Comparative Biochemistry and Physiology, Part C 236 (2020) 108769

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

Pets in peril: The relative susceptibility of cats and dogs to procoagulant snake venoms

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ARTICLE INFO

Keywords:
Elapid snake envenomation
Snake venom
Snakebite
Dogs
Cats
Coagulotoxins
Pseudonaja textilis
Clinical toxicology

ABSTRACT

Snakebite is a common occurrence for pet cats and dogs worldwide and can be fatal. In Australia the eastern brown snake (*Pseudonaja textilis*) is responsible for an estimated 76% of reported snakebite cases to domestic pets nationally each year, with the primary pathology being venom-induced consumptive coagulopathy. While only 31% of dogs survive *P. textilis* bites without antivenom, cats are twice as likely to survive bites (66%). Even with antivenom treatment, cats have a significantly higher survival rate. The reason behind this disparity is unclear. Using a coagulation analyser (Stago STA R Max), we tested the relative procoagulant effects of *P. textilis* venom—as well as 10 additional procoagulant venoms found around the world—on cat and dog plasma *in vitro*, as well as on human plasma for comparison. All venoms acted faster upon dog plasma than cat or human, indicating that dogs would likely enter coagulopathic states sooner, and are thus more vulnerable to procoagulant snake venoms. The spontaneous clotting time (recalcified plasma with no venom added) was also substantially faster in dogs than in cats, suggesting that the naturally faster clotting blood of dogs predisposes them to being more vulnerable to procoagulant snake venoms. This is consistent with clinical records showing more rapid onset of symptoms and lethal effects in dogs than cats. Several behavioural differences between cats and dogs are also highly likely to disproportionately negatively affect prognosis in dogs. Thus, compared to cats, dogs require earlier snakebite first-aid and antivenom to prevent the onset of lethal venom effects.

1. Introduction

Snakebite is a global issue not only for humans but also for pets. Symptomatic of this fundamental problem is the situation in Australia where snakebite envenomations can be deadly for humans but much more so for pets (Mirtschin et al., 1998; Welton et al., 2017). For example, of the estimated 6,123 people hospitalised by snakes annually in Australia (Welton et al., 2017), 2 people died per year over a 14-year period. In comparison, of the estimated 6,200 cats and dogs bitten by snakes annually in Australia, a minimum of 213 died as a result (Mirtschin et al., 1998), which is likely a gross underestimate, as it excludes pets not presented for treatment.

Survival rates of cats and dogs after snake envenomation differ considerably. In a questionnaire completed by 106 veterinarian surgeons, Mirtschin et al. (1998) found that cats have a much higher survival rate (66%) from snake envenomation without antivenom treatment compared to dogs (31%). This is despite the average time from bite to presentation at veterinary clinics reportedly being much longer (15 h) for cats than for dogs (6.5 h) (Barr, 1984). The higher survivability of cats is also reported with antivenom treatment: 91% of cats and 75% of dogs (Mirtschin et al., 1998).

A study comparing clotting times of cat and dog plasma from envenomed pets in Australia revealed only minimal or no change in clotting times for cats, but suggested this could result from a smaller dose of venom being received by cats (Holloway and Parry, 1989), rather than cats being physiological less susceptible to snake venom. Another study which introduced three Australian venoms (eastern brown snake (*Pseudonaja textilis*), tiger snake (*Notechis scutatus*), death adder (*Acanthophis antarcticus*)) to healthy cat and dog plasma suggested that the observed physiological difference in the plasma types (control values differing) was responsible for cats being less susceptible to the coagulant venoms (Crawford and Mills, 1985). However, the

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https://doi.org/10.1016/j.cbpc.2020.108769
Received 1 March 2020; Received in revised form 7 April 2020; Accepted 18 April 2020
Available online 03 May 2020

methods used to measure clotting times were inefficient (observing clotting by eye; one venom concentration only (2.5 μg/mL)), and the range of snake species studied similarly limited.

