# Taxon-selective venom variation in adult and neonate *Daboia russelii* (Russell's Viper), and antivenom efficacy

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## ABSTRACT

Major variations in venom composition can occur between juvenile and adult venomous snakes. However, due to logistical constraints, antivenoms are produced using adult venoms in immunising mixtures, possibly resulting in limited neutralisation of juvenile snake venoms. *Daboia russelii* is one of the leading causes of snakebite death across South Asia. Its venom is potently procoagulant, causing stroke in prey animals but causing in humans consumptive coagulopathy—a net anticoagulant state—and sometimes death resulting from hemorrhage. In this *in vitro* study, we compared the venom activity of—and antivenom efficacy against—six 2-week-old *D. russelii* relative to that of their parents. Using a coagulation analyser, we quantified the relative coagulotoxicity of these venoms in human, avian, and amphibian plasma. The overall potency on human plasma was similar across all adult and neonate venoms, and SII (Serum Institute of India) antivenom was equipotent in neutralising these coagulotoxic effects. In addition, all venoms were also similar in their action upon avian plasma. In contrast, the neonate venoms were more potent on amphibian plasma, suggesting amphibians make up a larger proportion of neonate diet than adult diet. A similar venom potency in human and avian plasmas but varying selectivity for amphibian plasma suggests ontogenetic differences in toxin isoforms within the factor X or factor V activating classes, thereby providing a testable hypothesis for future transcriptomics work. By providing insights into the functional venom differences between adult and neonate *D. russelii* venoms, we hope to inform clinical treatment of patients envenomated by this deadly species and to shed new light on the natural history of these extremely medically important snakes.

## 1. Introduction

Snakebite is a largely neglected health issue globally, with millions of people bitten annually, resulting in hundreds of thousands of human deaths and permanently injuries, and incalculable socio-economic burden (Fry, 2018; Gutierrez et al., 2017; Harrison et al., 2009, 2011; Harrison and Gutierrez, 2016; Kasturiratne et al., 2005, 2008, 2017). Most snakebites occur in India, Southeast Asia, Latin America, and sub-Saharan Africa where both poverty and snakes are common, but also unfortunately where access to antivenom, snakebite awareness, and appropriate treatment is largely inadequate (Chippaux, 2008, 2011, 2012; Gutierrez et al., 2014; Harrison et al., 2009; Kasturiratne et al., 2008, 2017).

Due to strong selection pressures, venom is accelerated in its evolution relative to other traits, undergoing extensive gene duplication and diversification (Fry, 2005). Over millions of years, this evolutionary process has resulted in neofunctionalisation of endogenous proteins into numerous venom toxins across the animal kingdom via variations on the molecular surface (Casewell et al., 2020; Dashevsky and Fry, 2018; Sunagar et al., 2013, 2014). One positive Darwinian selection pressure

upon venom composition is diet, with this link being well established in numerous snake species (Barlow et al., 2009; Daltry et al., 1996; Gibbs et al., 2011; Harris et al., 2020a, 2020b; Healy et al., 2019; Sousa et al., 2018; Youngman et al., 2019, 2021). With the abundance and diversity of prey types varying across habitats, variation in venom has repeatedly been linked to geography (Bourke et al., 2021; Chippaux et al., 1991; Sanchez et al., 2015; Senji Laxme et al., 2021; Sunagar et al., 2014). Differences in body size and ecological niches, both of which affect diet, can similarly drive divergent selection pressures upon different life-stages of snakes.

Ontogenetic differences in life history strategies can influence venom variation, resulting in neonates and adults possessing different venom composition (termed ontogenetic venom variation). This is important to quantify and understand because it can affect clinical envenoming syndromes, confuse diagnosis, and complicate treatment (Knudsen et al., 2021). Ontogenetic variation in venom composition has been shown for a wide range of venomous snakes, including the colubrid genus *Boiga* (Mackessy et al., 2006), the elapid genera *Pseudonaja* (Cipriani et al., 2017; Jackson et al., 2016) and *Suta* (Zdenek et al., 2019a), and the viperid genera *Bothriechis* (Pla et al., 2017), *Bothrops* e.g. (Freitas-de-Sousa et al., 2020; Rodrigues et al., 2021), *Crotalus* e.g. (Rokyta et al., 2017; Seneci et al., 2021), *Gloydius* (Gao et al., 2013), *Lachesis* (Gutierrez et al., 1990; Madrigal et al., 2012), and *Metlapilcoatlus* (Garcia-Osorio et al., 2020). Ontogenetic venom variation has been shown to affect envenomation syndromes. For example, coagulopathy was reportedly more pronounced for neonate envenomations by some *Bothrops* species (Bernal et al., 2020; Guercio et al., 2006; Milani Junior et al., 1997). Amongst the most extreme ontogenetic venom variation is that seen in the Australian elapid genus *Pseudonaja* (brown snakes), whereby neonates lack coagulotoxins present in adult venoms, being instead potently neurotoxic (Cipriani et al., 2017; Jackson et al., 2016). Ontogenetic venom changes potentially affect antivenom efficacy due to adult venoms being used in the immunising mixture for antivenom production because of logistical constraints of neonates producing much smaller venom yields. The widespread prevalence of ontogenetic changes in venom composition therefore illustrates the importance of investigating these features in highly venomous and medically significant species.

