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BoaγPLI from *Boa constrictor* Blood is a Broad-Spectrum Inhibitor of Venom PLA_2 Pathophysiological Actions

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Abstract

The use of venom in predation exerts a corresponding selection pressure for the evolution of venom resistance. One of the mechanisms related to venom resistance in animals (predators or prey of snakes) is the presence of molecules in the blood that can bind venom toxins, and inhibit their pharmacological effects. One such toxin type are venom phospholipase A_2s (PLA_2s), which have diverse effects including anticoagulant, myotoxic, and neurotoxic activities. BoaγPLI isolated from the blood of *Boa constrictor* has been previously shown to inhibit venom PLA_2s that induced myotoxic and edematogenic activities. Recently, in addition to its previously described and very potent neurotoxic effect, the venoms of American coral snakes (*Micrurus* species) have been shown to have anticoagulant activity via PLA_2 toxins. As coral snakes eat other snakes as a major part of their diet, neonate *Boas* could be susceptible to predation by this sympatric species. Thus, this work aimed to ascertain if BoaγPLI provided a protective effect against the anticoagulant toxicity of venom from the model species *Micrurus laticollaris* in addition to its ability shown previously against other toxin types. Using a STA R Max coagulation analyser robot to measure the effect upon clotting time, and TEG5000 thromboelastographers to measure the effect upon clot strength, we evaluated the ability of BoaγPLI to inhibit *M. laticollaris* venom. Our results indicate that BoaγPLI is efficient at inhibiting the *M. laticollaris* anticoagulant effect, reducing the time of coagulation (restoring them closer to non-venom control values) and increasing the clot strength (restoring them closer to non-venom control values). These findings demonstrate that endogenous PLA_2 inhibitors in the blood of non-venomous snakes are multi-functional and provide broad resistance against a myriad of venom PLA_2-driven toxic effects including coagulotoxicity, myotoxicity, and neurotoxicity. This novel form of resistance could be evidence of selective pressures caused by predation from venomous snakes and stresses the need for field-based research aimed to expand our understanding of the evolutionary dynamics of such chemical arms race.

Keywords PLA_2 inhibitor · Anticoagulation · Venom resistance · Endogenous inhibitors

Introduction

The co-evolution between competing species (Red Queen dynamics) creates selection pressures that often lead to evolutionary innovations. Constant antagonistic interactions modulate a rapid evolutionary adaptation in related species. This phenomenon is related to distinct ecological interactions, such as host-parasite interactions and venom resistance by predators or prey of venomous snakes (Arbuckle et al. 2017; Barchan et al. 1992, 1995; de Wit and Weström 1987; Drabeck et al. 2015; Fortes-Dias et al. 1991, 2016; Gibbs et al. 2020; Harris and Fry 2021; Jones et al. 2021; Khan et al. 2020; Lizano et al. 2003; Takacs et al. 2001, 2004; Van Valen 1973).

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Just as venom has been convergently evolved on a myriad of occasions (Casewell et al. 2013; Fry et al. 2009; Schendel et al., 2019), venom resistance is also a widely known phenomenon across the tree of life (Holding et al., 2016a, b). The interaction between venomous organisms and their predators/prey can lead to different mechanisms of resistance, and this dynamic was used to explain the high variation of predatory (venom composition) and defensive (biochemical venom resistance) traits (Arbuckle et al. 2017; Casewell et al. 2013; Gibbs et al. 2020; Holding et al. 2016a, b). The toxin resistance, for example, allowed access to different prey, such as the predation of poisonous toads by several toxin-resistant animals (Ujvari et al. 2015).

More specifically, venom resistance can occur in a wide range of mechanisms including toxin scavenging, in which molecules that compose the envenomated organism can bind to and inhibit the toxins; target-site insensitivity, whereby mutations on a receptor make the organism resistant; and off-target repurposing, whereby envenomated organisms alter the physiological effect of toxins, overcoming the toxicity (Arbuckle et al. 2017).

