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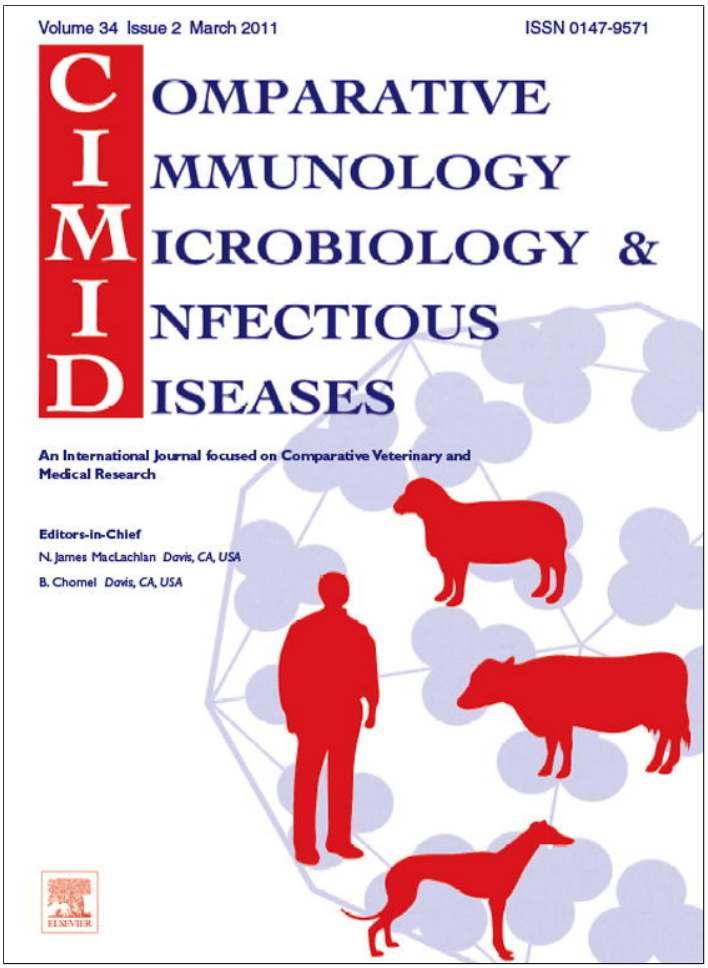

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Comparative Immunology, Microbiology and Infectious Diseases 34 (2011) 115–121

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Serum complement activity in the three-toed amphiuma (*Amphiuma tridactylum*)

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ARTICLE INFO

Article history:
Received 9 December 2009
Received in revised form 13 April 2010
Accepted 13 April 2010

Keywords:
Amphiuma
Innate immunity
Serum complement
Salamander

ABSTRACT

Some animals routinely endure serious injuries from predators or during intraspecific territorial conflicts. Such is the case for *Amphiuma tridactylum*, an aquatic salamander that lives in an environment rich in potentially infectious microbes, apparently with rare or no pathogenic infection. Some vertebrates possess innate immune mechanisms, but whether this is the case for *Amphiuma* is unknown. To assess this potential, plasma from 19 *A. tridactylum* was pooled and used for characterisation of serum complement activity. The ability of *A. tridactylum* plasma to hemolyse unsensitised sheep red blood cells (SRBCs) was titer-dependent, with low activity observed even at high plasma titers. The kinetic characterisation of SRBC hemolysis revealed that significant activity could be measured within 10 min of incubation, and maximal activity occurred within 60 min. The SRBC hemolysis by *A. tridactylum* plasma was also temperature-dependent, with maximal activity at 30 °C. In addition, this activity was sensitive to mild heat treatment, with 96% of activity inhibited by incubation at 56 °C for 30 min. The SRBC hemolysis could also be inactivated by pretreatment of the plasma with proteases, indicating that this activity was protein dependent. The activity required divalent metals ions, with activity inhibited by EDTA, citrate, or phosphate. However, the chelator-inhibited activity could be restored by the addition of excess Ca^{2+} or Mg^{2+}, but not Cu^{2+} or Ba^{2+}, indicating specificity of the divalent metal ion requirement. The sensitivity to heat, proteases, and divalent metal ion chelators strongly suggests that *A. tridactylum* plasma-mediated hemolysis of SRBCs is mediated by the serum complement system of proteins.

