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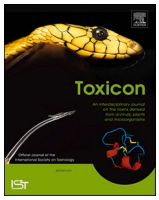

In vitro anticoagulant effects of *Bungarus* venoms on human plasma which are effectively neutralized by the PLA_2-inhibitor varespladib

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ABSTRACT

Bungarus (krait) envenomings are well-known for their life-threatening neurotoxic effects. However, their impact on coagulation remains largely unexplored experimentally or clinically. This study, examined the effect of begins to examine venoms from four *Bungarus* species—*B. caeruleus, B. candidus, B. fasciatus,* and *B. flaviceps* on human platelet poor plasma coagulation parameters using thromboelastography and coagulation inhibition assays. *B. flaviceps* completely inhibited clotting, while *B. caeruleus* only delayed clot formation. In contrast, *B. candidus* and *B. fasciatus* did not affect clotting. Subsequent examinations into the anticoagulant biochemical mechanisms demonstrated divergent pathophysiological pathways. *B. caeruleus* venom anticoagulant effects were prevented by the addition of an excess of phospholipids, with anticoagulation thereby the result of phospholipid depletion. In contrast *B. flaviceps* anticoagulation was not affected by the addition of an excess of phospholipids. Further investigations demonstrated that *B. flaviceps* mediates its anticoagulant toxicity through the inactivation of coagulation enzymes. The anticoagulant effects of both *B. flaviceps* and *B. caeruleus* were nullified by varespladib, a phospholipase A_2 (PLA_2) inhibitor, revealing the toxin class involved. These results uncover previously unrecognized and unexplored anticoagulant effects of *Bungarus* venoms.

1. Introduction

Snakes from the Elapidae and Viperidae families cause most of the fatal snakebite envenomings worldwide (Tednes and Slesinger, 2024). While elapid snakes have neurotoxicity as a basal trait, certain derived lineages may potently affect blood coagulation. The venoms Australian elapid snakes are unique in being primarily procoagulant through the use of Factor Xa and FVa recruited as toxins, with envenomings causing stroke in prey while producing consumptive coagulopathy in human bite victims (Zdenek et al., 2019a, 2019b; Lister et al., 2017; Sutherland and Tibballs, 2001). This is accomplished through the activation of Factor VII and, in some derived lineages, the additional activation of prothrombin (Chandrasekara et al., 2024). Other elapid venoms act in an anticoagulant manner. For example, metalloprotease enzymes in *Naja* venoms deplete fibrinogen levels through the destructive cleavage of α and β chains (Bittenbinder et al., 2019). Snake venom phospholipase A_2 (PLA_2) toxins exert myriad effects, including depletion of phospholipid levels (such as *Micrurus* and *Pseudechis* venoms), and inhibition of clotting enzymes (such as inhibition of Factor Xa and thrombin by *Naja* venoms) (Zdenek et al., 2020; Youngman et al., 2019; Chowdhury et al., 2021, 2022a; Dashevsky et al., 2021; Bittenbinder et al., 2018; Kini, 2003; Kerns et al., 1999; Sunagar et al., 2015).

The genus *Bungarus* are elapid snakes that evolved between 25 and 30 million years ago (Biakzuala et al., 2023). Commonly referred to as "Kraits," these consist of 12 *Bungarus* species, widespread across Southeast Asia. Their venoms are well-known to cause potent, flaccid paralysis with death due to respiratory failure when untreated

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Acronyms

MA	The amplitude of detectable clot
MRTG	The maximum rate of thrombus generation
PLA_2	Phospholipase A_2
PPL	Phospholipid
R	The time to initial clot formation where formation is 2 mm
SP	Split point (SP), the time taken until the fibrin clot begins to form
TGG	The total thrombus generation
TMRTG	the time to maximum rate of thrombus generation
Va	Varespladib

(Slowinski, 1994). Signs and symptoms of krait bites encompass ptosis, ophthalmoplegia with progressive blurring of vision, ataxia and life-threatening bulbar palsies represented by dysphonia, dysphagia and neck weakness. Headache, increased oral secretions, abdominal pain, vomiting, intercostal and diaphragmatic failure (Deshwal and Gupta, 2015; Law et al., 2014; Trinh et al., 2010; Mao et al., 2017; Kularatne, 2002; Wang et al., 2021; Faiz et al., 2010). Bites often occur at night and may be asymptomatic as many as 10 or more hours following a bite (Suraweera et al., 2020). *Bungarus* venoms contain both pre- and post-synaptically acting toxins that can disrupt neurotransmission. Presynaptically the β-bungarotoxin heterodimeric complexes composed of by a kunitz peptide covalently linked to a PLA_2 toxin can also blocks voltage-gated potassium channels to impede the release of acetylcholine (Sunagar et al., 2015; Xie et al., 2021). Postsynaptically, three finger toxins (3FTx)–α-bungarotoxins antagonistically bind to the alpha-1 subunit of the nicotinic acetylcholine receptor, preventing acetylcholine from binding and triggering muscular contraction (Fry et al., 2003). While neurotoxicity is the dominant feature clinically, anticoagulant toxins have been isolated from *Bungarus* venom (Chen et al., 2014; Utkin et al., 2015). However the genus has not been systematically investigated and the venom concentrations tested have been clinically implausible, e.g. 10 mg/mL (Utkin et al., 2015).

In light of the literature and clinical observations in patients (SPS, MRL, unpublished) we sought to begin filling this knowledge gap without clinical prejudice about its significance and investigate the coagulotoxic effects of four *Bungarus* venoms (*Bungarus caeruleus*, *B. candidus*, *B. fasciatus,* and *B. flaviceps*) spanning the geographic range and taxonomic diversity of this medically important genus (Fig. 1). Varespladib, a potent PLA_2 inhibitor varespladib was used to examine sPLA2-dependent effects of these venoms and their inhibition even as its primary use in krait-bite envenoming would be most likely for neurotoxicity, its prevention if given early and antivenom resistant treatment failure (Ghose and Faiz, 2015; Gutiérrez et al., 2020; Ranawaka et al., 2013; Prasarnpun et al., 2005; Lewin et al., 2022; Gerardo et al., 2024).