A classic example of toxin resistance is the resistance achieved by garter snakes (*Thamnophis* spp.) to tetrodotoxin present in their prey, where these snakes not only have modified sodium channels that make them insensitive to this powerful neurotoxin, but the snakes also sequester the toxins for defense against the snake's predators (Brodie et al. 2005; McGlothlin et al. 2014). Other examples of toxin resistance are opossums and squirrels being resistant to pit viper venoms via toxin scavenging (Biardi and Coss 2011; Rocha et al. 2002; Gibbs et al. 2020). An example of snake venom resistance by target-site insensitivity is the presence of mutations on the nicotinic acetylcholine receptors, which neurotoxin resistance to snake prey items and also predators of venomous snakes (Barchan et al. 1992; Harris and Fry 2021; Jones et al. 2021; Khan et al. 2020).

Toxin scavenging—whereby endogenous molecules within envenomated organisms can bind to and inactivate venom toxins—are a mechanism for resistance to snake venoms (Arbuckle et al. 2017; Fortes-Dias et al. 2016; Holding et al. 2016a, b). The largest number of scavenging molecules with known snake venom resistance are metalloproteinase inhibitors and phospholipase A_2 (PLA_2) inhibitors (PLIs) from plasma. These proteins can inhibit myotoxicity, neurotoxicity, acute inflammation, and anticoagulation (Bastos et al. 2016; Samy et al. 2012; Tanaka-Azevedo et al. 2003; Thwin and Gopalakrishnakone 1998). Besides their endogenous functions, they are also able to inhibit carrageenan-induced inflammation (Zhang et al. 2018), and showed *in vitro* antitumoral effects (Gimenes et al. 2017).

PLIs have been identified in snakes belonging to Boidae, Elapidae, and Viperidae families (Xiao et al. 2017), as well as in mammals, which can be predators or prey of snakes (Holding et al. 2016a, b; Lizano et al. 2003; Rocha et al. 2002; Thwin and Gopalakrishnakone 1998). These identified PLIs have been best studied for their inhibition of PLA_2 myotoxic and neurotoxic effects (da Costa Neves-Ferreira et al. 2009). Only a small number of PLIs have been evaluated regarding the inhibition of anticoagulation effects, mostly being related to protection against accidental self-envenomation (Oliveira et al. 2008, 2011; Santos-Filho et al. 2011).

Despite the PLIs being identified and characterized from numerous venomous snakes (Campos et al. 2016; Fortes-Dias et al. 2016), presumably as forms of autoresistance against their own venoms, only a small number of PLIs have been identified in non-venomous snakes (Fortes-Dias et al. 2020; Rodrigues et al. 2020; Shirai et al. 2009; Thwin et al. 2002; Xiong et al. 2017).

Resistance studies related to prey/predators have, in general, focused on mammals (Holding et al. 2016a, b; Rocha et al. 2002; Thwin and Gopalakrishnakone 1998). However, snakes wthatare prey for venomous snakes, or predators of venomous snakes, have also been shown to have evolved resistance. The occurrence of PLI in slow-moving species that are vulnerable to predation by sympatric cobras (Donnini et al. 2011; Thwin et al. 2000, 2002), provides evidence of the evolution of inhibitors against the toxins of predatory venomous snakes. Conversely study comparing the presence of PLI on two sympatric species from the genus *Elaphe* showed a quantitative difference in PLI content, in which the *E. quadrivirgata*, an ophiophagus (snake eating) snake (including on venomous species), has elevated PLI levels in comparison with *E. climacophora*, a non-ophiophagus snake (Shirai et al. 2009), which is suggestive of resistance to the toxins of venomous snake prey.