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1. Introduction

Wild animals often incur injuries from predators, intraspecific combat-related territoriality conflicts, and a variety of other sources. These animals often live in an environment that is rich in potentially infectious microbes, and thus are particularly vulnerable to infection when the injury breaches the integrity of the skin. However, some animals that routinely incur such injury rarely develop a serious infection. As a likely explanation, antibiotic properties of animal tissues have been documented in a variety of taxa including sharks [1], frogs [2], and mammals [3], and specifically in the blood of horseshoe crabs [4], lizards [5], crocodilians [6], birds [7], and mammals [8]. The biological molecules responsible for the antibiotic mechanisms vary widely between species from peptides, lipids, and alkaloids, to high concentrations of biliverdin, and depending on the type, defend against their respective microbial targets.

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0147-9571/$ – see front matter. Published by Elsevier Ltd.
doi:10.1016/j.cimid.2010.04.001

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Curiously, blood from the American alligator (*Alligator mississippiensis*), has shown a mechanism of resistance to a broad spectrum of microorganisms including bacteria [6], amoebae [9], viruses [10], and fungi [11]. Whether discovery of this defense arsenal is due to the relatively high number of studies focused on *A. mississippiensis* or simply the nature of the species, is yet unknown. Courtship in *A. mississippiensis* often involves intense male–male combat, which can result in open wounds. Given that this occurs in the particularly septic conditions of southeastern United States swamps and marshes, such an antimicrobial defense is likely a critical component of their evolutionary duration. While this scenario may be intuitive, there are other vertebrate animals in a similar situation, in the very same habitat.

Amphiuma tridactylum (three-toed Amphiuma) is a very large aquatic salamander that reaches a body length in excess of one meter and mass greater than 1 kg [12]. It inhabits roadside ditches, ponds, lakes, streams, and virtually any freshwater with a food supply. With formidable jaws and teeth, individuals routinely bite each other, presumably during territorial conflicts, which often results in deep cuts completely through the dermis and into the musculature [13]. However, these injuries heal quickly and do not seem to result in serious pathogenic infection in the lab or field (C. Fontenot, pers. obs.).

Several authors have shown that acquired immunity is less developed in ancient vertebrates such as fish [14] and crocodilians [15]. However, the innate immune systems of these ancestral ectotherms is thought to be more-developed than other their more recently evolved mammalian counterparts. The serum complement system of proteins has been described as a major component of innate immunity in ancient vertebrates and invertebrates [16,17]. Merchant et al. [6] showed that serum complement activity in crocodilians exhibited a much broader spectrum of antibacterial activity than human serum. In addition, Sunyer et al. [18] showed that teleost fish expressed at least five isoforms of the C3 serum complement protein component, and this was probably a major reason for the broad antibacterial activity in this group of vertebrates.

A. tridactylum has an elongate muscular body with four severely reduced limbs and three toes on each. It is abundant in the southeastern United States, with a geographic range that extends along the gulf coast from eastern Texas to central Alabama, and north to Missouri [19]. Courtship activities have not been confirmed, but studies of seasonal gonad gross anatomy and histological development suggest that breeding occurs December through March in Louisiana, when males annually reach peak testicular and cloacal development [20,21]. Females are reproductively biennial, and lay two beadlike strings of eggs in June, which are attended by an adult until they hatch in November [12]. Though apparently not required, hatching is influenced by inundation with water [22]. Hatchlings possess external gills for 7–9 days, and live in the cover of aquatic vegetation, where they feed on small aquatic insects. Their main food is crayfish and earthworms, depending primarily on availability, although they seem to eat anything that they can capture or scavenge, including insect larvae, mole crickets, fish, grasshoppers, water beetles, ground skinks, spiders, snails [23], and even small turtles [24]. *A. tridactylum* is largely nocturnal and hunts underwater with its head protruding from a crayfish burrow, waiting for passing prey. Small prey items are sucked into the mouth by bucco-pharyngeal expansion [25]. Alternatively, these salamanders can scavenge from large food sources by biting, then rolling its body in crocodile-fashion to pull a piece from the carcass. When feeding in the presence of other individuals, bumping each other often results in biting and rolling, and deep cuts through *Amphiuma* skin [13]. Whether the blood of these aquatic salamanders also contains antimicrobial properties is unknown. Thus, this study was conducted to characterise the serum complement system innate immunity defense in *A. tridactylum*.