2. Materials and methods

2.1. Venoms

Venom was sourced from licensed biological supply company MToxins, received under University of Queensland, Animal Ethics Approval March 15, 2021/AE000075, and work undertaken under UQ Biosafety Committee Approval #IBC/134B/SBS/2015. *Bungarus caeruleus*, *B. candidus*, *B. falviceps*, *B. fasciatus* and *Naja nigricolis* (positive control) were reconstituted to 1 mg/ml concentrated venom stock by adding 50% glycerol and deionized water from lyophilized venom. The liquid stock was stored at −20°C for further use after confirming the concentration by using Thermo Fisher Scientific™ NanoDrop 2000 UV–Vis Spectrophotometer (Thermofisher, Sydney, Australia).

2.2. Plasma and fibrinogen

Human-plasma work was performed under University of Queensland Biosafety Approval #IBC134BSBS2015 and Human Ethics Approval #2016000256. Australian Red Cross (44 Musk Street, Kelvin Grove, QLD 4059, Australia) supplied human platelet-poor plasma (3.2% citrated) under research approval #16- 04QLD-10. The plasma was aliquoted to 1.2 ml quantities after thawing and then flash-frozen in liquid nitrogen, stored at −80 °C until required for testing. Frozen aliquots were thawed at 37 °C in a Thermo Haake ARCTIC water bath during experiments. Fibrinogen was prepared to a concentration of 4 mg/ml by adding in Owen Koller (OK) buffer (Stago catalogue #00360) to 100 mg of fibrinogen (Lot#SLBZ2294 Sigma Aldrich, St. Louis, Missouri, United States), then aliquoted to 1 ml quantities, flash-frozen in

Fig. 1. Phylogenetic tree with the species studied in green with their corresponding distribution maps shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

liquid nitrogen, and stored at −80°C until further use.

2.3. Enzyme inhibitor

Small molecule PLA_2 inhibitor varespladib-HCl (LY315920) was provided by Ophirex, Inc., (Corte Madera, CA, 94925, USA and manufactured by ChemieTek, Inc. Indianapolis, Indiana Lot 001). The inhibitors arrived in powdered form and were first dissolved in 10% dimethyl sulfoxide (DMSO) and further diluted using deionized water to form 10 mM concentration main stocks. The final concentration of varespladib used in reactions was 0.56 mM.

2.4. Thromboelastography assays

To investigate the action of venom on human plasma, TEG5000 haemostasis analyzers (Haemonetics®, Haemonetics.com, catalogue #07033) was used to carry out thromboelastography. Automated measurement was done after consecutive addition of 72 μl of 0.025M $CaCl_2$ (Stago catalogue #00367), 72 μl phospholipid (Stago catalogue #00597), 20 μl of the OK buffer (Stago catalogue #00360), 7 μl of 1 mg/ml of venom, and 189 μl human plasma into the reaction cup. For negative control, venom was replaced by 7 μl 50% deionized water/glycerol. *Naja nigricollis* was used as a positive control due to its reliably potent anticoagulant action (Bittenbinder et al., 2018; Kini, 2003; Kerns et al., 1999). To test the inhibitor efficacy of varespladib, 10 mM was added to the reaction cup instead of the OK buffer (to achieve a final concentration of 0.56 mM in reaction mixture). To evaluate phospholipid dependence, two concentrations of phospholipid were tested; 100% phospholipid was made as per manufacturer instructions of adding 5 ml OK buffer to phospholipid vial, and separately a 500% concentration of phospholipid was prepared by adding 1 ml OK buffer rather than 5 ml to the phospholipid powder. Each reaction was run for 30 min. To ascertain direct effects upon fibrinogen, the assays were repeated using purified fibrinogen (Sigma) at a reaction concentration of 4 mg/mL in place of plasma. Tests for fibrinogen destruction was also carried out for *B. flaviceps* by adding thrombin (7 μl Stago catalogue #00673) after carrying out the normal test on fibrinogen for 30 min 7 of Liquid Fib was added to check if clots were formed (Dobson et al., 2019; Debono et al., 2019).

2.5. Enzyme inactivation assays

To understand further the mechanism of *B. flaviceps,* enzyme activation tests were performed using previously published protocols (Zdenek et al., 2019b) on the coagulation analyser STA-R Max® (Stago, Asnières sur Seine, France). 1 mg/ml venom stock was diluted with OK buffer to prepare a 100 μg/ml venom working stock. 25 μl of the working stock was loaded into cuvette automatically by the analyser (final concentration of venom in reaction mixture was 20 μg/ml) and other reagents were added automatically according to the test requirements and procedure as per table below Table 1. 50% glycerol/deionized water was used instead of venom, in the negative control for plasma studies. The mean values for venom-only and venom-plus-factor conditions were calculated. % increase relative to the control was determined using the formula:

*(((enzyme incubated with venom)/enzyme-only) - 1) * 100*. A value of 0 indicated no neutralization (no alteration in clotting time), while values > 0 indicated enzyme-inhibition, with values as % of enzyme-only control results.

Table 1
Inhibition assay protocols.