BoaγPLI is a PLA_2 inhibitor from the blood of *Boa constrictor* that has been shown to inhibitor local tissue damage (edema and myotoxicity) caused by PLA_2 toxins (Fortes-Dias et al. 2020; Rodrigues et al. 2020). This inhibitor however has not been tested against the venoms of coral snakes (*Micrurus* species), which are snake-specialist predators that occur sympatrically throughout the geographical range of *Boa constrictor* (Roze 1996). Thus, this study aimed to fill this knowledge gap by testing BoaγPLI for its ability to neutralize *Micrurus* venom effects. As our model *Micrurus* species, we selected. As the ability of BoaγPLI to neutralize coagulotoxic effects has not been investigated, we selected *M. laticollaris* as our model species. This species has a PLA_2 rich venom (Lomonte et al. 2016) that in addition to its previously characterized neurotoxic effects (Carbajal-Saucedo et al. 2013, 2014) has recently been shown to have anticoagulant toxicity (Dashevsky et al. 2021).

Methods and Materials

Venom and Inhibitor Collection and Preparation The BoaγPLI was purified from *Boa constrictor* plasma by ion-exchange chromatography (DEAE column) followed by affinity chromatography, with crotoxin coupled into the CNBr-activated Sepharose (Rodrigues et al. 2020) and lyophilized (Instituto Butantan Ethics Committee Approval #6,916,110,917). BoaγPLI CITES export permit number 20BR034548DF, CITES import number PWS2020-AU-001217. Venom from an adult *M. laticollaris* from the state of Colima, Mexico, was obtained by manual extraction, centrifuged to eliminate cellular debris, lyophilised, and stored at 4 °C as part of the venom bank at IBt, UNAM, until use. The samples were diluted with ultra pure water (water hereafter), vortexed, and the protein concentration was measured at 280 nm with a Thermo Fisher Scientific™ NanoDrop 2000 UV–Vis Spectrophotometer (Thermofisher, Sydney, NSW, Australia). Based on Nanodrop values, we corrected the concentration to make stocks of 1 mg/mL with 50% glycerol to prevent freezing at –20 ºC where working stocks were stored until use.

Plasma Preparation Citrated (3.2%) human plasma, collected from healthy donors, and obtained from the Australian Red Cross (Research agreement #18-03QLD-09; University of Queensland Human Ethics Committee Approval #2,016,000,256) was used. The plasma (pooled bag, label #A540020103540) was defrosted at 37 °C and then aliquoted into 1 ml tubes, flash-frozen in liquid nitrogen, and stored at − 80 °C until needed, at which time an aliquot was defrosted at 37 °C for 5 min. The plasma was replaced every 1 h during the analysis to avoid degradation.

Coagulation Tests The coagulation assays were performed on a Stago STA-R Max haemostasis analyser (Stago, Asnières Sur Seine, France), which measures clotting times with automated viscosity-based (mechanical) measurement at a maximum of 999 s. Different ratios of venom and inhibitor (1:2, 1:5, 1:10), with a final concentration of 10 µg/mL of venom: 20 µg/mL of BoaγPLI; 4 µg/mL of venom: 20 µg/mL of BoaγPLI; 2 µg/mL of venom: 20 µg/mL of BoaγPLI was prepared and incubated at 37 °C for 2 min (ThermoMixer comfort, Eppendorf) and then placed in the analyser. Then, 25 µL of the sample was added into a cuvette, with 50 µL of phospholipid (Stago catalog # 00,597) solubilised in Owren-Koller (OK) buffer (Stago catalog # 00,360), 50 µL of $CaCl_2$ 25 mM (Stago catalog # 00,367), 25 µL of OK buffer and 75 µL of plasma. The total volume in the cuvette was 250 µL. After 2 min at 37 °C, 25 µL of Factor Xa (used according to Stago kit; catalog # 00,311) was added to induce coagulation (Zdenek et al. 2020). To perform the negative control, 1:1 water:glycerol was used as a sample. Also, the BoaγPLI without the venom was used as a sample to investigate whether the inhibitor alters the normal clotting time. As a positive control, the *M. laticollaris* venom without the inhibitor was used as a sample. All the experiments were conducted in triplicate.