2. Materials and methods

Chemicals and biochemicals: Isoflurane was purchased from Sigma (St. Louis, MO). Sheep red blood cells (10%, washed pooled) were purchased form Rockland Immunochemicals (Gilbertsville, PA).

Treatment of animals: Nineteen *A. tridactylum* were collected between 1 May and 30 July, 2009, 18 of which were from East Baton Rouge Parish, and one from Livingston Parish, LA. These animals were collected from drainage ditches and ponds using Promar (Gardena, CA) collapsible polyethylene minnow traps, baited with partially opened cans of cat food. The traps also contained a sealed empty plastic bottle to maintain part of the trap above the water line so that trapped animals could reach the water surface to breath.

Immediately prior to blood sampling, each individual was placed into a body-size-matched transparent plastic tube plugged at both ends with a rubber stopper and anesthetised by a 20 min exposure to a cotton ball saturated with 1–2 mL (as needed) of isoflurane. For most individuals, this was sufficient time to induce anesthesia, indicated by a lack of righting response to placing the animal upside-down (ventrum up), and general lack of muscular tension and responsiveness. A few of the larger individuals required an additional 1 mL of isoflurane and 10 min to achieve anesthesia.

An incision was made through the dermis and hypaxial musculature to expose the heart and associated vessels. Blood was then slowly drawn from the post-caval vein into heparinised syringes (1000 USP units sodium heparin/mL) until no more blood was available or the heart stopped beating. Whole blood was centrifuged at $2500 \times g$ for 5–10 min at ambient temperature. Plasma was then transferred with a pipette to fresh tubes and stored at store at $-80\,^{\circ}C$. All sacrificed individuals used in this study were deposited in the Southeastern Louisiana University Vertebrate Museum. These methods were approved by Southeastern Louisiana University IACUC, Protocol #0015.

SRBC hemolysis assay: The SRBC hemolysis assay was conducted using a modified method of Mayer [26]. For experiments in which the effects of plasma titer on SRBC hemolysis were investigated, various volumes of *A. tridactylum* plasma (0–500 μL) were diluted to 500 μL using 100 mM HEPES buffer (pH 7.4). Five hundred microliters of

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2% unsensitised SRBCs were added to each diluted plasma sample and incubated at ambient temperature for 30 min. The samples were centrifuged at 5000 × *g* for 5 min, and 200 μL of the supernatant was placed into each well of a 96-well microtiter plate. The Abs_{540} for each was determined using a Benchmark Plus™ (BioRad, Hercules, CA) microtiter plate reader. Total SRBC hemolysis was achieved by adding 2 μL of Triton-X 100 detergent to a 1 mL solution of 1% SRBCs. The optical density (540 nm) of each sample was expressed as a percentage of the absorbance of the maximum hemolysis control. The data are expressed as the means ± standard deviations of four independent determinations for each data point.

For experiments in which the kinetic characteristics of *A. tridactylum* serum complement activity were investigated, 20 mL of 2% SRBCs were mixed with 8 mL of HEPES buffer and 12 mL of *A. tridactylum* plasma. Aliquots were removed and centrifuged (5000 × *g* for 5 min.) at specific time points, and the absorbance of each sample at 540 nm was determined as described above.

To examine the effects of temperature on *A. tridactylum* serum complement activity, 200 μL of plasma were diluted with 300 μL of 100 mM HEPES buffer (pH 7.4). Different aliquots were incubated at various temperatures (5–40 °C), along with aliquots (500 μL) of 2% SRBCs, for 10 min to establish thermal equilibrium. The diluted plasma samples were then added to the SRBCs and incubated for 30 min at the different temperatures, and then the absorbance of each sample at 540 nm for each sample was determined as described above.

To determine the effects of heat treatment on *A. tridactylum* serum complement activity, plasma was incubated for 1 h at 56 °C [27]. In addition, the effects of proteases were tested by incubation of *A. tridactylum* plasma with 10 units of either trypsin or chymotrypsin for 30 min at ambient temperature. To determine the requirement for divalent metal ions, the plasma was treated with 0.5, 2, 10, or 30 mM EDTA (final concentration). Attempts to reconstitute EDTA-depleted serum complement activity occurred by incubating *A. tridactylum* plasma with 2 mM EDTA, followed by the addition of 10 mM $CaCl_2$, $MgCl_2$, $BaCl_2$, or $CuCl_2$. The samples were assayed for SRBC hemolysis as described above.