Assay	Procedure
Enzyme inhibition assays	Step 1: 25 μL venom +50 μL 0.025 M calcium +25 μL OK buffer +50 μL PPL +25 μL of enzyme [quantities used to replicate physiological conditions present in plasma were: 1.5 μg/mL FVIIa Prolytix #HCVIIA-0031; 15 μg/mL FIXa Prolytix #HCIXA-0050; 15 μg/mL FXIa Prolytix #HCXIA-0160; or FXa Stago #00311; or thrombin Stago #00611]. Step 2: 120 s incubation at 37 °C Step 3: Addition of 75 μL human plasma
Prothrombinase complex inhibition assay	Step 1: 25 μL venom +50 μL 0.025 M calcium +25 μL OK buffer +50 μL PPL +75 μL human plasma Step 2: 120 s incubation at 37 °C Step 3: Addition of 25 μL Factor Xa (Stago #00311)

2.6. Statistical analyses

All assays were run in triplicate. GraphPad PRISM 10.0.0 (GraphPad Prism Inc., La Jolla, CA, USA) was utilized for data visualization and statistical analyses.

3. Results

To determine the impact of *Bungarus* venoms on plasma clotting, they were first analyzed by thromboelastography under normal conditions and various clotting parameters were analyzed (Figs. 2A and 3). As expected, the positive control (*Naja nigricollis* venom) prevented clot formation throughout the maximum time limit of 1800 s. The effects of *Bungarus* venoms on plasma clotting were highly variable. *B. candidus* venom did not significantly affect any of the clotting parameters including: split point (SP), the time taken until the fibrin clot begins to form; R, the time to initial clot formation where formation is 2 mm; MA, the amplitude of detectable clot; MRTG, the maximum rate of thrombus generation; TGG, the total thrombus generation; or TMRTG, the time to maximum rate of thrombus generation. *B. fasciatus* also did not have significant effects upon clotting parameters. In contrast, *B. caerulus* venom displayed moderate anticoagulant effect by impeding the time to clot formation extending SP, R, and TMRTG by ~100% relative to the spontaneous clotting control≥ with low-level effects upon MA, MRTG, and TGG. These patterns are consistent with impedance of the clotting cascade. *B. flaviceps* however was potently anticoagulant, completely prevented clot formation, with all parameters (SP, R, TMRTG, MA, MRTG, and TGG) dramatically affected. This is indicative of a strong impact upon the clotting cascade.

Further tests were conducted to ascertain where in the clotting cascade *B. caerulus* and *B. flaviceps* venoms were acting to produce their respective anticoagulant effects. We first evaluated the phospholipid dependency of the venom (Figs. 2B and 4). To test whether the mode of anticoagulation was due to phospholipid destruction, we increased the phospholipid (PPL) concentration by five-fold (500%) in the test. The excess of phospholipid restored normal clotting parameters for *B. caeruleus* venom, revealing that this species' venom mediates its anticoagulant action through the depletion of phospholipids, which are essential cofactors for endogenous clotting enzymes. However, the *B. flaviceps* anticoagulant effect was not impacted by the increased phospholipid levels, indicating that the underlying biochemical mechanisms lay elsewhere. Subsequent tests revealed *B. flaviceps* inhibited multiple clotting enzymes (Fig. 5). The extrinsic pathway enzyme FVIIa

Table 2
Welch and Brown-Forsythe ANOVA tests of clotting factor inhibition from Fig. 5.

	FVIIa	FIXa	FXa	FXIa	P.C.	Thrombin
FVIIa		0.0174	0.0008	0.0012	0.3785	<0.0001
FIXa	0.0174		0.0109	0.6779	0.2278	0.0042
FXa	0.0008	0.0109		0.0025	0.1022	0.0518
FXIa	0.0012	0.6779	0.0025		0.2186	0.0003
P.C.	0.3785	0.2278	0.1022	0.2186		0.128
Thrombin	<0.0001	0.0042	0.0518	0.0003	0.128	

P.C. = Prothrombinase Complex.
p-**values** are Welch and Brown-Forsythe ANOVA.

