Gram-negative (*E. coli*) and one Gram-positive (*E. cloacae*) species was inhibited at a concentration of 25%. However, concentrations of 75 and 100% hemolymph were very effective at killing all of the bacterial species examined.

It was noted that for some of the bacterial species, that the control cultures that contained no hemolymph had absorbance values far lower than for culture treated with hemolymph. This occurred primarily in bacterial species for which the hemolymph was less effective as an antibiotic agent. For example, the values for 10, 25, and 50% hemolymph in *S. pyrogenes* showed substantially higher absorbance values than that for 0% hemolymph. This is likely a result of hemolymph being both relatively ineffective at lower concentrations, and also a rich source of protein and other nutrients, such that the bacteria grow more efficiently in the lower concentration of hemolymph than in the unaltered nutrient broth. This was supported by our CFU data showing the *S. pyrogenes* cultures that contained 10, 25, and 50% hemolymph had more colonies, when plated, than the control cultures that contained no hemolymph (data not shown). In addition, some of the OD_{610} values for cultures, and particularly those with higher concentrations of hemolymph, are well below zero. Hemolymph is blue in color due to the copper content of hemocyanin (Van Holde et al. 2001) thus, the decrease in absorbance is due to the reduction in this protein due to metabolism by the bacteria or thermal instability at 37°C during the experiment.

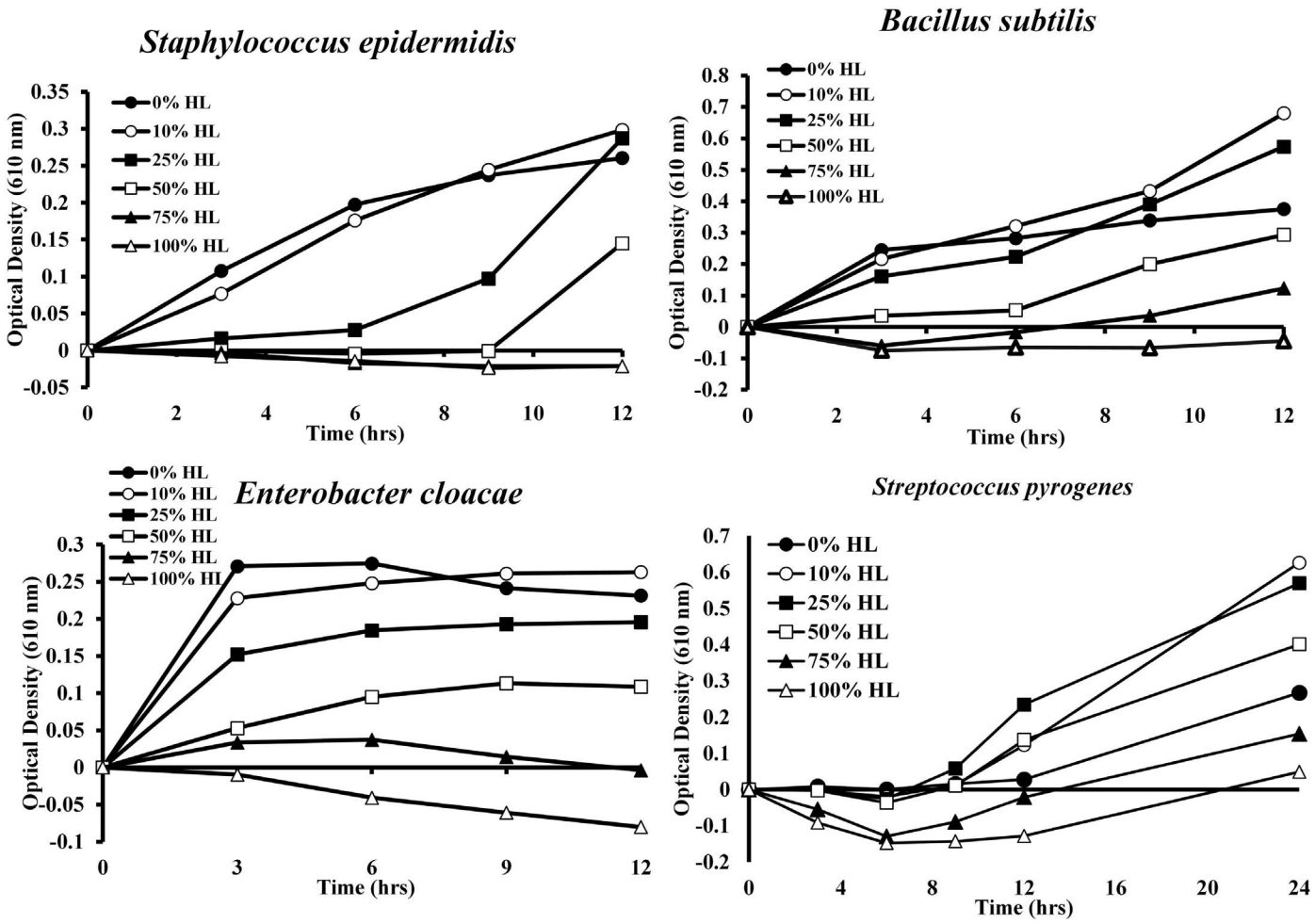

Figure 2.—Antibacterial activities against Gram-positive bacteria. Liquid cultures were maintained in 384-well microtiter plates at 37°C and the OD_{610} was measured in a microtiter plate reader at various times. The results represent the means of five replicates for each hemolymph dilution. (HL = hemolymph)