Thromboelastography (TEG) Tests To measure whether the BoaγPLI can inhibit the anticoagulant activity of *M. laticollaris* venom by altering the clot velocity and strength, a Thrombelastogram® 5000 Haemostasis analyser (Haemonetics®, Haemonetics Australia Pty Ltd., North Rdye, Sydney 2113, Australia) was used, with natural cups and pins. Briefly, 15 µL of BoaγPLI (1 mg/mL concentration), 2 µL of *M. laticollaris* venom (1 mg/mL concentration) were added to 3 µL of OK buffer (Stago catalog # 00,360) and incubated for 120 s at 37 °C (ThermoMixer comfort, Eppendorf). Then, the mixture was placed in the cup with 72 µL of $CaCl_2$ 25 mM (Stago catalog # 00,367) and 72 µL phospholipids diluted into OK buffer (Stago catalog # 00,597). Finally, 189 µL of plasma was added, the mixture was pipette-mixed and 7 µL of Factor Xa (Stago kit; catalog # 00,311) was used as a coagulation activator. The volumes in this assay were chosen to match the same ratios in coagulation tests on the STA-R Max analyser.

The entire procedure—from adding the plasma to the beginning of the reading—was performed in 15 s for standardization. The experiments were carried out in triplicate. TEG measurements occurred for 30 min. As negative controls, 1:1 water:glycerol replaced the venom incubated with inhibitor. Also, the BoaγPLI without the venom was used as a sample to investigate whether the inhibitor alters the normal clotting time induced by FXa. As a positive control, the *M. laticollaris* venom without the inhibitor was used as a sample.

Statistical Analysis Statistical analyses were performed in GraphPad PRISM 8.4.3 software (GraphPad Prism Inc., La Jolla, CA, USA), using $p < 0.05$ as statistical significance. The experiments were performed in triplicate, using the same venom and PLI, which does not represent a population sample. The coagulation test was compared using a one-way ANOVA with Sidak as a post hoc test. The venom values and the venom incubated with the BoaγPLI within the same ratios were compared.

For the Thromboelastography tests, One-way ANOVA with Tukey as a post hoc test for multiple comparisons was used to compare the different parameters: clotting velocity (SP – split point (seconds), R—time until detectable clot – 2 mm (seconds), TMTRGG—time to maximum rate of thrombus generation (minutes)), and the strength of the clot (MA – maximum amplitude of clot (millimeters),

MRTGG—maximum rate of thrombus generation (dynes/cm^2/s), TGG—total thrombus generated (dynes/cm^2)) between the venom, the venom with BoaγPLI, only BoaγPLI and the negative control. All the values were shown as mean ± standard deviation. All raw data are included in Supplementary File 1.

Results and Discussion

BoaγPLI was shown to be a dose-dependent, potent inhibitor of the *M. laticollaris* venom anticoagulant action (Figs. 1 and 2). Consistent with a previous report (Dashevsky et al. 2021), *M. laticollaris* venom extended the clotting time to 129.3 ± 2.1 s at the highest concentration tested, 5.8 times higher than the control (22.3 ± 1.1 s). Crucially, BoaγPLI did not interfere with the clotting times when incubated alone with the plasma (21.5 ± 0) but did neutralise the venom anticoagulant activities (Fig. 1). Additional studies using thromboelastography assay were performed to ascertain the venom action upon clot strength, and the relative inhibition of this toxic function by BoaγPLI. Corroborating the results obtained on the other assay platform (Fig. 1), *M. laticollaris* venom potently inhibited clot formation (Fig. 2). When incubated with the venom, the BoaγPLI restored the clotting at almost the negative control. Considering the parameters obtained by the thromboelastographic analysis, the