*Daboia russelii* and *D. siamensis* are cryptic ambush predators, reaching lengths of 100–120 cm, and giving birth to 20–60 live young (ovoviviparous). Adults are thought to eat mostly rodents and gerbils (*Tatera indica*), while juveniles reportedly eat anything 'they can overpower', including conspecifics, land crabs, scorpions, beetles, lizards, and anurans (toads and frogs) (Wüster, 1998). They inhabit 'fairly open areas' that are grassy or bushy, including rice fields and other agricultural lands (Wüster, 1998). Being mostly nocturnal (although see below) and occurring throughout 10 South Asian countries, *D. russelii* and *D. siamensis* are the leading cause of fatal snakebite in Pakistan, Sri Lanka, India, Bangladesh, Burma and Thailand (Alirol et al., 2010; Warrell, 1989). Despite being sluggish and reclusive, flooding events and habitat invasion by humans bring *D. russelii* in contact with humans. For example, a recent 9-mo radiotelemetry study of 18 *D. russelii* revealed they ambush foraged during daytime throughout the year and spent a substantial amount of time in plantations, which make up a significant proportion of the landscape in the human-dominated rural landscape in Karnataka, India (Glaudas, 2021). As a result, unfortunately the human-snakebite conflict regarding *D. russelii* is acute.

Common clinical manifestations of *D. russelii* bites include local swelling (edema), blood disturbances (coagulopathy), neuromuscular paralysis, myotoxicity, and subsequent renal failure. Pituitary heaemorrhage has been reported from patients in Burma and southern India, resulting in life-changing hormonal abnormalities (e.g. hoarse voice, decreased libido, and failure of penile erection) for some victims (Warrell, 1989). It should be acknowledged that, while effects of *D. russelii* venom can be deadly, the venom is a useful bioresource that can, counterintuitively, improve human health. That is, because

*D. russelii* venom is resistant to the widely used interfering anticoagulant heparin, dRVVT (dilute Russell's Viper Venom Time) is the most widely used clotting test to diagnose lupus anticoagulants (LA), a debilitating and sometimes deadly auto-immune disease (Hillarp et al., 2020; Jacquot et al., 2019; Triplett, 2000).

Coagulopathy, or disturbances to blood coagulation, is difficult to treat and is a primary pathology leading to death in many snakebite victims. Adult *D. russelii* venom reduces blood coagulability via P-IIId snake venom metalloprotease enzymes activating the blood clotting component factor X, which in turns converts prothrombin to endogenous thrombin (Chowdhury et al., 2021). Thrombin in turn converts fibrinogen into fibrin, resulting in strong, stable fibrin clots (Chowdhury et al., 2021; Gowda et al., 1994; Kini and Koh, 2016; Kisiel et al., 1976; Morita, 1998; Schiffman et al., 1969; Siigur and Siigur, 2010; Suntravat et al., 2010; Takeya et al., 1992; Yamada et al., 1997). The venom also activates factor V via serine proteases (Mukherjee, 2014). In prey, this venom activity results in rapid incapacitation by stroke due to a higher venom-to-blood ratio (Loria et al., 2003; Martin, 1893). Conversely, in humans the dilution of venom into a much larger blood volume results in the consumption of fibrinogen and/or other factor deficiencies, reducing coagulability, and causing internal bleeding (Maduwage and Isbister, 2014).

As discussed, because diet is strongly linked to venom composition, if diet differs between life stages in a species, then venom composition would also likely differ. In the case of the *D. russelii*, the diet has been shown to differ between life stages, but it is unknown whether this corresponds to ontogenetic differences in venom activity, clinical effects, and antivenom efficacy. While most studies that investigate variation in *D. russelii* venom focus on geographical venom variation (Faisal et al., 2021; Pla et al., 2019; Senji Laxme et al., 2021), this study focuses on ontogenetic venom variation. We set out to test the coagulotoxic action of—and antivenom efficacy against—venoms from a family of *D. russelii*. We performed *in vitro* tests upon human plasma to provide a medical perspective and also on avian and amphibian plasma to provide an evolutionary perspective.

## 2. Materials and methods

### 2.1. Venom and plasma stocks

All venom and plasma work were undertaken under the University of Queensland biosafety approval IBC134BSBS2015 and animal ethics approval SBS/019/14/ARC. Individual venoms from two captive Pakistan locality adult (female and male) *D. russelii* and their six progeny were included in the study. Venoms were extracted from all specimens on the same day, when the neonates were 14 days old. Venoms were flash-frozen with liquid nitrogen and then freeze-dried (lyophilised) for stable storage. Working stocks of venom were made to 1 mg/mL concentration with 50% deionised water and 50% glycerol to prevent freezing at −20 °C.

Avian plasma was collected from healthy, domestic chickens (*Gallus domesticus*) without antibiotics in their system. Whole blood was extracted from the brachial wing vein, immediately dispensed into MiniCollect tubes (Greiner Bio-One Ref: 450,413 (now discontinued)) with 3.2% citrate, and centrifuged (2000 RCF for 10min, then the supernatant centrifuged again (10,000 RCF for 10min). Tubes were then flash-frozen in liquid nitrogen and stored at −80 °C until pooling. Cane Toad (*Rhinella marina*) plasma was collected and handled in the same manner except dispatched as an end result. Cane Toads were used as a model for amphibian because of their large size, and also because they a feral status in Australia, making euthanasia more ethically acceptable than using smaller, threatened native species.

Two bags of 3.2% citrated human plasma (Label # 4,133,302 O+ and 6,187,639 A+) were obtained from the Australian Red Cross and pooled together. The plasma was then aliquoted to 1 mL quantities, flash-frozen in liquid nitrogen, and stored at −80 °C until required. For testing,

aliquots were defrosted for 5 min in at 37 °C in a Thermo Haake ARCTIC water bath with a SC150-A40 circulator. Defrosted plasma aliquots were used for a maximum of 1 h and then replaced.