In Australia, the eastern brown snake (*Pseudonaja textilis*) is a highly venomous snake (Broad et al., 1979; Zdenek et al., 2019a) that is well adapted to human-disturbed habitats, and is thus common in suburban and rural areas across its range in southern and eastern Australia. This species is responsible for the majority of bites to humans (63%) and to pets (76%) (Mirtschin et al., 1998; Welton et al., 2017). The leading cause of death from envenomation by *P. textilis* to dogs, cats, and humans is the disruption of blood homeostasis via potent procoagulant toxins (Lalloo et al., 1995; Sutherland and Tibballs, 2001; Isbister et al., 2010; Chaisakul et al., 2013). Despite similar clinical syndromes produced by *P. textilis* envenomations in humans and dogs (Heller et al., 2007; Sprivulus et al., 1996), some differences exist. While *P. textilis* venom contains highly potent neurotoxins (Barber et al., 2012; Coulter et al., 1983), which are relatively more abundant in juveniles than adults (Cipriani et al., 2017; Jackson et al., 2016), neurotoxicity is uncommon in human envenomings (Barber et al., 2012), but is a feature in dog envenomings (Heller et al., 2007), likely due to the smaller body size of dogs resulting in a relatively higher concentration of the neurotoxins.

Due to coagulopathy being the key feature in *P. textilis* envenomations, as well as in envenomations by many other medically significant Australian elapid species (Isbister, 2009; Isbister et al., 2010), physiological differences in plasmas could greatly affect relative survivability between taxa. The precedence for this presumption is that multiple studies have found different plasmas have varying susceptibility to different procoagulant venoms (Maduwage et al., 2016; Sousa et al., 2018; Youngman et al., 2018). Furthermore, intra-species differences in plasma may also occur according to differences in ecology, morphology, and phylogeny (Tella et al., 2004).

People and families often care deeply about their pets, yet very little research has investigated the effects of snakebite on cats and dogs in particular, and their relative susceptibility. Although there are several epidemiological case-studies on cat/dog snakebite in Australia (Judge, 2015; Padula and Winkel, 2016a, 2016b, 2016c; Holloway and Parry, 1989), tests have been limited, and comparable studies are lacking for snake species from other parts of the world.

We set out to test the relative physiological susceptibility of cat and dog plasma to the effects of *P. textilis* venom and 10 additional procoagulant snake venoms from across the world. For informative comparison and public interest, we also tested the venoms on human plasma. To elucidate susceptibility of different plasmas to particular coagulotoxins, included venoms were chosen based on coagulotoxin biochemistry type (venom-expressed FXa, venom expressed FVa:FXa complex, and snake venom metalloproteases) and known coagulotoxic action (Factor X activation and prothrombin activation). Our findings provide a better understanding of the clinical pathologies displayed in envenomated cats and dogs and therefore have implications in reducing cat and dog deaths worldwide from snakebite.

2. Methods

2.1. Plasma collection and handling

We used 30 mL surplus plasma (total, not per animal) obtained from cats and dogs by Dr. Joshua Llinas at Unusual Pets Vets (Queensland registration #: 4184) during routine veterinary check-ups of 10 cats and 10 dogs of multiple breeds and ages, with no prior snakebite history (UQ animal ethics approval numbers ANRFA/149/20 and SBS/019/14/ARC). Consent forms were obtained from each owner explaining that any surplus material may be used for research. Care was taken to ensure gentle handling of whole blood so as to prevent lysis of red blood cells and contamination of plasma. Any hemolysed samples (visually assessed by pink colour) were discarded. Blood samples were extracted using a 22-gauge needle and 5 mL syringe; the needle was removed from the animal and the blood was gently emptied into 1 mL MiniCollect Coagulation tubes (ref. #: 450413; Greiner Bio-One). Once blood collection reached the fill-line on the tube, the sample was immediately gently inverted 20 times to homogenise the anticoagulant agent (3.2% citrate) throughout the blood sample to prevent clotting. The samples were gently spun for 10 min at 2000 RCF and the supernatant spun for an additional 10 min at 10,000 RCF. The supernatents were then transferred to cryotubes and flash-frozen in liquid nitrogen (−180 °C) and stored at −80 °C until later pooled for use in testing. To pool all individual plasma samples, all samples were simultaneously thawed at 37 °C in an immersion bath circulator (Thermo Haake ARCTIC SC150-A40), gently mixed together, and then aliquoted into 2 mL eppendorf tubes. Once pooled, all tubes were simultaneously flash-frozen within 15 min of thawing using liquid nitrogen and then stored at −80 °C until required.