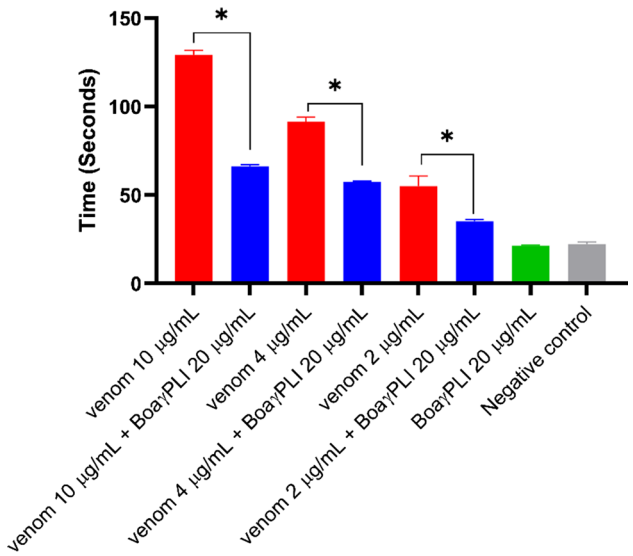

Fig. 1 Clotting times (seconds) of the *M. laticollaris* venom alone (red) or incubated with 20 µg/mL of BoaγPLI (blue) in different concentrations (10 µg/mL; 4 µg/mL; 2 µg/mL). The FXa was used as a clotting activator (see methodology Sect. 2.3 for details). The inhibitor significantly reduced the clotting time when incubated with the venom (*) (One-way ANOVA with Sidak as a post hoc test; $p < 0.05$ was considered significant). Each bar is the mean of $n = 3$ with error bars represented by the standard deviation

inhibition with the BoaγPLI and the *M. laticollaris* venom clearly and significantly reduced the clotting times (SP, R, and TMRTGG) and increased the strength of the clot (MA, MRTGG, and TGG). As with the other assay platform, BoaγPLI did not interfere with the clotting times when incubated alone with the plasma.

The present study that the endogenous PLA_2 inhibitor BoaγPLI circulating in the blood of non-venomous snake *Boa constrictor* effectively neutralizes the PLA_2-driven anticoagulant activity of the venom of the model ophiophagus species *M. laticollaris*. The observed inhibitory action involved restoring clotting time and clot strength in a dose-dependent manner. This supports previous work showing BoaγPLI inhibited other PLA_2-driven toxic actions. This indicates that BoaγPLI, and homologous proteins in other snakes, are broad-spectrum inhibitors capable of neutralizing a diversity of PLA_2 toxic activities.

The interaction between predators and prey promotes selective pressures via coevolution, with offensive and defensive traits being selected according to the efficacy of predation or resistance to predation. Venom resistance is a prime example of this phenomenon (Arbuckle et al. 2017; Casewell et al. 2013; Gibbs et al. 2020; Harris and Fry 2021; Holding et al. 2016a, b). The presence of endogenous toxin inhibitors, such as PLIs, leads to venom resistance (Fortes-Dias et al. 2016) by toxin scavenging (Arbuckle et al. 2017). Endogenous PLIs have been previously classified as myotoxic or neurotoxic inhibitors (da Costa Neves-Ferreira et al. 2009). Previous studies showed the efficacy of BoaγPLI in inhibiting the edematogenic and myotoxic activity of different PLA_2s (Rodrigues et al. 2020), but the ability to impede anticoagulant toxicity had not been investigated. In contrast, the inhibition of the PLA_2s anticoagulant effects by the PLIs as a form of auto-resistance to one's own venom had been investigated in a small number of studies on vipers being immune to their own venoms (*B. jararacussu* γPLI and αPLI, *B. alternatus* αPLI, for example) (Oliveira et al. 2008, 2011; Santos-Filho et al. 2011).

While previous studies were conducted with isolated PLA_2s, we used the crude venom of the ophiophagus model species *M. laticollaris* because the crude venom effects would be exerting the selection pressure for the evolution of inhibitors in sympatric prey snake species. *Micrurus laticollaris* was chosen as a model species for study because its venom has a high percentage of PLA_2s in its composition, with diverse activities including anticoagulant and neurotoxˆc effects (Lomonte et al. 2016; Dashevsky et al. 2021).