Statistics and controls: The results displayed represent the means ± standard deviations of four independent determinations. The results of each experiment were expressed as the % maximum of a positive lysis control, which was obtained by treating 1% SRBCS with 0.1% Triton-X detergent (v/v), and passing the sample repeatedly through a TB syringe (10 passes) until all of the SRBCs had been disrupted, as determined by observation under 400× magnification. The statistical significance between treatment groups was determined by analysis of variance using Duncan's post hoc comparisons.

3. Results

Fig. 1 shows the titer-dependent hemolysis of SRBCs by *A. tridactylum* plasma. The curve appears sigmoidal in nature, with linear activities from 0 to 250 μL, and from 300 to 500 μL. The incubation of 1% SRBCs with 50, 100, 150, and 200 μL of plasma resulted in 1.2, 2.6, 3.4, and 4.5% of maximal hemolysis activity, respectively. Likewise, incubation with 300, 350, 400, 450, and 500 μL of plasma resulted in 15.7, 16.8, 17.6, 20.6, and 22.5% of positive control values, respectively. However, there was a disproportional, but reproducible, increase in activity from 250 to 300 μL of plasma, producing a sigmoidal relationship between plasma titer and SRBC hemolysis. These data suggest that there is a critical concentration for the serum complement cascade to perform optimally. Furthermore, the data suggest positive cooperativity between the enzymes involved in the serum complement cascade.

Fig. 2 shows the sensitivity of *A. tridactylum* serum-mediated SRBC hemolysis to EDTA, a potent chelator of divalent metal ions. Pretreatment of EDTA with 0.5, 2, 10 or 30 mM EDTA for 5 min at ambient temperature resulted in a 43.6, 88.9, 96.8, or 97.6% inhibition of activity, relative to untreated serum. In addition, treatment of *A. tridactylum* plasma with 5 mM EDTA, phosphate, or citrate resulted in 94, 98, or 89% reduction in hemolytic activities, respectively, compared to untreated plasma (Table 1). These

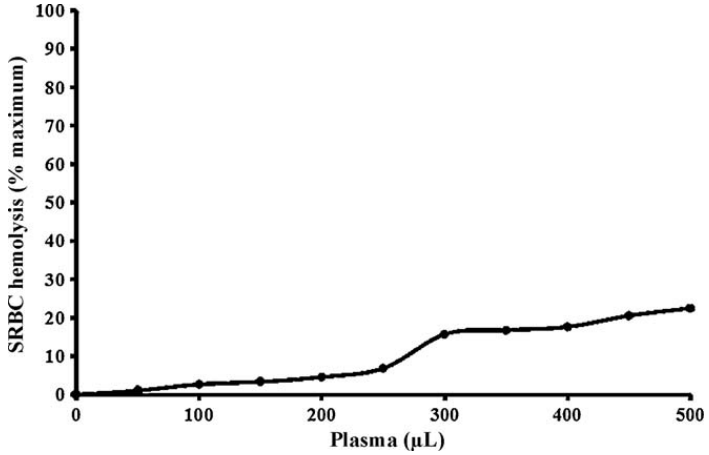

Fig. 1. Plasma titer-dependent hemolysis of SRBCs by serum from *A. tridactylum*. *Amphiuma tridactylum* serum was incubated with 1% unsensitised SRBCs for 30 min at ambient temperature. The samples were centrifuged to remove insoluble materials, and the optical densities of the resulting supernatants were determined. The results were compared to a SRBC hemolysis positive control, and are expressed as % maximum hemolysis, and represent the means ± standard deviations for four independent experiments.