Human plasma (3.2% citrate) was pooled from healthy individuals and donated by the Australian Red Cross (Research approval #16-04QLD-10; 44 Musk Street, Kelvin Grove, Queensland 4059). Two bags (~200 mL each) of pooled human plasma [Label #3991594 (O-) and label #3985833 (B+)] were further pooled, aliquoted, and stored in the same manner as cat and dog plasma.

For experimentation, one plasma aliquot at a time was thawed in an immersion bath circulator and used immediately. Plasma aliquots were replaced every 30 min at maximum to maintain freshness.

All plasma work was undertaken under University of Queensland Biosafety Approval #IBC134BSBS2015.

2.2. Venom collection and handling

We were interested in determining the relative susceptibility of cats and dogs to multiple procoagulant snake venoms with different coagulotoxic activities (Table 1). Eleven snake species from across the world were included (Fig. 1). All venom work was undertaken under

Table 1
Snake venoms used, selected based on known coagulotoxic activities.

Scientific name	Common name	Distribution	Coagulotoxic activity	Source
Bothrops atrox	Lancehead Viper	South America	FX and PT activating	Sousa et al. (2018)
Calloselasma rhodostoma	Malayan Pit Viper	SE Asia; Indo.	FX activating	Debono et al. (2019b)
Cerastes cerastes	Horned Viper	North Africa; Middle East	FX activating	Yamada et al. (1997)
Daboia siamensis	Siamese Russell's Viper	S. China; SE Asia; Indo.	FX activating	Zhu et al. (2015)
Echis coloratus	Painted Saw-scaled Viper	Middle East and Arabian Peninsula	PT activating	Morita and Iwanaga (1978)
Echis ocellatus	West African Saw-scaled Viper	West Africa	PT activating	Hasson et al. (2003)
Notechis scutatus	Tiger Snake	South and SE Australia	PT activating	Kini (2005)
Oxyuranus scutellatus	Coastal Taipan	Coastal Northern Australia	PT activating	Walker et al. (1980)
Pseudechis porphyriacus	Red-bellied Black Snake	South and SE Australia	PT activating	Maddock et al. (2017)
Pseudonaja textilis	Eastern Brown Snake	Eastern & Southern Australia	PT activating	Masci et al. (1988)
Thelotornis mossambicanus	Eastern Twig Snake	Eastern Africa	PT activating	(Debono et al., 2019a, b)

FX = Factor X; PT = Prothrombin.