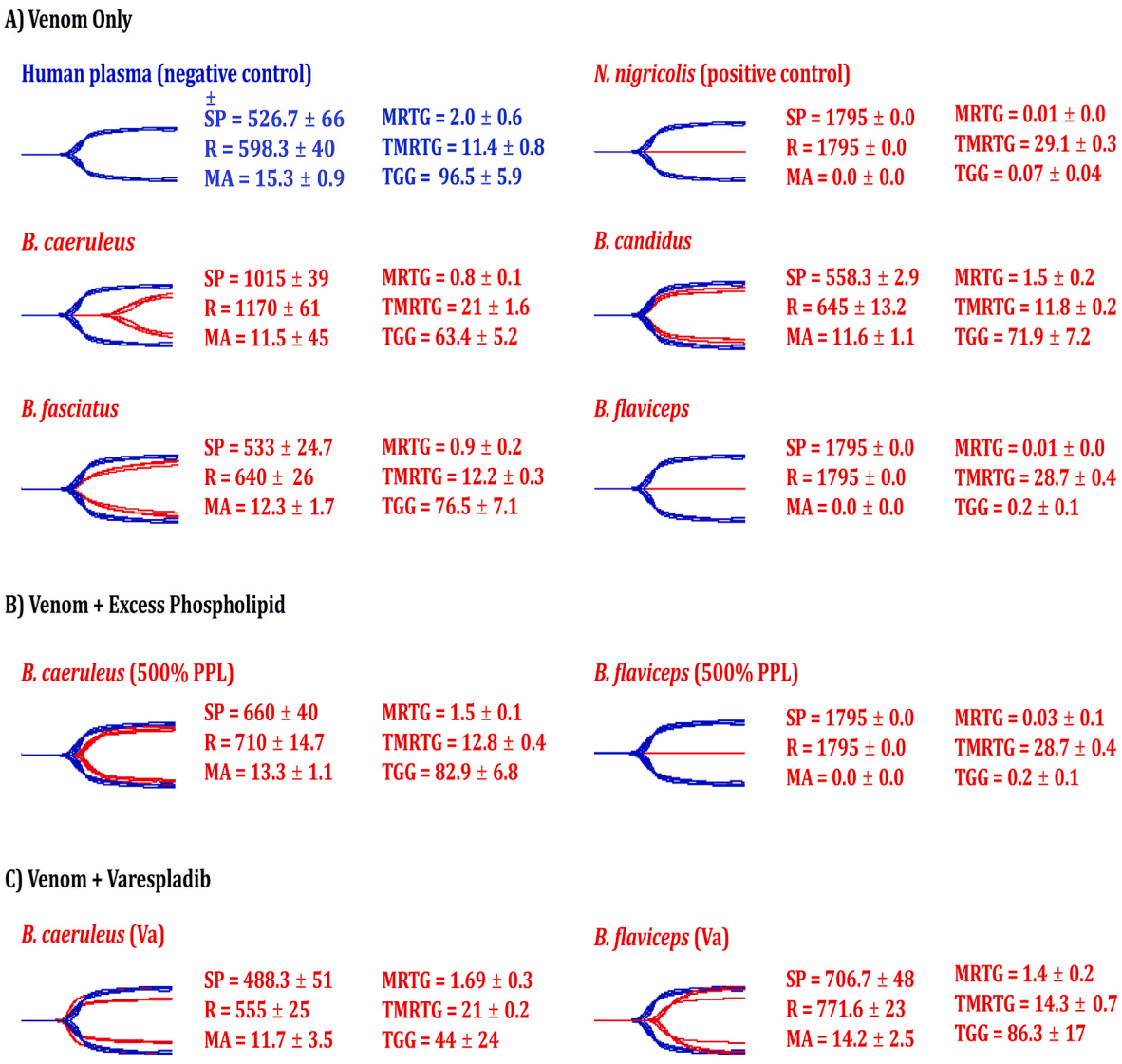

Fig. 2. Overlaid thromboelastography traces showing effects of venoms on platelet-poor plasma. Blue traces = spontaneous clot controls, red traces = plasma treated with venom, phospholipid (PPL), or varespladib (Va). SP = split point, time taken until clot begins to form (min). R = time to initial clot formation where the fibrin clot is 2 mm (min). MA = amplitude of detectable clot (mm). MRTG = maximum rate of thrombus generation (dynes/cm^2/s). TMRTG = time to maximum rate of thrombus generation (min). TGG = total thrombus generation (dynes/cm^2). Values are N = 3 mean ± standard deviation. R = 1800 indicates that *N. nigricollis* and *B. flaviceps* venom both prevented clotting within the machine maximum measurement time of 1800 s. Further tests of the two anticoagulant *Bungarus* venoms to determine if the venom effect was due to B) degradation of phospholipids and C) if the anticoagulant mechanism (whether due to phospholipid degradation or otherwise) was mediated by PLA_2 toxins and could be blocked by the phospholipase A_2 specific inhibitor varespladib (**Va**). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

clotting time was increased by 103.56 ± 2.65% increase in clotting time. The intrinsic pathway enzymes were also affected: FIXa clotting time increased by 76.11 ± 0.49% increase, and FXIa clotting time increased by 73.49 ± 2.10%. The common pathway was also affected, with FXa clotting time increased by 17.88 ± 4.48%, prothrombinase complex clotting time increased by 178.66 ± 38.97%; and thrombin clotting time increased by 36.96 ± 1.82% in (Fig. 5). The prothrombinase complex is made up of by FXa and FVa, with phospholipid an essential cofactor for its activity. As the impact upon the prothrombinase complex effect by *B. flaviceps* venom was much stronger than that upon FXa, and phospholipid degradation was not a feature for this venom, this leaves binding to FVa as the mechanism of inhibition for the effect upon prothrombinase complex and therefore a major site of action. The cumulative effect of acting upon multiple enzymatic processes results observed for this venom might be having a particularly strong net anticoagulant effect.

Subsequent tests were undertaken to ascertain whether the anticoagulant activities of *B. caeruleus* and *B. flaviceps* were driven by PLA_2-toxins. This was accomplished through the use of the PLA_2-specific small-molecule enzyme-inhibitor varespladib. Despite their divergent effects, varespladib was effective in reversing the anticoagulant activity induced by both *B. caeruleus* and *B. flaviceps*. In both case, there was a restoration of thromboelastography clotting parameters to levels (Figs. 2C and 6), thereby revealing that the anticoagulant actions of these venoms are mediated by PLA_2 toxins. As illustrated by the site of action studies, the PLA_2 toxins may be exerting their observed anticoagulant effects in radically different ways. In this experimental system, the *B. caeruleus* anticoagulation is due to enzymatic degradation of phospholipids. In contrast, the *B. flaviceps* anticoagulation appears to be due to non-enzymatic actions by the PLA_2 toxins, with them instead acting as enzyme inhibitors.