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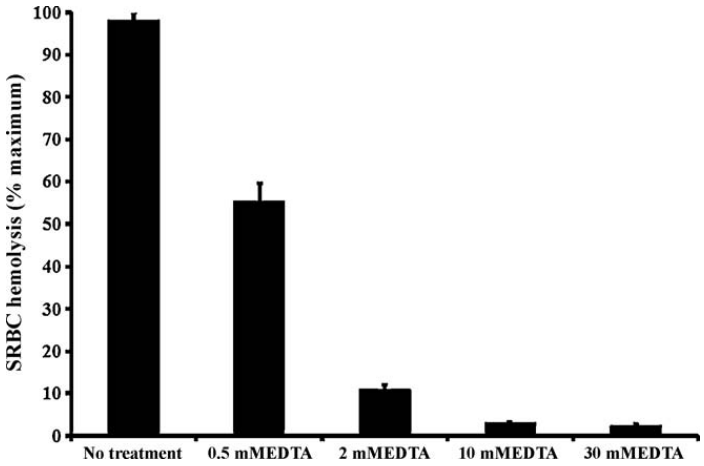

Fig. 2. Concentration-dependent effects of EDTA on SRBC hemolysis by *A. tridactylum* plasma. Plasma was incubated with 1% unsensitised SRBCs for 30 min at ambient temperature. The samples were centrifuged to remove insoluble materials, and the optical densities of the resulting supernatants were determined. The results were compared to a SRBC hemolysis positive control, are expressed as % maximum hemolysis, and represent the means ± standard deviations for four independent experiments.

results indicate that the inhibition of hemolytic activity by EDTA is not specific to this compound, but is a general effect of the sequestration of divalent metal ions. Further treatment of plasma with 5 mM EDTA and 10 mM Ca^{2+} or Mg^{2+} resulted in near complete restoration of hemolytic activity. However, treatment of plasma with 5 mM EDTA and either 10 mM Cu^{2+} or Ba^{2+} had no effect on the EDTA inhibition of SRBC hemolysis. These results indicate that the *A. tridactylum* serum complement activity has a requirement for specific divalent metals ions.

Table 1 displays the effects of mild heat treatment and various divalent metal ion chelators on the ability of *A. tridactylum* plasma to hemolyse SRBCs *in vitro*. Incubation of *A. tridactylum* plasma at 56 °C for 30 min resulted in a 96% reduction in SRBC hemolysis, relative to plasma incubated at ambient temperature for the same amount of time. Preincubation of *A. tridactylum* serum with 5 mM EDTA, citrate, or phosphate (all chelators of divalent metal ions) for 5 min resulted in 94.6, 95.7, and 91.2% inhibition or hemolytic activity. Incubation of the serum with either trypsin or chymotrypsin for 30 min (37 °C) resulted in a strong depletion (96.1 and 97.1%, respectively) of SRBC hemolysis, indicating that this activity is mediated by plasma proteins. The data shown in Fig. 2 and Table 1, taken together, strongly suggest a role of serum complement activity in the amphiuma-mediated hemolysis of SRBCs.

Table 1
Effects of mild heat treatment, proteases, and divalent metal chelators on the hemolysis of SRBCs by *A. tridactylum* plasma. The effects of classical inhibitors of serum complement activity (mild heat, proteases, and metal chelators) were tested on the *A. tridactylum* plasma-mediated hemolysis of SRBCs. The samples were centrifuged to remove insoluble materials, and the optical densities of the resulting supernatants were determined. The results were compared to a SRBC hemolysis positive control, and are expressed as % maximum hemolysis, and represent the means ± standard deviations for four independent experiments.

Plasma treatment	Plasma SRBC hemolysis activity (% maximum)
None	98.2 ± 3.2
56 °C, 30 min	3.2 ± 0.3[a]
10 U trypsin, 30 min, 37 °C	3.8 ± 0.1[a]
10 U chymotrypsin, 30 min, 37 °C	2.9 ± 0.1[a]
5 mM phosphate	8.6 ± 0.9[a]
5 mM citrate	4.2 ± 0.5[a]
5 mM EDTA	5.3 ± .02[a]
5 mM EDTA + 10 mM $CaCl_2$	89.4 ± 3.6[b]
5 mM EDTA + 10 mM $MgCl_2$	92.6 ± 2.7[b]
5 mM EDTA + 10 mM $CuCl_2$	4.6 ± 0.9[c]
5 mM EDTA + 10 mM $BaCl_2$	5.9 ± 0.4[c]

[a] Statistically lower than untreated serum ($p < 0.001$).
[b] Statistically higher than EDTA-treated serum ($p < 0.001$).
[c] Statistically lower than EDTA-treated serum ($p < 0.001$).