## 2.2. Clotting times

Clotting times were measured using the STA-R Max® (Stago, Asnières sur Seine, France) coagulation analyser and adapted from validated coagulation assay protocols (Chowdhury et al., 2021; Lister et al., 2017; Rogalski et al., 2017; Zdenek et al., 2019b). 1 mg/mL venom stocks (50% glycerol/50% deionised water) were diluted 1:10 with OK Buffer (sterilised physiological saline, Stago catalogue #00360) and loaded into the analyser to run concentration curves with the following dilutions: 1, 1:2, 1/5:, 1:12.5, 1:30, 1:80, 1:160, and 1:400. Final venom concentrations were: (µg/mL) 20, 10, 4, 1.6, 0.66, 0.25, 0.125, 0.05). For each test, the following were added to the plastic cuvette (250 µL total volume): 50 µL of diluted venom, 25 µL OK buffer, 50 µL 0.025 M calcium chloride (Stago catalogue # 00367), and 50 µL phospholipid (cephalin prepared from rabbit cerebral tissue adapted from STA C·K Prest standard kit, Stago Catalog # 00597, solubilised in 5 mL of Owren Koller (OK) Buffer [Stago Cat# 00360])). The cuvette was then briefly shaken by the machine to mix the contents and was then incubated for 2 min at 37 °C. Then 75 µl of undiluted plasma was added after incubation, and clotting time was recorded immediately.

Clotting time (seconds) of human plasma was automatically measured via a viscosity-based (mechanical) detection system whereby opposing magnets oscillate a small, spherical pellet inside the test cuvette until a clot is formed. Avian and amphibian plasmas were not run on this assay due to limited supply of these plasmas. Reagents were kept at 15–19 °C in the machine during experimentation and otherwise stored at 4 °C. Venom samples in the analyser were replaced every 15–30 min to minimise enzymatic degradation.

Coagulation activator kaolin (Stago C·K Prest standard kit, Stago catalogue #00597) was used as a daily positive control to check for consistent plasma clotting responses. For the negative control, 1:1 deionised water/glycerol was used in place of venom in the assay to determine spontaneous clotting time.

## 2.3. Antivenom efficacy

The ability of Serum Institute of India (SII) antivenom (details below) to neutralise coagulotoxic venom effects *in vitro* was tested by repeating the above mentioned concentration curves and replacing the 25 µL of OK buffer (added to the cuvette before incubation) with 25 µL of OK buffer and SII antivenom (final cuvette concentration of 0.5%). We used a variable venom concentration and fixed antivenom concentration so that the concentration curves of venom-only could be compared with dilution curves of venom incubated with antivenom.

SII antivenom is an equine F (ab')2 antivenom prepared by hyper-immunising horses to the venom of *Echis carinatus, Daboia russelli, Naja naja* and *Bungarus caeruleus*. The package insert claims that every mL of antivenom neutralises no less than the following dry weights (mg) of the venoms, respectively: 0.45, 0.60, 0.60, and 0.45. While SII antivenom is no longer being produced, a similar relative pattern of antivenom efficacy of adults to neonates compared to current antivenoms may be expected because most venom for antivenom production in India is sourced from the same region: Chennai in the southeastern Indian state of Tamil Nadu (Pla et al., 2019; Sharma et al., 2015; Whitaker and Whitaker, 2012).

Antivenom efficacy against venom was determined using the area under the curve (AUC) (calculated by GraphPad) values in an X-fold shift formula in Excel:

$$\frac{AUC \; of \; venom \; incubated \; with \; antivenom}{AUC \; of \; venom} - 1$$

An X-fold shift of zero indicates no neutralisation (no change of clotting time curve); values > 0 indicate venom neutralisation (change in clotting time curve).

## 2.4. Factor X activation assay

Fluoroskan Ascent™ (Thermo Scientific, Vantaa, Finland) was used to detect factor X activation by all venoms. Reagents mentioned in Table 1 were manually plated in 384-well plates (black, lot#1171125, Nunc™ Thermo Scientific, Rochester, NY, USA), followed by automated pipetting of 70 µl of enzyme buffer without calcium (150 mM NaCl, and 50 mM Tri-HCl, pH 7.3) and Fluorogenic Peptide Substrate, (ES011Boc-Val-Pro-Arg-AMC. Boc: t-Butyloxycarbonyl; 7-Amino-4-methylcoumarin; R & D systems, Cat# ES011, Minneapolis, Minnesota) in 500:1 ratio to start the reaction. The plate was warmed to 37 °C, then shaken for 3 s before each measurement. The reaction was measured every 10 s for 300 times at 390/460 nm (excitation/emission). The fluorescence was generated by the cleavage of the substrate and measured by Ascent® Software v2.6 (Thermo Scientific, Vantaa, Finland). To obtain results of interest, blank (background) values were subtracted from all other reactions, followed by subtraction of "venom without zymogen" values from "venom with zymogen" values. This was done to subtract the venom's action directly on the substrate. The resultant values were