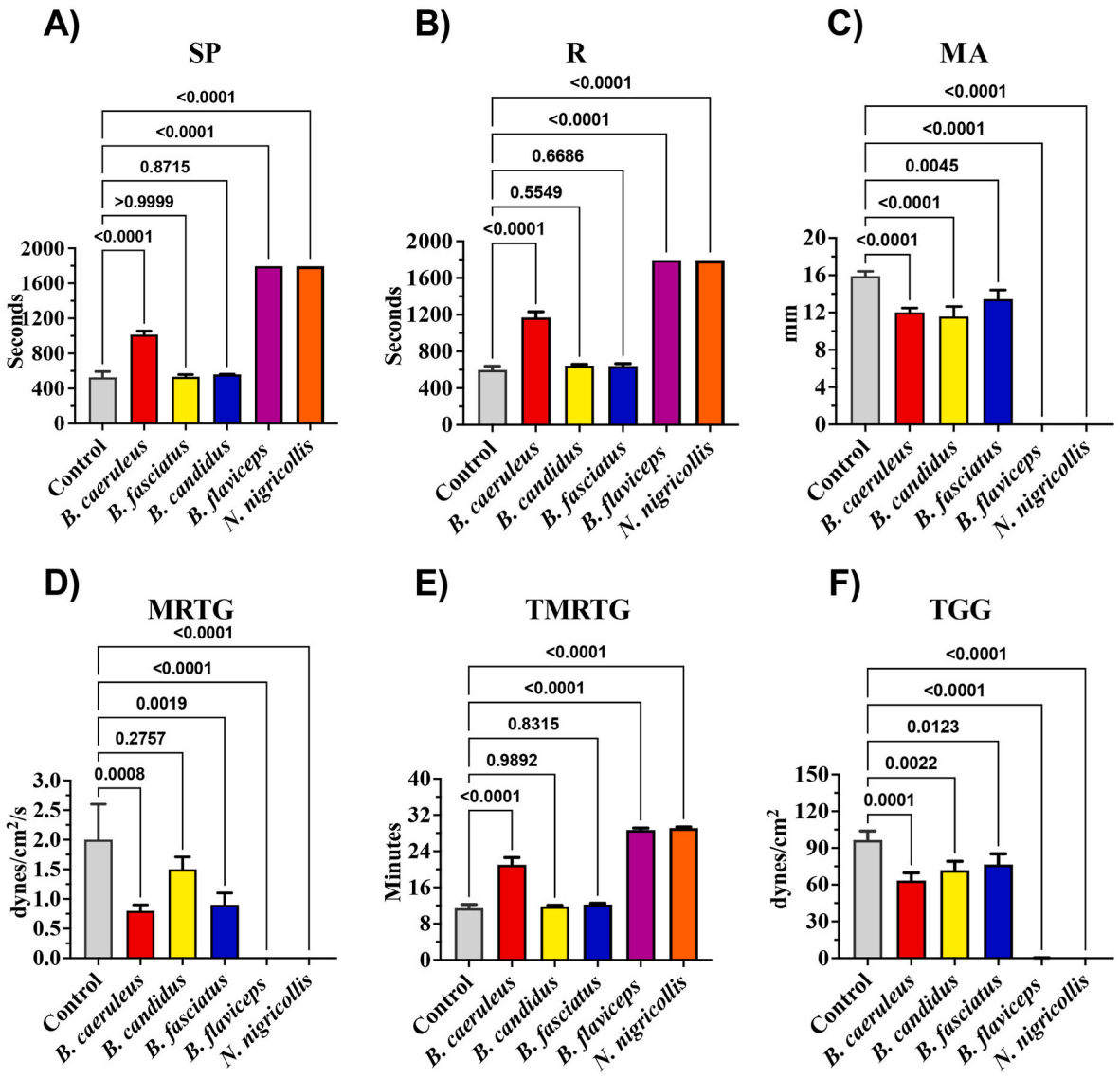

Fig. 3. Thromboelastography traces depicted as histograms showing effects of venoms (only) on plasma. A) SP = split point, time taken until clot begins to form (min). B) R = time to initial clot formation where fibrin clot is 2 mm (min). C) MA = amplitude of detectable clot (mm). D) MRTG = maximum rate of thrombus generation. E) TMRTG = time to maximum rate of thrombus generation. F) TGG = total thrombus generation. Spontaneous plasma = clotting time without venom. Note for A) and B) *N. nigricollis* and *B. flaviceps* venom both prevented clotting within the machine maximum measurement time of 1800 s. Values are N = 3 mean ± standard deviation. *p*-values are Welch and Brown-Forsythe ANOVA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

Human plasma *in vitro* data in this studied documented differential coagulotoxicity in the venoms of four *Bungarus* species (*B. caeruleus*, *B. candidus*, *B. fasciatus*, and *B. flaviceps*) of elapid snakes. In this *in vitro* *B. candidus* and *B. fasciatus* did not have any significant effects upon coagulation parameters, *B. caeruleus* had a moderate anticoagulant effect, while *B. flaviceps* venom displayed profound anticoagulant attributes. and isolated anticoagulant toxins present in the venom in only low concentrations (Chen et al., 2014). Our findings support this as *B. fasciatus* had negligible effects in this study, which tested venom at < 1/500th the concentration than previous work using extremely high concentrations of venom (e.g. 10 mg/mL) (Utkin et al., 2015).

As a whole, krait venoms are rich in three-finger toxins, kunitz-type peptides, and PLA_2-scaffolds, all of which contribute to the stereotypical neurotoxicity of these bites (Xie et al., 2021; Alam et al., 2023; Ali et al., 2013). While all four venoms tested are known to contain abundant PLA_2s, only *B. caeruleus* and *B. flaviceps* demonstrated strong anticoagulant effects *in vitro*. Normalization of coagulation by the addition of the PLA_2-inhibitor varespladib supports a role for venom PLA_2 toxins in mediating these effects in both venoms. This efficacy of varespladib was consistent with previous publications on the effectiveness of this inhibitor against other PLA_2-mediated venom actions (Zdenek et al., 2020; Chowdhury et al., 2021, 2022a; Dashevsky et al., 2021; Lewin et al., 2016a, 2016b, 2022; Youngman et al., 2020, 2022; Salvador et al., 2019; Bryan-Quirós et al., 2019). However, while the anticoagulant actions of *B. caeuruleus* and *B. flaviceps* were dependent on venom PLA_2 toxins, the mechanisms of action appear distinct from each other. *B. caeruleus* created an anticoagulant state through phospholipid destruction, thereby depleting the levels of this essential cofactor used by endogenous clotting enzymes. By increasing the available phospholipid by 500%, normal clotting parameters were restored, an anticoagulant pattern previously observed for the elapid species *Micrurus laticollaris* (Dashevsky et al., 2021). In contrast, raising the phospholipid levels did