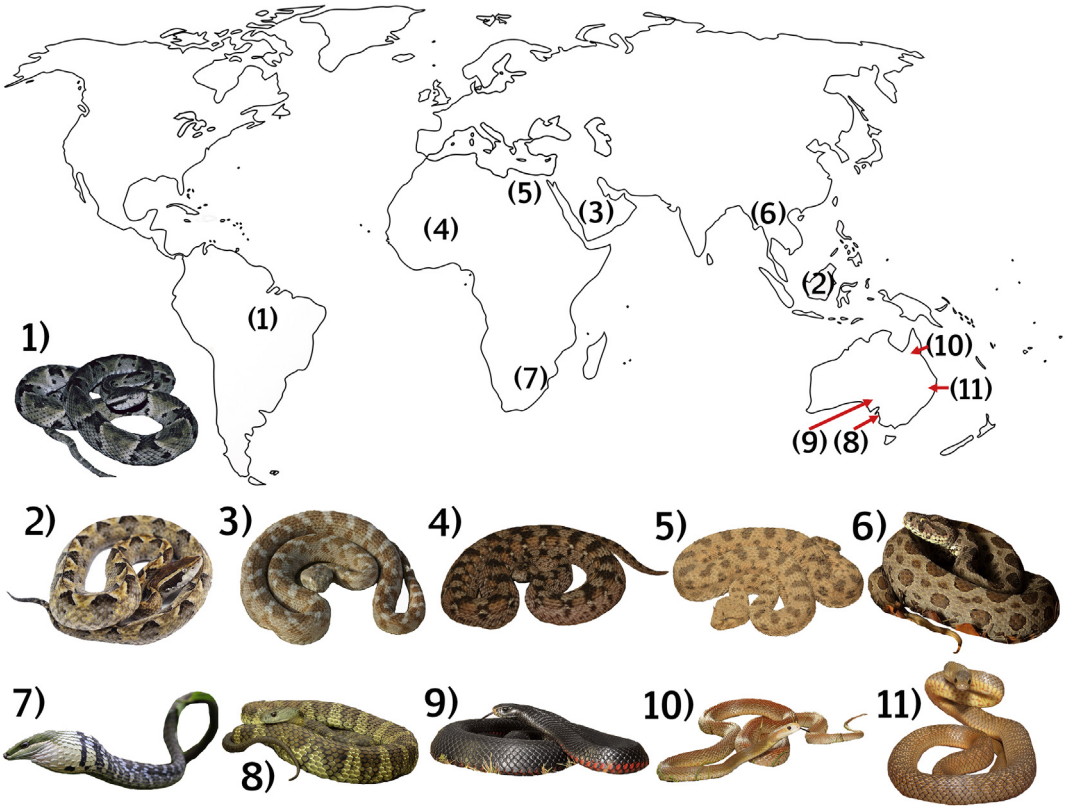

Fig. 1. A world map of the localities from which all 11 snake venoms originated. 1) *Bothrops atrox* (Santarém, Brazil), 2) *Calloselasma rhodostoma* (estimated locality based on species range), 3) *Echis coloratus* (Saudi Arabia), 4) *Echis ocellatus* (Mali), 5) *Cerastes cerastes* (estimated locality based on species range), 6) *Daboia siamensis* (Myanmar), 7) *Thelatornis* sp. (estimated locality based on species range), 8) *Notechis scutatus* (Lake Alexandrina, SA), 9) *Pseudechis porphyriacus* (South Australia), 10) *Oxyuranus scutellatus* (Julatten, QLD) 11) *Pseudonaja textilis* (Gold Coast, QLD). Photographers: Chris Hay, Wolfgang Wüster, Tom Charlton, and Tino Antilla.

University of Queensland Biosafety Approval #IBC134BSBS2015.

Venoms were sourced (from individual adult snakes) from either the long-term cryogenic collection of the Venom Evolution Laboratory, or donated by Nathan Dunstan of Venom Supplies Pty Ltd. or Chris Hay of The Australian School of Herpetology. *Bothrops atrox* venom was exported under license from Brazil (export permit: 17BR026238/DF) to Australia (import permit: IP 15016115). Venom extraction was conducted via traditional (membrane) methods or via the pipette-tip method (for low-yielding *Pseudonaja* snakes) as described by Mirtschin et al. (2006), then immediately placed in a liquid nitrogen dry shipper for transport, and later stored at −80 °C.

All venoms were lyophilised and reconstituted in deionised water, centrifuged (4 °C, 5 min at 14,000 RCF), and the supernatant made into a 'working stock' (1 mg/mL) with 50% glycerol to prevent freezing at −20 °C. Protein concentrations were determined in triplicate using a NanoDrop 2000 UV–Vis Spectrophotometer (Thermofisher, Sydney, NSW, Australia) at an absorbance of 280 nm.

2.3. Coagulation experiments

Venom-induced clotting times of dog, cat, and human plasmas were automatically measured using a STA-R Max® analyser (Stago, Asnières sur Seine, France) in an 8-point dilution series of decreasing venom concentration (20 μg/mL to 0.05 μg/mL). Once the venom sample was loaded into the machine, pipetting into small cuvettes (250 μL max. Vol.) occurred automatically, according to pre-programmed experimental methodologies (Table 2). As a comparison to venom-induced clotting speeds, the maximum speed of fibrinogen clotting was measured by introducing a vast excess of thrombin to fibrinogen (Table 2), whereby thrombin cleaves fibrinogen into fibrin strands to form a clot.