**Table 1**
Factor X activation assay conditions*.

| | |
|---|---|
| Blank | 20 µL of enzyme buffer without calcium [(150 mM NaCl, and 50 mM Tris-HCl (pH 7.3)] |
| Positive Control with Activated factor X (FXa) or Thrombin | 10 µL of enzyme buffer without calcium + 10 µL of 10 µg/mL FXa (Haematologic Technologies catalog # HCXA-0060) or 1 µg/mL thrombin (Haematologic Technologies catalog # HCT-0020)) |
| Control with factor X Zymogen or prothrombin | 10 µL of enzyme buffer without calcium + 10 µL of (10 µg/mL FX (Haematologic Technologies catalog # HCX-0050) or 1 µg/mL prothrombin (Haematologic Technologies catalog # HCP 0010)) |
| Venom without factor X Zymogen or prothrombin | 10 µL of enzyme buffer without calcium + 10 µL venom of 1 µg/mL FX (Haematologic Technologies catalog # HCX-0050) activation or 0.1 µg/mL for prothrombin (Haematologic Technologies catalog # HCP 0010)) activation |
| Venom with factor X Zymogen or prothrombin | 10 µL zymogen (10 µg/mL FX or 1 µg/mL prothrombin) + 10 µL venom of 1 µg/mL FX (Haematologic Technologies catalog # HCX-0050) activation or 0.1 µg/mL for prothrombin (Haematologic Technologies catalog # HCP 0010)) activation |
| Blank | 20 µL of enzyme buffer without calcium [(150 mM NaCl, and 50 mM Tris-HCl (pH 7.3)] |
| Positive Control with activated factor X (FXa) or Thrombin | 10 µL of enzyme buffer without calcium + 10 µL of 10 µg/mL FXa (Haematologic Technologies catalog # HCXA-0060) or 1 µg/mL thrombin (Haematologic Technologies catalog # HCT-0020)) |
| Control with factor X Zymogen or prothrombin | 10 µL of enzyme buffer without calcium + 10 µL of (10 µg/mL FX (Haematologic Technologies catalog # HCX-0050) or 1 µg/mL prothrombin (Haematologic Technologies catalog # HCP 0010)) |
| Venom without factor X Zymogen or prothrombin | 10 µL of enzyme buffer without calcium + 10 µL venom of 1 µg/mL FX (Haematologic Technologies catalog # HCX-0050) activation or 0.1 µg/mL for prothrombin (Haematologic Technologies catalog # HCP 0010)) activation |
| Venom with factor X Zymogen or prothrombin | 10 µL zymogen (10 µg/mL FX or 1 µg/mL prothrombin) + 10 µLvenom of 1 µg/mL FX (Haematologic Technologies catalog # HCX-0050) activation or 0.1 µg/mL for prothrombin (Haematologic Technologies catalog # HCP 0010)) activation |

*All assays had 10 µL of phospholipid + the remaining well volume (up to 100 µL total) being 'substrate with calcium'. See Methods for details.

**Table 2**
Statistical results comparing the function activity of adult and neonate venoms.

| Assay | Mann-Whitney $U$ tests (unequal sample size, non-Gaussian) | | |
|---|---|---|---|
| | U-value | N-value | P-value |
| Clotting time (sec) | 0 | $n_1 = 6$, $n_2 = 18$ | **<0.0001** |
| Clotting time (AUC) | 0 | $n_1 = 6$, $n_2 = 18$ | **<0.0001** |
| FX activation | 0 | $n_1 = 6$, $n_2 = 18$ | **<0.0001** |
| PT activation | 52 | $n_1 = 6$, $n_2 = 18$ | 0.93 |
| Antivenom efficacy | 45 | $n_1 = 6$, $n_2 = 18$ | 0.58 |
| TEG avian SP | 39.5 | $n_1 = 6$, $n_2 = 18$ | 0.34 |
| TEG amphibian SP | 0 | $n_1 = 6$, $n_2 = 18$ | <0.0001 |
| | **Welch's *t*-test** (unequal sample size, Gaussian) | | |
| | **T-value** | **df** | **P-value** |
| TEG human SP | 0.00 | 14.58 | >0.99 |
| TEG human MA | 20.43 | 17.50 | **<0.0001** |
| TEG avian MA | 2.179 | 6.47 | 0.069 |
| TEG amphibian MA | 5.612 | 18.07 | **<0.0001** |

Significant results are bolded. Gaussian refers to Gaussian distribution.
SP=Split Point (clotting time); MA = Maximum amplitude (clot strength).

normalised as a percentage relative to the positive controls FXa or thrombin (see Table 2).

### 2.5. Thromboelastographic analyses

To evaluate the clot strength produced by the venoms on human, avian (domestic chicken as the model species), and amphibian (cane toad as the model species) plasma, investigations was carried out on TEG5000 haemostasis analysers (Haemonetics®, Haemonetics.com, catalogue # 07–033). Although clotting times of human plasma were measured on the coagulation analyser, this variable was measured again to compare with that of our avian and amphibian plasmas on this assay. This assay mirrored the reagents and ratios of the coagulation analyser assay. Specifically, it included consecutive addition of 72 µl of 0.025 M CaCl₂, 72 µl phospholipid, 20 µl of the OK buffer, 7 µl of 1 mg/mL of venom (50% glycerol), and 189 µl plasma to the reaction cup, followed by automated measurement for 30 min.

The positive control for human and avian plasmas was Thrombin (STA – Liquid FIB standard kit, Stago, cat#00673): 7 µL Thrombin replaced 7 µL of venom. Because Thrombin does not clot amphibian plasma (Sousa et al., 2020), we instead used Kaolin (a clay mineral (5 mg of Kaolin/mL) (Stago kit; cat. # 00597) to initiate a clot.

The mechanism for measurement is as follows: contents of the assay sit inside a small cup which is rotated gently left and right by 4° 45′, six times a minute. Movement of the cup imitates sluggish venous flow and activates coagulation. At the top of the plastic cup, a sensor probe sits inside a plastic shaft, where the clot forms around, causing resistance that is measured by the metal probe.

### 2.6. Statistical analyses

All tests were run in triplicate. Data were organised in Excel and then analysed in GraphPad PRISM 8.1.1 (GraphPad Prism Inc., La Jolla, CA, USA). Correlation between adult vs neonate venom effects was ascertained using the Pearson's product-moment correlation test in R Studio. For statistical tests of factor activation and TEG data to compare neonates and adults (which had unequal sample sizes), we used the Shapiro-Wilk test to test for normality. When the data passed the test, Welch's t-tests were used (equal SDs not assumed); when failed, unpaired Mann-Whitney $U$ tests were used.