The kinetic character of SRBC hemolysis by *A. tridactylum* plasma is displayed in Fig. 3. Amphiuma *A. tridactylum* plasma showed little hemolytic activity within the first 10 min of exposure to SRBCs. At 15, 20, and 30 min, the plasma hemolytic activity reached 2.2 ± 2.2, 4.5 ± 0.4, and 11.8 ± 1.8% of the maximal hemolysis, respectively, relative to the positive control. The activity slowly climbed to the maximum of 20.8 ± 1.8% at 60 min.

The effects of temperature on SRBC hemolysis by *A. tridactylum* plasma are illustrated in Fig. 4. Incubation of *A. tridactylum* serum with SRBCs at 5, 10, 15, or 20 °C had a significant negative effect, resulting in only 35, 25, 45, or 64% of the optimal activity ($p < 0.05$), respectively, which was observed at 50 °C. SRBC hemolysis by *A. tridactylum* blood was also significantly compromised at elevated temperatures, with reduced activity at 40 °C (26% of optimal, $p < 0.05$).

4. Discussion

A. tridactylum plasma exhibits similar serum complement activity relative to other herpetological species. For example, the activity of *A. tridactylum* plasma (Fig. 1) is similar to that of *Phrynops geoffroanus* (Geoffroys side-necked turtle) [28], and *Pantherophis obsoletus* (Texas ratsnake, Mark Merchant, unpublished data). However, this activity is far less than that observed in crocodilian species [29–32]. Despite the propensity of *A. tridactylum* to inflict deep wounds during territorial disputes and aggressive

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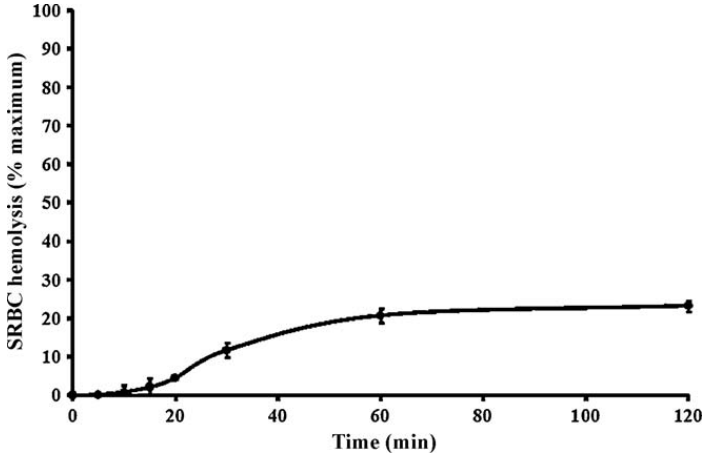

Fig. 3. Time-dependent hemolysis of SRBCS by *A. tridactylum* plasma. Plasma was incubated with 1% unsensitised SRBCs for different amounts of time at ambient temperature. The samples were centrifuged to remove insoluble materials, and the optical densities of the resulting supernatants were determined. The results were compared to a SRBC hemolysis positive control, and are expressed as % maximum hemolysis, and represent the means ± standard deviations for four independent experiments.

feeding behaviors [13], these aquatic amphibians do not seem to have evolved the potent complement system that is observed in modern crocodilians. However, the serum complement system is only one of several potential antimicrobial mechanisms that remain to be investigated.

The analysis of the dynamics of SRBC hemolysis showed a slow rise in activity during the first 20 min of exposure to SRBCs, followed by a more substantial increase between 20 and 30 min (Fig. 3). Maximal activity was reached at 60 min, a kinetic profile is similar to that observed in aquatic turtles in Brazil [28]. However, this is in contrast to that observed in crocodilians, where the SRBC hemolytic activity is detected within 2–5 min, and maximal activity is recorded within 20–30 min [29–32]. In addition, the increases in hemolytic activities observed in *A. tridactylum* serum were much lower than that observed in the crocodilian species. This slow activation of complement activity could either be due to a low K_m of the enzymatic system, but is more likely due to low concentrations of complement proteins relative to other species examined.