In this study we documented potent inhibition of *M. laticollaris* anticoagulant activity by the BoaγPLI in a dose-dependent manner, showing that this inhibitor can protect against anticoagulant toxins from the venom of ophiophagus snakes. This supports previous work showing that BoaγPLI

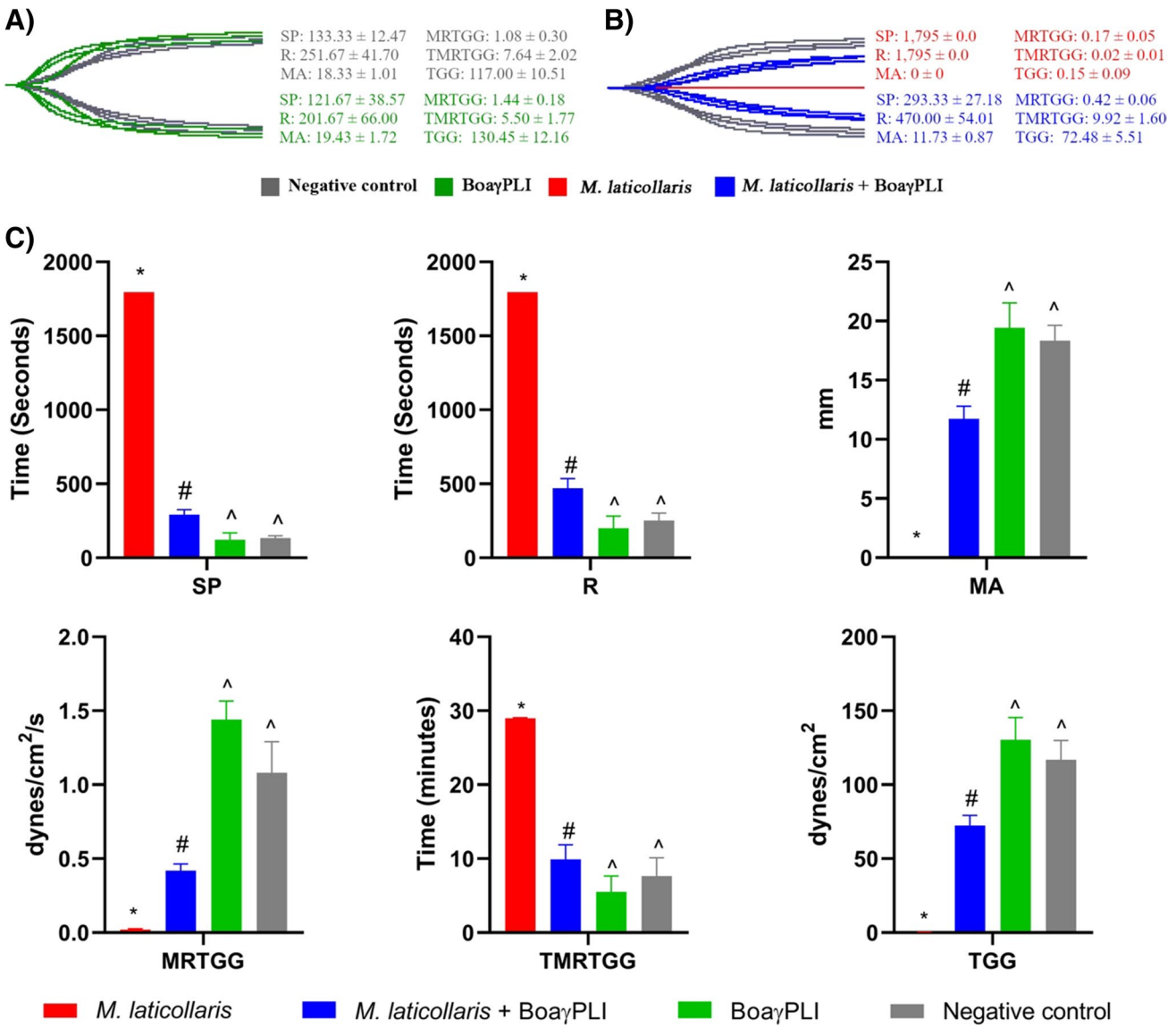

Fig. 2 Overlaid traces **A** Showing that spontaneous clotting control (gray) is not affected by BoaγPLI (green) and **B** Showing that spontaneous clotting (gray) is blocked by *M. laticollaris* venom (red), and BoaγPLI impedes the venom effect (blue). **C** Thromboelastography parameters of *M. laticollaris* venom (Red), *M. laticollaris* with BoaγPLI incubated (blue), BoaγPLI (green), and Negative Control (grey). The parameters assess the velocity of the clotting were: A = amplitude of detectable clot (mm); MA = maximum amplitude of clot (mm); SP = split point, time taken until clot begins to form (sec); R = time to initial clot formation where formation is 2 mm (sec); MRTGG = maximum rate of thrombus generation (dsc, dynes/cm^2/s); TMRTG = time to maximum rate of thrombus generation (min); and TGG = total thrombus generation (dynes/cm^2). Each experiment was performed in triplicate and the values are shown as mean ± standard deviation. Different symbols above the error bars indicate statistical significance (One-way ANOVA with Tukey as a post hoc test). Values of $p < 0.05$ were considered significant

was able to inhibit myotoxicity, edema, and enzymatic activity of isolated venom PLA$_2$s (Rodrigues et al. 2020). Studies suggest that PLA$_2$s can inhibit blood clotting through a myriad of ways, ranging from the hydrolysis of procoagulant phospholipids that are required for various biochemical reactions of the clotting cascades (Kini 2003; Saikia and Mukherjee 2017) through to binding to clotting factors directly (Bittenbinder et al. 2018; Faure et al. 2011; Saikia and Mukherjee 2017). Correspondingly, inhibitors may act upon a catalytic or non-catalytic site, such as auto-resistance inhibitor αBaltMIP from *Bothrops alternatus,* which impedes both the anticoagulant and catalytic activities of *B. alternatus* venom (Santos-Filho et al. 2011). Elucidating the specific mechanism of action of BoaγPLI is beyond the scope of this paper and should be the subject of future research. Thus, the relative binding site of BoaγPLI on the