Cofactors calcium and phospholipid (cephalin prepared from rabbit cerebral tissue) were included in all assays, as they are required for clotting, usually required for coagulotoxic venom activity (Nakagaki et al., 1992; St. Pierre et al., 2005), and can affect venom function (Rogalski et al., 2017; Sousa et al., 2018; Zdenek et al., 2019b). Clotting time (seconds) was automatically measured via a viscosity-based (mechanical) detection system: opposing magnets oscillate a small metal spherical pellet inside the test cuvette (250 μL total volume) until a clot is formed. Reagents were kept at 15–19 °C in the machine during experimentation and otherwise stored at 4 °C. Venom samples were replaced every 15–30 min to minimise enzymatic degradation. All tests were performed in triplicate.

2.4. Data analysis

AUCs (Area Under the Curve) of venom-induced clotting time curves were calculated using GraphPad Prism software (v.7.0). Two-way ANOVA Tukey tests were performed in this program to make multiple comparisons of every AUC mean with every other AUC mean so as to determine significance levels of venom-induced plasma clotting times of different plasma types. Specifically, three comparisons per snake venom were made: cat vs. dog; cat vs. human; dog vs. human. *P*-values $\leq .05$ were considered statistically significant.

The phylogenetic tree used for phylogenetic comparison of the normalised AUC data was based on timetree.org and input into Mesquite (version 3.2). This tree was then used for a comparative analysis in Rstudio using the APE package for basic data manipulation (Paradis et al., 2004). In order to investigate the evolutionary relationships of the AUC trait (i.e. clotting times produced by different snake species), and to compare these relationships between cat and dog plasmas, ancestral states were estimated over the tree using maximum likelihood in the contMap function of the R package phytools (Revell,

Table 2
Assay methodologies.

Assay	Methodology
Negative control (spontaneous clotting)	**Step 1**: 50 µL 50% glycerol stock diluted with Owren–Koller (OK) Buffer (isotonic saline) (Stago catalog # 00360)) + 50 µL 0.025 M calcium (Stago catalog # 00367) + 50 µL phospholipid (made up according to Stago kit; catalog #00597) + 25 µL OK buffer **Step 2**: 120 s incubation at 37 °C **Step 3**: Addition of 75 µL plasma (as a start reagent to then immediately begin measurement reading)
Venom curves	**Step 1**: 50 µL 0.1 µg/mL venom (from 1 mg/mL 50% glycerol stock diluted with OK buffer) (diluted by machine for 8-pt curve) + 50 µL 0.025 M calcium + 50 µL phospholipid +25 µL OK buffer **Step 2**: 120 s incubation at 37 °C **Step 3**: Addition of 75 µL plasma
Maximum speed of fibrinogen clotting	**Step 1**: 50 µL 6 mg/mL human fibrinogen (Sigma Aldrich Cat#F3879) + 50 µL 0.025 M calcium + 50 µL phospholipid +25 µL OK buffer **Step 2**: 120 s incubation at 37 °C **Step 3**: Addition of 50 µL thrombin (Stago Cat#00673)

2012).

3. Results

We conducted 117 8-point dose recf9wn in Table 3, along with maximum venom-induced clotting times at 20 µg/mL venom concentration. The relative potency and statistical comparisons of all snake venoms across all plasma types are illustrated via comparing AUCs in Fig. 2. Dog plasma consistently clotted quicker than cat plasma across all snake venoms (Fig. 3). *Pseudonaja textilis* venom clotted all plasmas quicker than all other snake venoms. The largest difference in venom effects produced on cat versus dog plasma was observed within *Echis*. Cat and human plasma clotted faster than each other, depending on the venom tested.