### 3. Results and discussion

We studied the functional activity of adult and neonate *D. russelii* venoms *in vitro* regarding antivenom efficacy and coagulation disturbances upon human, avian, and amphibian plasmas.

Neonates and adult venoms of *D. russelii* produced similar clotting

times on human plasma in concentration curves (Fig. 1). While there was a statistically significant ontogenetic difference of clotting times at 20 µg/mL venom concentration, with neonates being more potent (Mann-Whitney $U = 0$, $n_1 = 6$, $n_2 = 18$, p < 0.0001), the differences between the venom potencies (as low as 15%) were not biologically noteworthy in our opinion. In contrast, the relative activation of factor X displayed a very strong ontogenetic signal, with neonate venom exhibiting a potency that was highly significantly greater than adult venom (Mann-Whitney $U = 0$, $n_1 = 6$, $n_2 = 18$, p < 0.0001) (Fig. 2, Table 2).

Importantly, the observed activation of factor X is only one mechanism of the procoagulant activity: *D. russelii* venom has been shown also activate factor V (Mukherjee, 2014). Such a discordance between net procoagulant effect and specific action upon factor X has been noted previously for *Macrovipera* venoms, with the discordance hypothesised to be due to the venoms being less active on factor X and having a proportionally greater activation activity upon factor V (Chowdhury et al., 2021). Due to limited venom quantities, it was beyond the scope of this study to ascertain the relative effect of the included *D. russelii* venoms upon factor V, but this should be the subject of future research. However, in terms of net coagulotoxic effect, while Mukherjee et al. (2014) found proteases in Indian *D. russelii* venom exhibited a dose-dependent defibrinogenation and anticoagulant activity in a mouse model (Mukherjee, 2014), a pooled sample of the two adult Pakistan venoms used in this study was found to have no direct action on fibrinogen (Chowdhury et al., 2021), thus suggesting regional variation in this trait (Chowdhury et al., 2021). In addition, the proteases in the Mukherjee et al. (2014) study were purified and in and of themselves were minor components in the venoms (Mukherjee, 2014). The activation of prothrombin was negligible for all venoms, with no ontogenetic signal (Mann-Whitney $U = 52$, $n_1 = 6$, $n_2 = 18$, p = 0.93) (Fig. 2). For antivenom efficacy, there was likewise no ontogenetic signal (Mann-Whitney $U = 45$, $n_1 = 6$, $n_2 = 18$, p = 0.58) (Fig. 2).

We investigated clot strength within and between the plasma types, focusing on two clotting parameters: Clotting Time = time to initial clot formation (SP, Split Point), and Maximum Clot Firmness = maximum amplitude of formed clot (mm) (MA, Maximum Amplitude). For human plasma, SP had no ontogenetic signal (Welch's t = 0, df = 14.58, p > 0.99), but MA had a highly significant ontogenetic signal, with neonates producing stronger clots (Welch's t = 20.43, df = 17.50, p < 0.0001) (Fig. 3, Table 2). The strong ontogenetic signal for MA on human plasma was congruent with neonates having greater ability to activate factor X. Thus, MA and FX-activation were strongly associated with each other (correlation of 0.99, with a p-value of <0.0001). For avian plasma, there was no ontogenetic signal for any of the clotting parameters: SP: Mann-Whitney $U = 39.5$, $n_1 = 6$, $n_2 = 18$, p = 0.34; and MA Welch's t = 2.179, df = 6.47, p = 0.069 (Table 2).

For amphibian plasma, there was a highly significant ontogenetic signal, with neonates clotting the plasma quicker (SP: Mann-Whitney $U = 0$, $n_1 = 6$, $n_2 = 18$, p < 0.0001) (Fig. 3). Likewise, neonates produced significantly stronger clots than adults (MA: Welch's t = 5.612, df = 18.07, p < 0.0001). These results are of particular interest, as previous studies have shown that, due to variations in the sequences of clotting enzyme zymogens, different animal plasmas have varying susceptibility to coagulotoxins due to variation in the type and structure of clotting factors present (Maduwage et al., 2016). This suggests that action upon amphibian plasma is accomplished by toxins selective for amphibian clotting factors (Seneci et al., 2021; Sousa et al., 2018; Youngman et al., 2021). Therefore, potent venom action by neonate venoms upon amphibian plasma is suggestive of amphibians included in their diet. While our amphibian model was a toad, frogs may similarly be targeted by neonates because toads and frogs are much more genetically related to each other (7 million years taxonomic distance) compared to avians/humans (40 million years taxonomic distance) (timetree.org). Regardless, field observations (and/or gut analysis of museum specimens) are required to confirm amphibian predation by neonates.