Physiological function increases exponentially with temperature to an upper limit determined by enzymatic function. In amphibians, that critical upper limit (35–40 °C) is relatively low compared to other tetrapods (>40 °C) [33]. The thermal profile for *A. tridactylum* shows that optimal temperature for complement activity is near 30 °C (Fig. 4). The same thermal optimum has been observed for *A. mississipiensis* [29], an ectothermic semi-aquatic vertebrate that inhabits the same geographic range as *A. tridactylum*. However, the preferred body temperature for *A. tridactylum* in the laboratory is 26 °C (C. Fontenot, unpublished data). Preferred body temperatures for amphibians are typically toward the higher end of their tolerance range [34], but they are not necessarily expected to match the complement activity temperature optimum. Achieving optimum complement activity temperature in an individual may be reserved for times of pathogenic infection. All vertebrates, including amphibians, are capable of producing fever in response to microbial infection or inflammatory pyrogens [35]. For salamanders and frogs, this is a behavioral increase

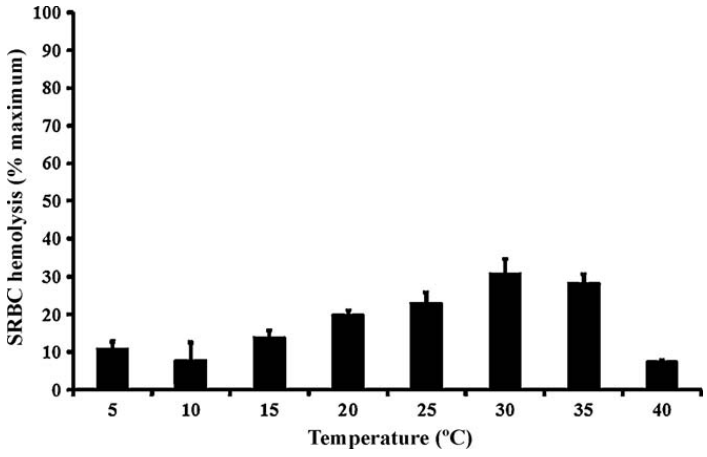

Fig. 4. Temperature-dependent hemolysis of SRBCs by *A. tridactylum* plasma. Plasma was incubated with 1% unsensitised SRBCs for 30 min at different temperatures. The samples were centrifuged to remove insoluble materials, and the optical densities of the resulting supernatants were determined. The results were compared to a SRBC hemolysis positive control, and are expressed as % maximum hemolysis, and represent the means ± standard deviations for four independent experiments.

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in body temperature of 2–5 °C, depending on the species [33].

As an example of what is available to *Amphiuma*, maximum weekly field water temperatures ranged from 2 to 36 °C seasonally in 1989 at a study site ditch near Ville Platte, LA, with temperatures above the preferred body temperature (26 °C) available from mid-march through mid-November, and optimum complement activity temperature (30 °C) available May–October (C. Fontenot, unpublished data). Because temperatures were collected repeatedly from a single point in the deepest water possible (~0.5 m), warmer temperatures were certainly available in shallower water. This suggests that individual *A. tridactylum* could behaviorally thermoregulate to optimum complement activity temperatures during most of the year (but not continuously because of daily environmental temperature variation). In addition, because *A. tridactylum* has a very large lung capacity [36], there is potential to increase body temperature above that of the water by ventilation when air temperatures are greater than water temperatures.

Despite the differences in the potencies, kinetic profiles, and temperature optima of SRBC hemolytic parameters in different ectothermic species, the biochemical mechanisms are very similar, as shown by the sensitivity to the classical inhibitors of serum complement pathways (Fig. 2, Table 1). In addition, these characteristics are shared with complement pathways in more modern vertebrates, including humans [37,38]. However, one difference is that human complement has an absolute requirement for both Mg^{2+} and Ca^{2+}, while the complement system in more ancient vertebrates, including *A. tridactylum* (Fig. 2, Table 1) and crocodilians [39], appear to require either Ca^{2+} or Mg^{2+} for activity. While this may seem to be a small difference, it could be an indication of significant differences in the mechanisms and regulation of this important component of the innate immune system.

To our knowledge, this study represents the first description of the innate immune system in any member of the genus *Amphiuma*. The activities observed, although similar to some aquatic reptiles and less potent than others, does exhibit many of the biochemical characteristics (heat and protease sensitivity, Ca^{2+} or Mg^{2+}-dependence) previously observed in other herpetological fauna.

Acknowledgements

We thank Matthew Indest for field assistance, and Lauren Smith, Michael Rodriguez, and Roldán Valverde for technical lab assistance with plasma collection and processing.

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