4. Discussion

We investigated the relative speed of clotting times produced by 11 procoagulant venoms from across the world on cat, dog, and human plasmas. All venoms produced the quickest clots in dog plasma. *Pseudonaja textilis* venom clotted all plasma types faster than any other venom, and among Australian venoms *P. porphyriacus* was least potent Congruent with these results, *P. textilis* was shown to consume clotting factors in humans quicker than *N. scutatus* (Isbister et al., 2010), and, even though venom potency is one of a multitude of factors which affects pathology and prognosis (Mirtschin and Davis, 1982), a mathematical model also predicted FXa toxins like those in *P. porphyriacus* and *N. scutatus* to produce less severe VICC (Venom-induced Consumption Coagulopathy) than prothrombinase toxins (Gulati et al., 2013) like those in *P. textilis* and *O. scutellatus*.

In the present study, *P. textilis* venom was clearly the most potent across all plasma types compared to all other venoms, making this venom possibly the fastest-acting procoagulant venom in the world. In fact, the speed at which *P. textilis* clots human plasma in our assay (5.67 ± 0.16 s) approaches our maximal recorded clotting speed for a vast excess of thrombin in fibrinogen tests (3.2 ± 0.1 s). Our *in vitro* finding of *P. textilis* venom being the most potent on all plasmas compared to other venoms is consistent with clinical reports, with envenomations from this species being rapidly life-threatening for humans (in as little as fifteen minutes) (Sutherland and Tibballs, 2001), and difficult to neutralise (with antivenom) in humans and dogs, requiring the largest antivenom dosage to ameliorate venom effects (Sprivulus et al., 1996).

The reported higher survival rate of cats vs. dogs (66% vs. 31%) to elapid snakebites in Australia (Mirtschin et al., 1998) could be due to physiological and behavioural factors acting synergistically or independently. Regarding physiological effects, the present study found that spontaneous clotting times of recalcified plasma (without venom added) varied vastly between plasma types (Table 3), with dog plasma naturally clotting quicker than cat and human plasma in negative control tests and all venom tests. These results were congruent with another study that similarly found quicker control clotting times in dogs

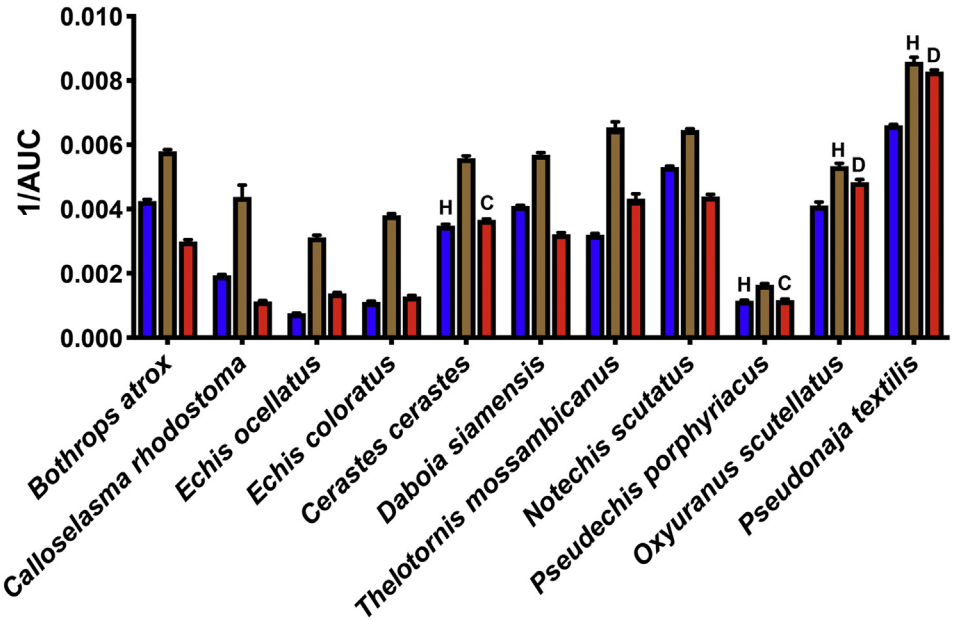

Fig. 2. Relative potency of 11 procoagulant snake venoms in 8-pt dilution curves across cat (blue), dog (brown), and human (red) plasma. AUC (Area Under the Curve) values were inverted (1/AUC) to illustrate more potent venoms with larger bars and vice versa for lesser potent venoms. All clotting time comparisons using a two-way (plasma type vs. venom type) ANOVA Tukey's multiple comparisons test were significant (p .05) *except* bars labelled with a letter (C = Cat; D = Dog; H = Human) above the bar; *C. cerastes* cat vs human, 0.23; *P. porphyriacus* cat vs human, 0.22; *O. scutellatus* dog vs human 0.07; *P. textilis* dog vs human 0.87. Data are average means from n = 3 AUCs, and error bars represent standard error.