It is not surprising that hemolymph derived from *H. arizonensis* exhibits broad-spectrum antibacterial activities. It is surprising, however, that the activity is very low at temperatures of 35 and 40°C (Fig. 4). This scorpion species is most active during the very hot months of late June–September (Hadley 1974; Merchant, unpublished observations), when daytime temperatures often reach 46–47°C and nighttime lows only drop to 36–38°C. Like most scorpions, *H. arizonensis* is almost completely nocturnal, and thus only active at times during coolest portion of the diurnal cycle; however, this would still mean that this scorpion is active during times when their immune systems seem to be unequipped to deal with potential microbial threats (Fig. 4). These hazards could potentially come from ingestion of microbes during feeding (Grabowski & Klein 2017), wounds inflicted by potential predators (Nisani & Curiel 2019), or even conspecific aggressions (Tallarvoric 2000). Previous studies have found that scorpions injected with *Bacillus typhosus* and *Bacillus shiga* bacteria survived the infection when maintained at room temperature (18–22°C) but exhibited 100% mortality when maintained at 37°C after infection (Grasset et al.1945). Collectively, these results indicate that the immune mechanisms of *H. arizonensis* do not show high activity at higher temperatures (35–40°C). Although these animals are active at these temperatures at night during the hot summer months, it is possible that bacterial infections are not a problem at these temperatures in the extremely low humidity environments in which these scorpions live. Burrow temperatures at depth may also be mitigating factors.

The kinetic profile showed that antibacterial activities of hemolymph from *H. arizonensis* exhibits very rapid activity (Fig. 3). Activities with this type of fast action are consistent with the employment of AMPs as a defense mechanism. Since AMPs have been identified, isolated, and characterized in a variety of scorpion species (Ehret-Sabatier et al. 1996; Hmed et al. 2013), it is likely that *H. arizonensis* also expresses these proteins. However, most AMPs are small, with uncomplicated tertiary structures, and thus typically exhibit high levels of thermal stability (Ebbensgard et al. 2015). Given the fact that the antibacterial activities of *H. arizonensis* are relatively thermally labile (Fig. 4), it seems unlikely that the activities are due to the presence of AMPs. In addition, many scorpion antimicrobial peptides exhibit hemolytic activity toward eukaryotic erythrocytes, but no hemolytic activity toward

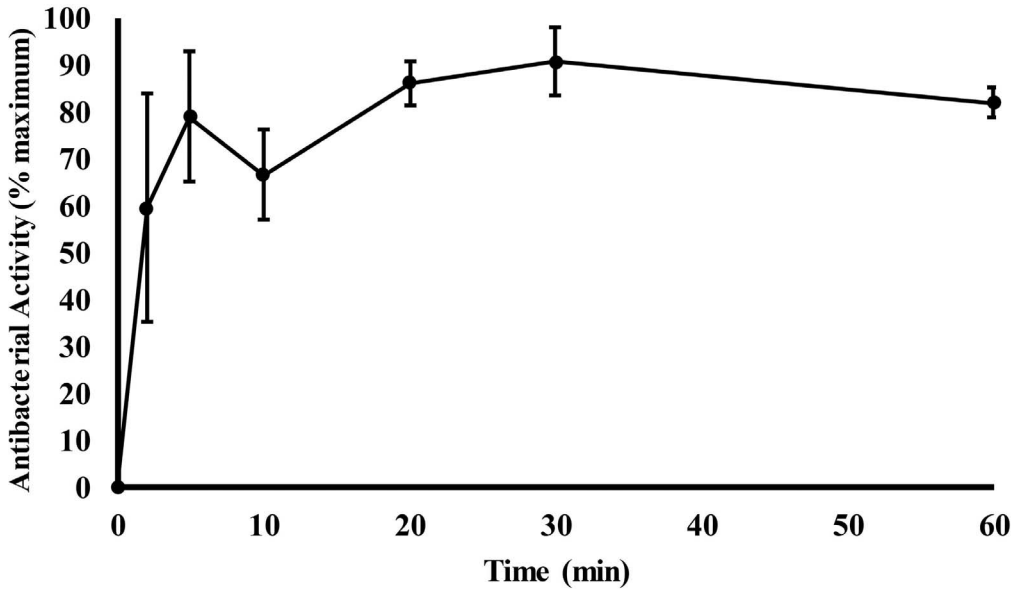

Figure 3.—Kinetic profile of antibacterial activity of scorpion hemolymph against *E. coli* bacteria. Log- phase bacterial cultures were incubated with 50% hemolymph (v/v, diluted with nutrient broth) and aliquots (20 μL) were plated onto nutrient broth agarose and incubated overnight at 37°C as described in the Materials and Methods. Each data point represents the means ± standard deviations of six replicates for the percentage of bacteria that were killed by the hemolymph.