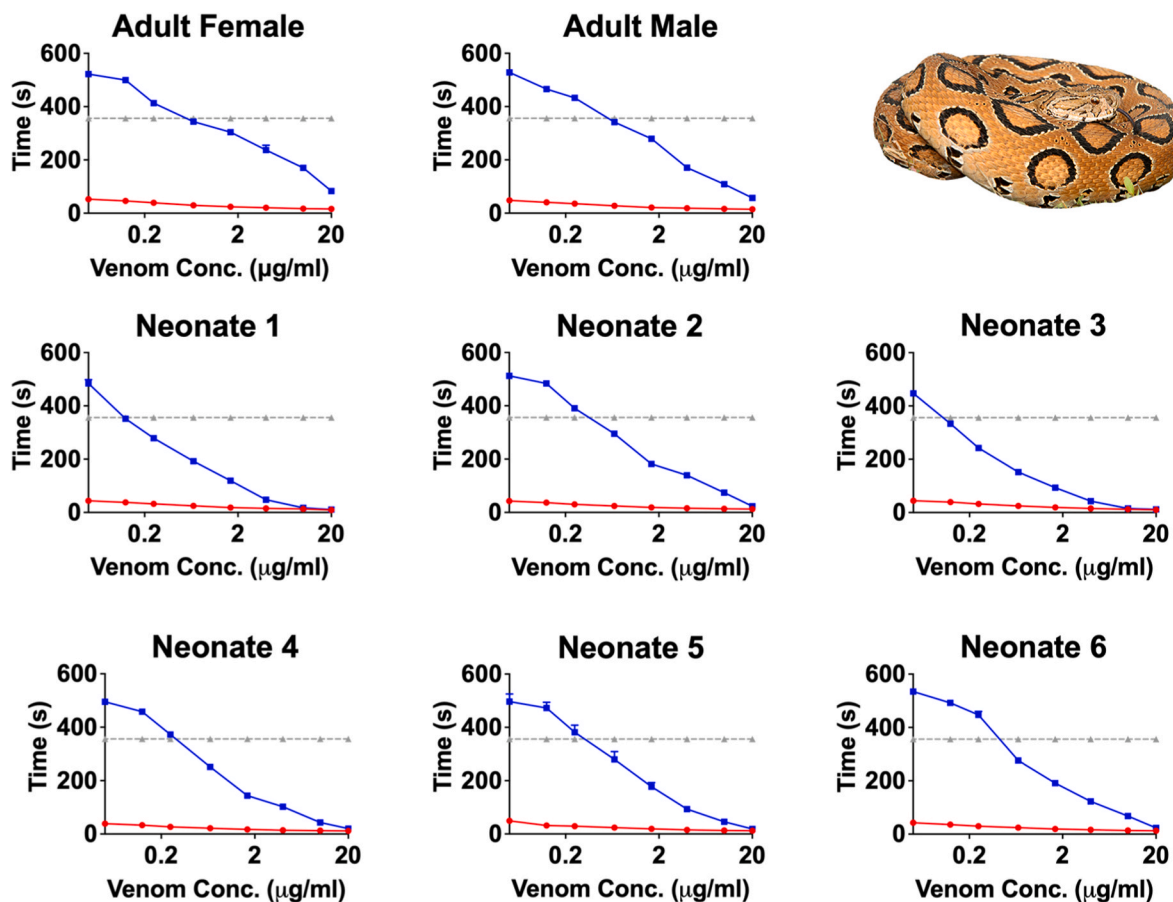These significant differences in venom function between neonates

**Fig. 1.** Concentration curves, with venom-induced clotting time on human plasma (red), and with Serum Institute of India antivenom (final concentration 0.5%) (blue), compared to negative control values of spontaneous clotting (356.06 ± 5.7 s) (grey). For each species, linear graphs are presented on the left and logarithmic views on the right. Conc. = Concentration. Values are n = 3 mean and SEM error bars (some smaller than the symbol). *Daboia russelii* image: © W. Wuster. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and adults suggest ontogenetic variation in venom-either via the presence of different isoforms, or the proportion of different toxin types in the venoms. Supporting evidence for the presence of different isoforms between the different age classes is a rare case following a bite from a juvenile *D. russelii* in a 16-year-old male in Tamil Nadu, India, where a bite to the ankle resulted in a painful erection (priapism), which only subsided with antivenom treatment (Senthilkumaran et al., 2021). No bites from adult *D. russelii* to date have been reported to cause this painful symptom, suggesting some differences in juvenile *D. russelii* venom compared to that of adults. Both this report and our results of same overall biochemical action being preserved, including potency on human and avian plasmas, yet variable potency on amphibian plasma, suggest the presence of ontogenetic variations of toxin isoforms within toxin classes (factor X activation by P-IIId SVMP or factor V activation by serine proteases). More common ontogenetic venom variation occurs via different toxin classes being present in the venoms of different life-stages, such as *Crotalus culminatus* being procoagulant as neonates but anticoagulant as adults, or at the most extreme with coagulotoxins being absent in the venom of juvenile *Pseudonaja* species, with juveniles being neurotoxic instead (Cipriani et al., 2017; Jackson et al., 2016; Seneci et al., 2021). Future work should use transcriptomics to determine which genes are being expressed to produce the presumed different isoforms of FX and FV activators observed in adult and neonate *D. russelii* venoms.

As the leading cause of fatal snakebite in Pakistan, Sri Lanka, India, Bangladesh, Burma and Thailand (Alirol et al., 2010; Warrell, 1989), *D. russelii* is a highly medically significant snake. Our *in vitro* data suggest that neonate specimens are as capable as adults—drop for drop of

venom—for their potential to cause severe consumptive coagulopathy. The data also suggests that SII antivenom is equally effective against adult and neonate venom. An important caveat here is that different antivenoms currently in production in India may have different relative and absolute efficacy against these venoms. Another caveat is that adult *D. russelii* produce nearly triple the venom yield than juvenile snakes (adults: 127 ± 13 mg, mean ± SE; juveniles: 45 ± 7 mg) (Tun and Khin Aung, 1986), meaning that bites from adult snakes are likely to produce more severe envenomation than neonates. Our study can guide future work such as *in vivo* studies that may provide addition insights into variations in pathophysiological effects, including regarding other venom-induced syndromes besides coagulotoxicity (e.g. myotoxicity and neurotoxicity). Thus, future research should investigate ontogenetic differences for other pathophysiological effects of *D. russelii* venoms.