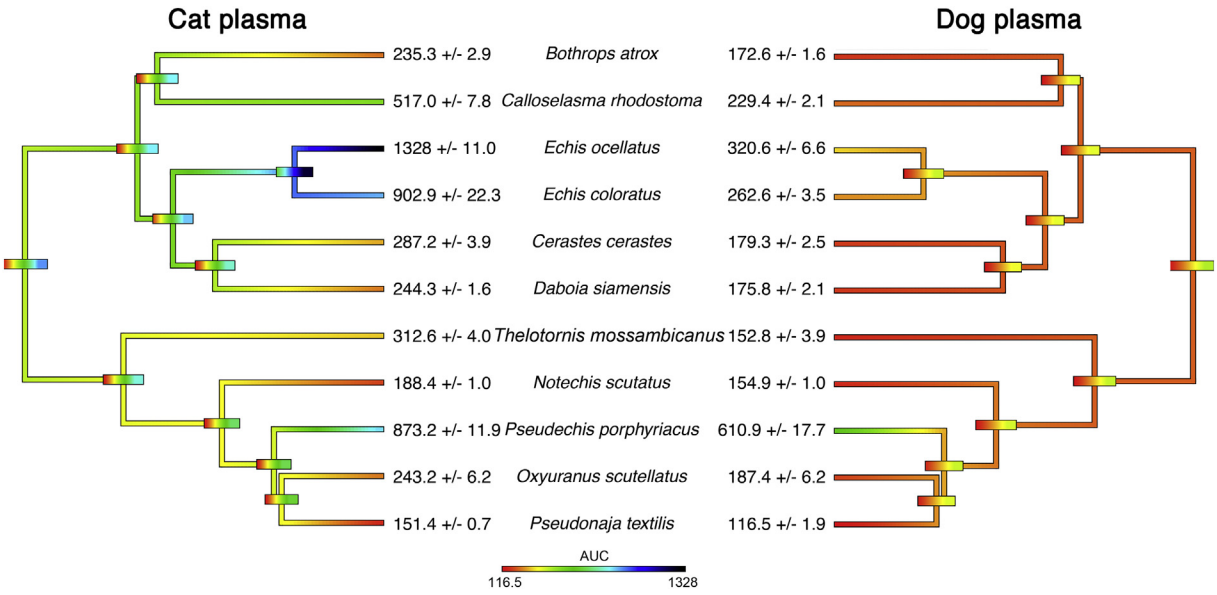

Fig. 3. Ancestral state reconstruction of AUC (Area Under the Curve) values (± SE) derived from 8-pt venom-dilution curves (in triplicate). Warmer colours represent more rapid clotting of the plasma (e.g. *P. textilis* venom most potent on both cat and dog plasmas), with the same colour scale for each venom, based on the lowest and highest venoms across the both sets (cat and dog). Error bars indicate 95% confidence intervals for the estimate at each node. Phylogenetic tree based on timetree.org.

Table 3
Clotting times (AUCs ± SE) of negative controls and venom maximum velocity tests across all plasma types, based on methodologies in Table 2.