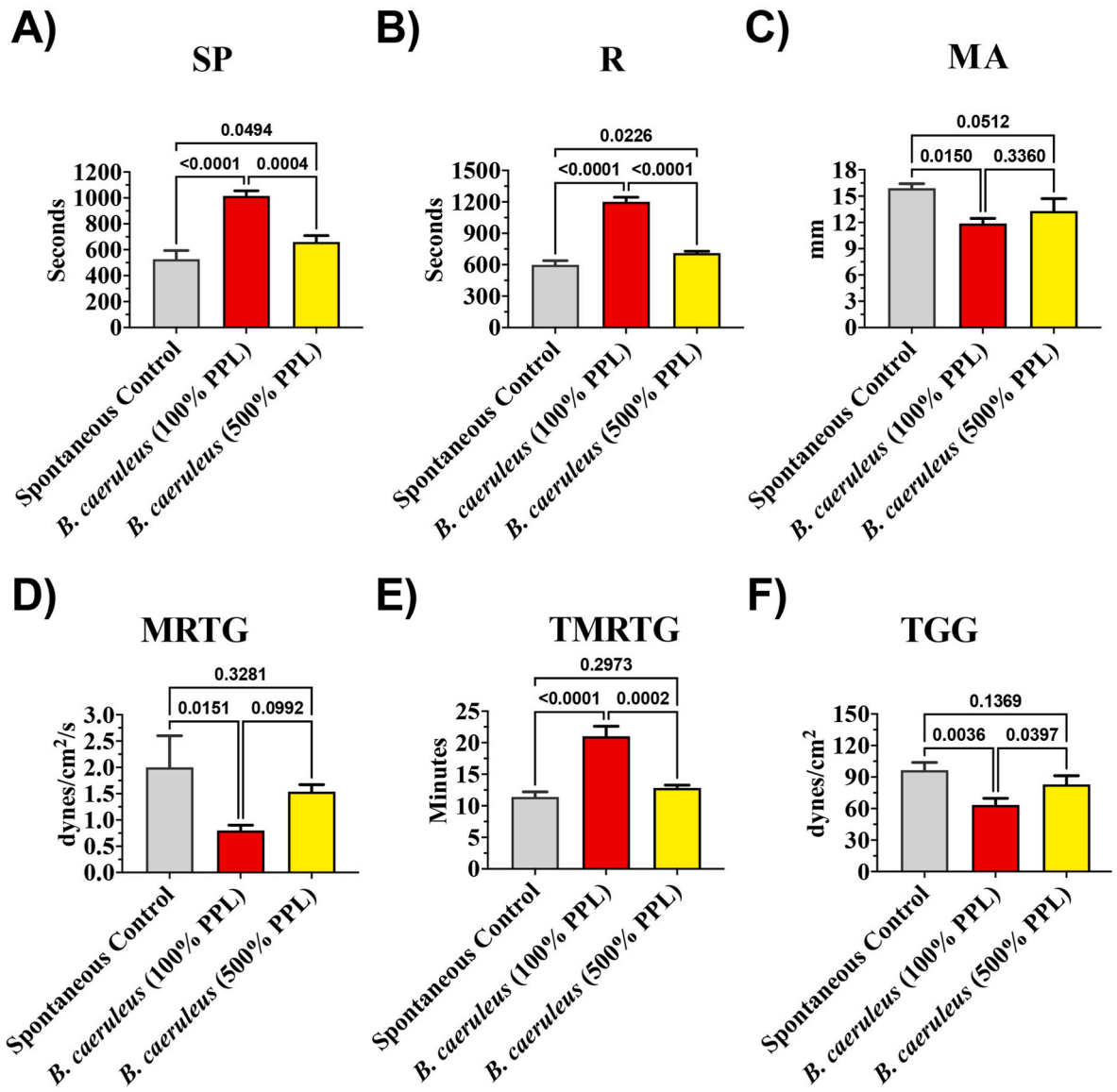

Fig. 4. Thromboelastography traces depicted as histograms showing effects of *B. caeruleus* venom on human plasma mixed with normal test concentration of phospholipid (100%) and a five-fold higher (500%) phospholipid concentration A) SP = split point, time taken until clot begins to form (min). B) R = time to initial clot formation where formation is 2 mm. C) MA = amplitude of detectable clot. D) MRTG = maximum rate of thrombus generation. E) TMRTG = time to maximum rate of thrombus generation. F) TGG = total thrombus generation. Spontaneous plasma = the clotting time without venom. PPL= Phospholipid. Values are N = 3 mean ± standard deviation. *p*-values are Welch and Brown-Forsythe ANOVA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

not negate the anticoagulant effects of *B. flaviceps.* Instead, this venom phenotype is apparently anticoagulant by toxins built upon PLA_2-scaffolds using non-enzymatic mechanisms to inhibit a myriad of clotting enzymes, with the rank order of FVIIa > FIXa = FXIa > thrombin > FXa. However, even more potently affected was the formation of the prothrombinase complex, with the results suggesting this is due to binding to FVa to prevent it from forming a complex with FXa. Inhibiting clotting factors including the prothrombinase complex has been observed for other elapid snakes (Zdenek et al., 2020; Bittenbinder et al., 2018).