sheep red blood cells was detected (data not shown). However, it is possible that the rapid antibacterial action is due to the presence of hemocyanin, as this protein has been shown to have important immune function (Coates & Nairn 2014).

Antimicrobial peptides in the venom of *Hadrurus sp.* (Torres-Larios et al. 2001; Schwartz et al. 2007; Rokyta & Ward 2017) almost certainly exert antibacterial properties that protect these animals from infection. The expression of these peptides in other scorpion species are inducible by microbial challenge and were also shown to be under the control of immune responsive elements in the promoter region (Gao et al. 2007), illustrating the immunological importance of these proteins. These peptides could potentially exert antimicrobial effects on prey items prior to ingestion. In addition, some scorpions are known to spray venom on themselves, thus possibly utilizing the fluid as an immune mechanism to cleanse saprophytic microbes from their external surfaces (Cesa-Luna et al. 2019).

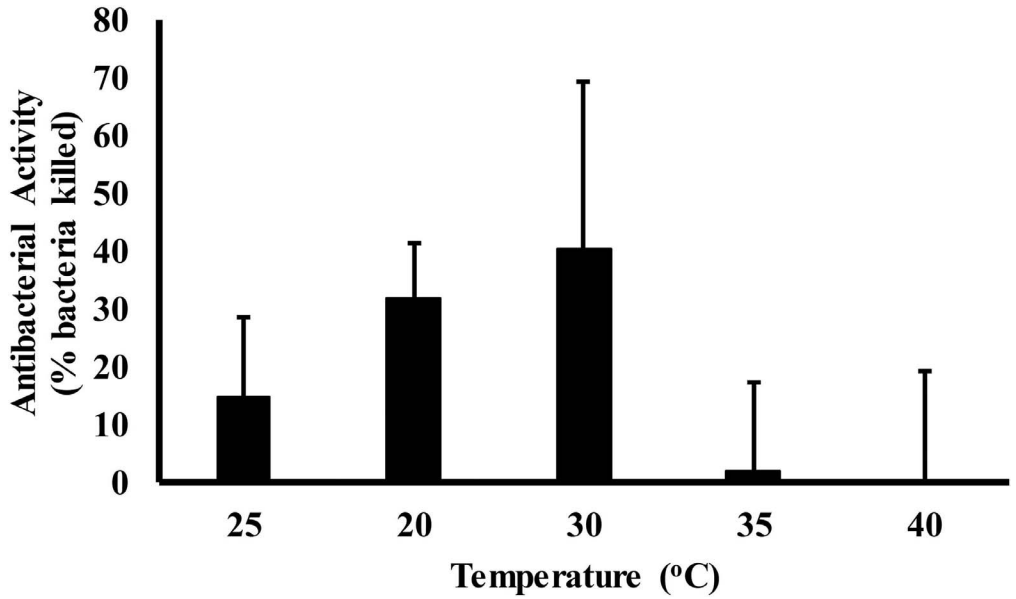

Figure 4.—Thermal profile of antibacterial activity of scorpion hemolymph against *E. coli* bacteria. Log-phase bacterial cultures were incubated with 50% hemolymph (v/v) for 30 min. at the indicated temperatures. The cultures were then plated (20 μL) onto nutrient broth agarose and incubated overnight at 37°C as described in the Materials and Methods. The results indicate the percentage of bacteria that were killed by the hemolymph, and represent the means ± standard deviations of eight replicates for each temperature.

Melanization is a well-characterized innate immune response during which internal threats are encased (Cerenius et al. 2007). It is interesting that the hemolymph from *H. arizonensis* did not display any type of melanization response to bacterial challenge. In addition, puncture of the opisthosoma for the purposes of hemolymph collection did not result in melanization as a wound healing response, as we observed no localized black pigmentation in areas of puncture as has been observed with other arthropods (Bilandzija et al. 2017). The immunological melanization response is present in all classes of Arthropods (Ratcliffe et al. 1982) but is known to have been lost in several lineages (Bilandzija et al. 2017). Therefore, it is plausible that some clades or scorpion species, including *H. arizonensis*, have lost the melanization response to infections and injury. Alternatively, a melanization response in *H. arizonensis* might not be responsive to bacterial challenge or puncture injuries such as the ones caused during the collection of hemolymph, but could be reactive to other stimuli.

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