Test	Cat	Dog	Human
Control			
Spontaneous clotting (negative control)	215.57 ± 4.04	108.53 ± 1.62	456.73 ± 5.89
Venom (20 μg/mL) maximum velocity			
Bothrops atrox	10.37 ± 0.15	7.43 ± 0.12	12.97 ± 0.67
Calloselasma rhodostoma	15.13 ± 0.68	7.9 ± 0.36	18.06 ± 0.64
Cerastes cerastes	11.53 ± 0.06	7.93 ± 0.12	11.37 ± 0.06
Daboia siamensis	11.00 ± 0.26	8.56 ± 0.21	12.57 ± 0.15
Echis coloratus	35.10 ± 4.26	10.60 ± 0.53	29.53 ± 3.70
Echis ocellatus	48.50 ± 1.77	11.93 ± 0.76	24.07 ± 1.67
Notechis scutatus	9.33 ± 0.15	7.8 ± 0.1	11.00 ± 0.30
Oxyuranus scutellatus	9.96 ± 0.37	7.67 ± 0.31	8.80 ± 0.20
Pseudechis porphyriacus	34.87 ± 0.78	24.20 ± 0.78	36.70 ± 3.37
Pseudonaja textilis	6.80 ± 0.00	5.10 ± 0.10	5.67 ± 0.16
Thelotornis mossambicanus	9.20 ± 0.00	5.13 ± 0.06	7.83 ± 0.40

than cats (Crawford and Mills, 1985). Varying speeds of spontaneous clotting times are probably attributed to elevated levels of coagulation factors in dogs compared to humans. For example, FVIII, the activated version of which activates Factor X to Factor Xa, is present in dogs at levels 8-fold that of humans (Giles et al., 1982). Domestic dogs, which evolved from the wolf (*Canus lupus*) (Leonard et al., 2002), may have evolved to clot quickly due to historically pack-hunting large and dangerous prey (e.g. wild boar, male deer, elk) that can inflict large wounds. Baseline clotting times would also be affected by the enzymatic *speed* of coagulation factors (not just their *concentration*), but this is unknown and has not been measured directly.

Variation in the natural clotting times of different plasmas may contribute to susceptibility to procoagulant snake venoms through a faster depletion of clotting factors and thus a quicker onset of the potentially fatal VICC condition (Isbister et al., 2010). This condition can persist until blood clotting factors have been replaced (resynthesised) by the body, which, in humans, requires ~14 hours (Isbister et al., 2009).

Physiological differences of plasma aside, another major variable which would affect venom-induced pathology in cats vs. dogs is their behaviour. Behavioural differences could affect the bite site location on the body, as well as the speed of venom travel throughout the body. Dogs typically investigate with their nose and mouth, which are highly vascularised areas, whereas cats typically swat with their paws. A bite to the paw would likely delay venom effects relative to a bite on the muzzle, as is evidenced by extensive human case reports which indicate a slower absorption of venom in limbs versus the face and core (Sutherland and Tibballs, 2001). Furthermore, dogs are typically more physically active than cats, which would lead to relatively quicker circulation of venom (and thus its effects) throughout the dog's body due to an increased heart rate. Consistent with this, the levels of venom in the blood were lower for cats than dogs (Moisidis et al., 1996).. Moreover, if behaviour among cats and dogs is consistently different, then this could help explain the reported (Mirtschin et al., 1998) greater survival in cats and thus have major implications for patient outcome.

Future research should test the potency of procoagulant venoms on other domesticated animals of economic importance across the world such as llamas, pigs, horses, and cows.

5. Conclusion

Our results indicate that dog plasma is particularly susceptible to procoagulant snake venoms compared to cat and human plasma. Our findings suggest that procoagulant snake venoms produce a more rapid onset of pathophysiological effects in dogs due to the inherent trait of dog plasma to naturally clot quicker. Thus, our observation that dog plasma consistently clotted quicker than cat plasma in both control and venom tests may explain the observed greater clinical susceptibility of dogs to procoagulant snake venoms. Behavioural differences between cats and dogs likely exacerbate the greater susceptibility of dogs to snakebite, making rapid snakebite first-aid and antivenom treatment particularly important for envenomated dogs.

Acknowledgments

CNZ and JSD were recipients of PhD scholarships administered by the University of Queensland. BGF was funded by Australian Research Council Discovery Project DP190100304. We thank Alana Morcus for assisting JL in cat and dog plasma collection.

Author contributions

CNZ conceived the experiments; CNZ and BGF designed the experiments; CNZ performed the experiments; CNZ analysed the data; JL, ND, LA, and BGF contributed reagents and materials; CNZ, BGF, JSD, LA, and JL wrote the paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared.

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