This is the first study to investigate ontogenetic differences of venom function in the *D. russelii*, and it guides future work on the venom and ecology of *D. russelii*, particularly across snake age classes. We hope our study helps to inform evidence-based design of clinical management strategies for the envenomed patient, as well as provide insights to evolutionary biology., and the results of this study inform in the management of envenomation and provide insights into evolutionary biology.
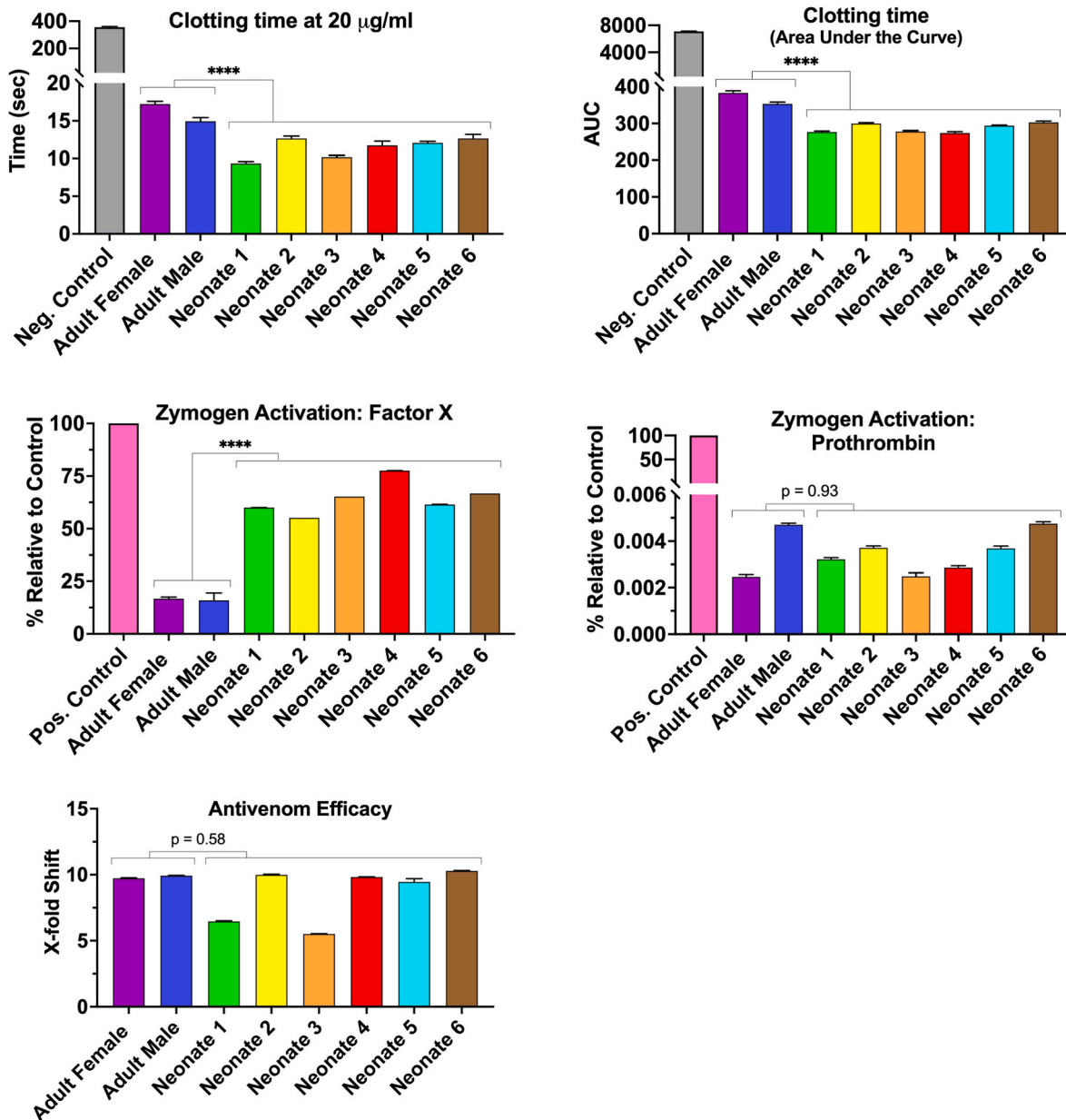
**Fig. 2.** Relative venom activity for the ability to clot human plasma, activate clotting factor zymogens, and antivenom efficacy. Values are n = 3 mean with SD error bars. Neg. = negative; Pos. = positive. Negative controls for top row were a blank in replace of venom. Positive controls for middle row were the activated factor (e.g. FXa or Thrombin). For clotting times and AUC, smaller values indicate greater potency. For zymogen activation and antivenom efficacy, larger values indicate greater activation or efficacy, respectively. Antivenom efficacy was calculated as per the Methods, whereby a value of 0 indicates no shift (no neutralisation by antivenom), while >0 indicates neutralisation by antivenom. Values are mean ± SD of N = 3. Statistical tests are Mann-Whitney U tests, with **** indicating $p < 0.0001$. Clotting time (sec) stats: Mann-Whitney $U = 0$, $n_1 = 6$, $n_2 = 18$, $p < 0.0001$. Clotting time (AUC): Mann-Whitney $U = 0$, $n_1 = 6$, $n_2 = 18$, $p < 0.0001$. FX activation stats: Mann-Whitney $U = 0$, $n_1 = 6$, $n_2 = 18$, $p < 0.0001$. Prothrombin stats: Mann-Whitney $U = 52$, $n_1 = 6$, $n_2 = 18$, $p = 0.93$. Antivenom efficacy stats: Mann-Whitney $U = 45$, $n_1 = 6$, $n_2 = 18$, $p = 0.58$.