In recent years, several investigators have examined the effects of direct toxin inhibitors (DTIs) such as varespladib for the purpose of early intervention and reversal of toxic PLA_2 effects (Gerardo et al., 2024; Bulfone et al., 2018; Williams et al., 2019). These therapeutics are being intensively investigated as potential treatment options for venom sPLA2-induced toxicity and may be suitable for field treatments alone or in combination with other similarly orally bioavailable toxin inhibitors (Lewin et al., 2016a; Bulfone et al., 2018; Puzari et al., 2021; Albulescu et al., 2020). As the bites from such venomous species occur more in rural or remote areas where fast medical interventions are required but mostly delayed, the importance of these inhibitors as first-aid interventions cannot be overstated. With respect to *Bungarus* venoms that typically have substantial proportions of PLA_2s, varespladib has been of particular interest to prevent, abrogate or reverse neurotoxicity (Gutiérrez et al., 2020; Puzari et al., 2021; Oh et al., 2017; Patra et al., 2019; Chapeaurouge et al., 2018; Hia et al., 2020). DTIs such as prinomastat, marimastat and varespladib have shown promising effect in curbing the coagulotoxicity of snake. The results of this study further suggest the utility of varespladib in negating certain coagulotoxic effects (Bittenbinder et al., 2019; Zdenek et al., 2020; Chowdhury et al., 2021, 2022a; Dashevsky et al., 2021; Lewin et al., 2016a, 2016b, 2022; Youngman et al., 2020, 2022; Salvador et al., 2019; Bryan-Quirós et al., 2019; Murphy et al., 2024; Howes et al., 2007; Bourke et al., 2020; Jones et al., 2022). In addition to the previously demonstrated experimental utility for reversing the effects of presynaptic neurotoxins, but do not in

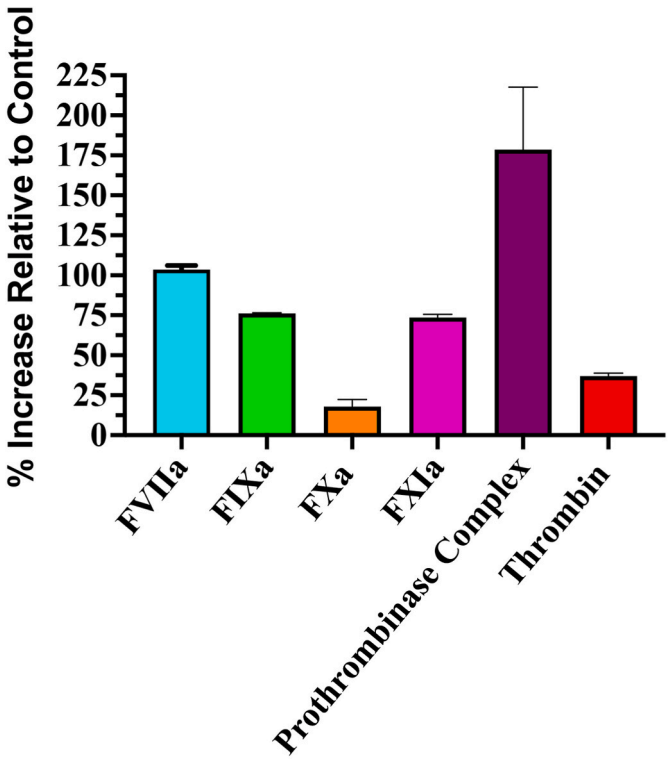

Fig. 5. Relative inhibition of specific points upon the clotting cascade by *B. flaviceps* venom. A value of 0 indicates no alteration in clotting time relative to control, and hence no inactivation of the pathway being assessed. Values are N = 3 ± standard deviation. Welch and Brown-Forsythe ANOVA tests *p*-values are shown in Table 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

any way imply that this is an important factor in the current clinical management of krait bite envenoming (Gutiérrez et al., 2020; Murphy et al., 2024).

Several limitations of the study are worthy of note: Only four *Bungarus* species were included, and all four species occupy large ranges, future work should investigate intra-species variations to ascertain regional, ontological and treatment efficacy variations (Alirol et al., 2015). In addition, the full range of *Bungarus* species should be investigated to ascertain their relative coagulotoxicity, especially in light of the extreme variation in relative potency and sites of action observed in this study. In addition, future work through *in vivo* studies is required to confirm the *in vitro* effects observed in this study and platelet rich plasma, whole blood or more complete hematological preparations should be considered for investigation.

Reports on human envenomings by *Bungarus* species generally focus on stereotypical neurotoxic effects, duration of recovery, antivenom as well as other treatment resistance and unusual CNS manifestations. However, changes in blood coagulation parameters are not routinely measured in clinical cases (Law et al., 2014; Trinh et al., 2010; Mao et al., 2017; Kularatne, 2002; Oh et al., 2017; Silva et al., 2016; Tongpoo et al., 2018; Chaisakul et al., 2017). Given the paucity of documented coagulopathy in relation to *B. caeruleus* and particularly that of *B. flaviceps* venoms, our study highlights the rare, but potential significance of this aspect where patients might already be vulnerable from other causes such as malnutrition, alcoholic liver disease, or chronic parasitic diseases that impact hepatic function (Boyer et al., 2015). This study does not consider or opine the contexts in which coagulation testing might be routinely done or beneficial in these bites, but rather highlights the potential and aims to raise awareness that there are unanswered questions and unrecognized phenomena relating to many envenoming syndromes. By analogy, an example of observational bias in the field of snakebite envenoming research has been highlighted in recent review of neurotoxic effects of viperid snakebite where hemotoxic envenoming is the most prominently observed and taught feature. Yet, many who treat Russell's viper bites in southern India will have encountered severe neurotoxicity in patients which also have life-threatening venom-induced consumption coagulopathy (Chowdhury et al., 2022b; Lay et al., 2023; Lay and Hodgson, 2024) Our study's notable findings relate to the strong anticoagulant effect of *B. flaviceps* on human plasma and the raising of questions and awareness for new avenues of basic and clinical research. Finally, these findings contribute to a holistic understanding of venom diversity.