### Con/flicts of interest/Competing interests

None.

### Availability of data and material

All data are in figures.

### Code availability

No code used.

### Ethics approval

University of Queensland biosafety approval IBC134BSBS2015 and animal ethics approval SBS/019/14/ARC.

### Credit author statement

Concept BGF and FJV. Resources: BGF, FJV, AV, RF, TC. Laboratory investigation: CNZ, AC, GYHH, BGF, Data analysis: CNZ, AC, BGF. Writing – primary draft: CNZ, AC, BGF. Wiring – editing: all authors, Christina N. Zdenek[1], Abhinandan Chowdhury[1], Grace Y. H. Haw[1], Aude Violette[2], Rudy Fourmy[2], Thomas Christ[3], Freek J. Vonk[4,5@], Bryan G.

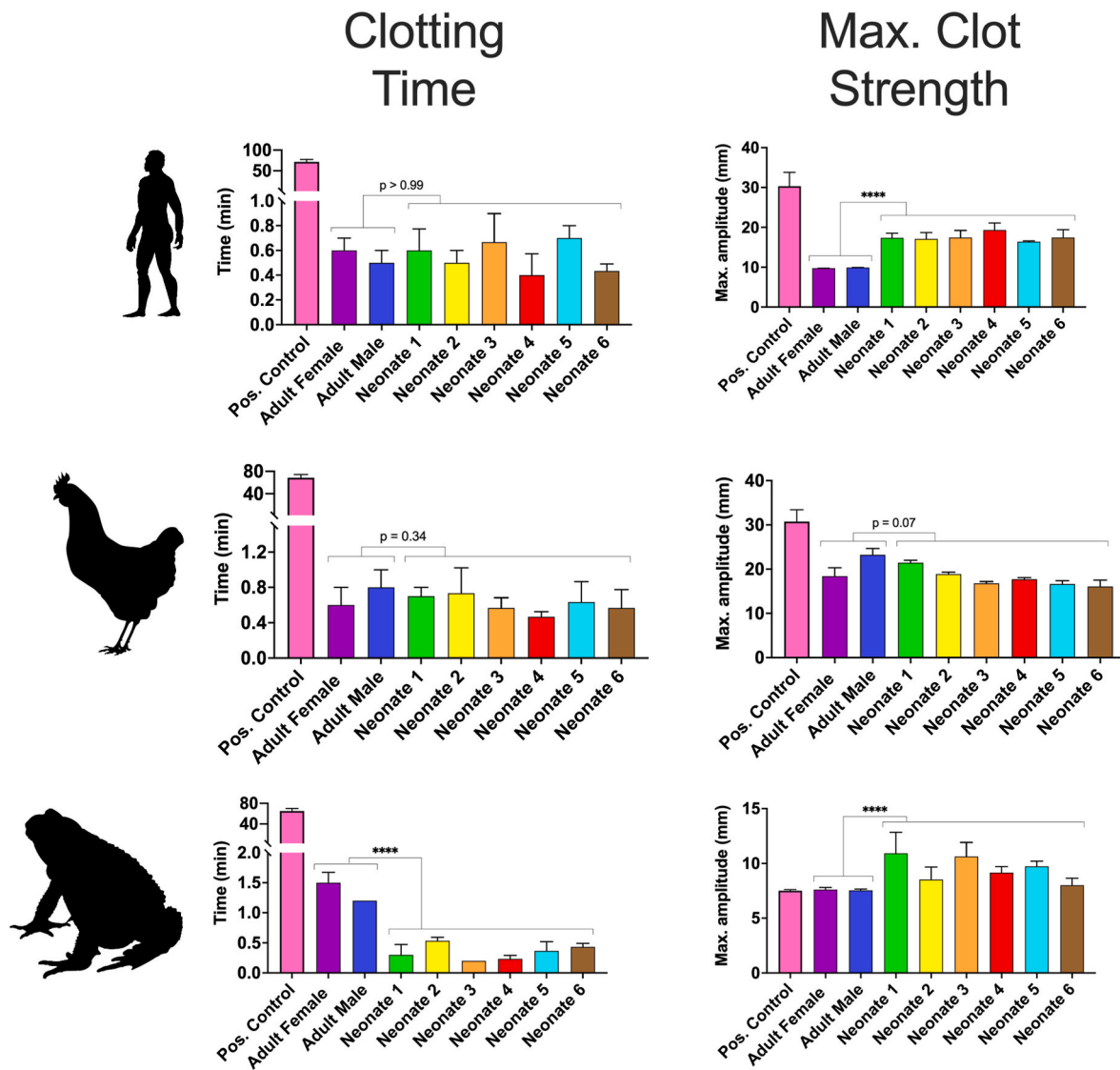**Fig. 3.** Thromboelastographic comparison of *D. russelii* venoms (19.8 μg/mL) on human (top row), avian (middle row), and amphibian (bottom row) plasmas for the clot parameters: Clotting Time (left column) = time to initial clot formation (SP, Split Point); Maximum Clot Firmness (right column) = maximum amplitude of formed clot (mm) (MA, Maximum Amplitude). Values are n = 3 mean with SD error bars. Pos. = Positive. Thrombin was used as a positive control for human and avian plasma, and Kaolin was used for amphibian plasma (see Methods). For Clotting Time, higher numbers = slower action. For Max. Clot Firmness, higher numbers = stronger clots. Animal silhouettes: copyright-free from Phylopic.org.

Fry[1@].

### Ethical statement

All venom and plasma work were undertaken under the UQ approval IBC134BSBS2015 and UQ Animal Ethics approval SBS/019/14/ARC.

### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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