Ethical statement

Hereby, I, Abhinandan Chowdhury consciously assure that for the "***In vitro* examination of anticoagulant effects of *Bungarus* (krait) venoms on human plasma**." the following is fulfilled:

1) This material is the authors' own original work, which has not been previously published elsewhere.
2) The paper is not currently being considered for publication elsewhere.
3) The paper reflects the authors' own research and analysis in a truthful and complete manner.
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CRediT authorship contribution statement

Abhinandan Chowdhury: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Bryan G. Fry:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Conceptualization. **Stephen P. Samuel:** Writing – review & editing, Validation, Formal analysis. **Ashish Bhalla:** Writing – review & editing, Formal analysis. **Sakthivel Vaiyapuri:** Writing – review & editing, Formal analysis. **Parul Bhargava:** Writing – review & editing, Formal analysis. **Rebecca W. Carter:** Writing – review & editing, Formal analysis. **Matthew R. Lewin:** Writing – review & editing, Visualization, Supervision, Resources, Formal analysis, Conceptualization.

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.

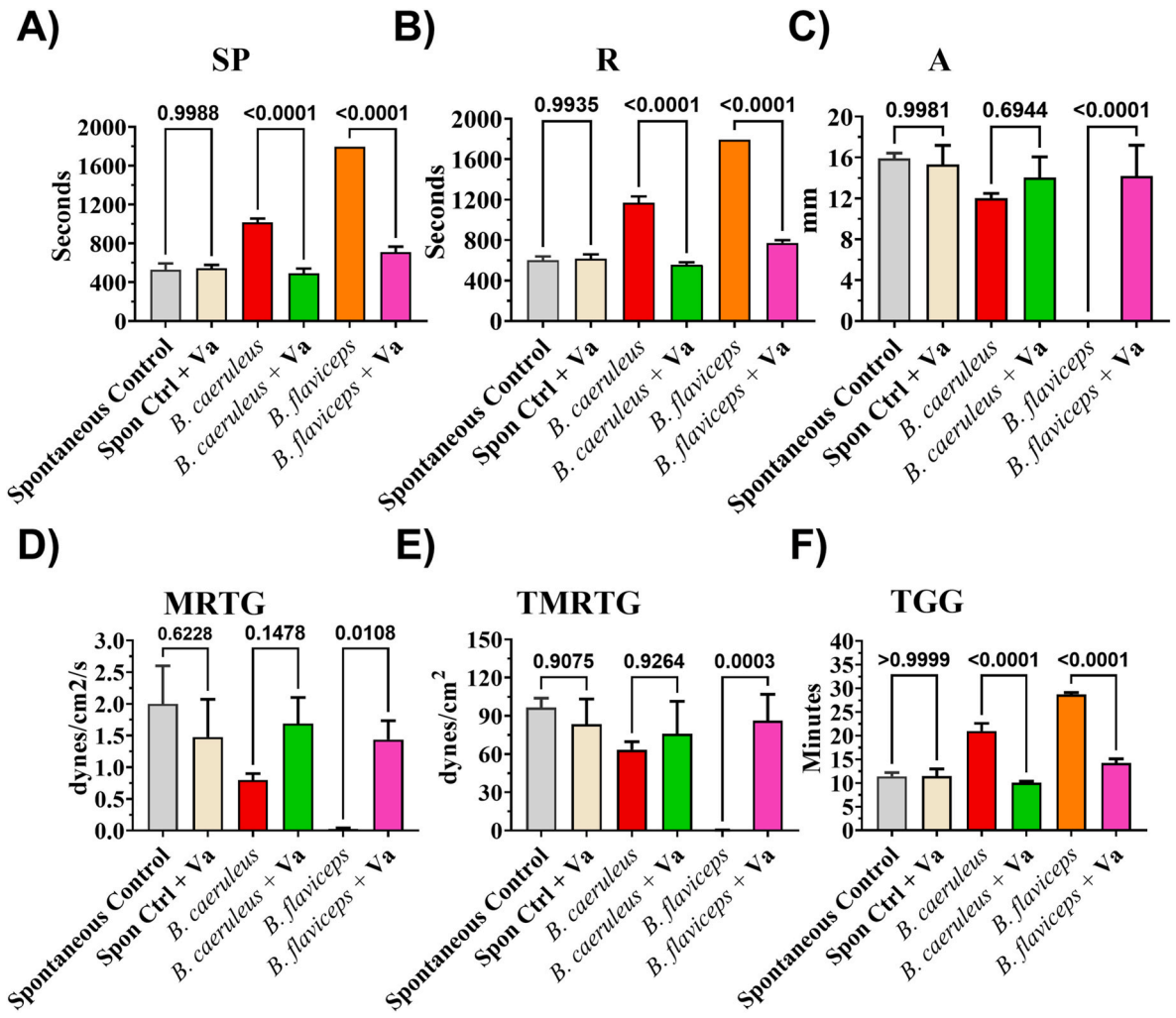

Fig. 6. Histograms showing comparisons of thromboelastography parameters affected by the addition of 0.56 mM varespladib. **A)** SP = split point, time taken until clot begins to form. B) **R** = time to initial clot formation where formation is 2 mm. C) M**A** = amplitude of detectable clot. D) MRTG = maximum rate of thrombus generation. **E)** TMRTG = time to maximum rate of thrombus generation. **F)** TGG = total thrombus generation. Spontaneous plasma = clotting time without venom (negative control). **Va** = Varespladib. Values are N = 3 mean ± standard deviation. *p*-values are Welch and Brown-Forsythe ANOVA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Data availability

Data will be made available on request.

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