affected PLA_2 is a rich area for future structural biology studies to ascertain if the binding to the enzymatic pocket is responsible for the broad-spectrum inhibitory action. Such work should examine a broader range of venoms and inhibitors to capture the full molecular diversity of interactions.

Boa species are slow moving snakes not able to escape fast-moving predators such as *Micrurus* species. As the *Boa* genus co-exists sympatrically across its range with the ophiophagus-specialist genus *Micrurus,* these results suggest that predatory selection pressures have led to the evolution of the inhibitors While adult specimens are too large to be predated upon, small specimens are vulnerable, particularly against large species of *Micrurus* in South America which may 1.5 m in size, such as *M. spixii*. PLIs have been identified in other slow-moving vulnerable species, including *Malayopython reticulatus* and *Python sebae,* which would also be vulnerable when young to predation by sympatric ophiophagus snakes (Donnini et al. 2011; Thwin et al. 2000, 2002). The evolution of venom resistance in slow-moving reptiles that are a risk of predation by sympatric venomous snakes has been noted for other toxin types, such as the convergent evolution of resistance in the lamprophid snake *Pseudaspis cana,* the pythonid snake *Python bivittatus,* and the varanid lizard *Varanus exanthematicus* against the three-finger neurotoxic peptides in the venoms of sympatric cobras (Harris and Fry, 2021; Jones et al., 2021). As such, further research with evolutionary approaches should investigate the origin and role of the endogenous inhibitors that promote venom resistance in vulnerable prey items. Future field ecology studies are necessary to reveal the selection pressure imposed by ophiophagus snakes on such slow-moving species, as this area is currently data deficient. This study, therefore, opens intriguing new avenues of natural history research.

In summary, BoaγPLI is an endogenous PLA_2 inhibitor isolated from the non-venomous snake *Boa constrictor* which inhibits diverse PLA_2-driven anticoagulant toxicities. This result further contributes to the knowledge about this inhibitor and reinforces the theory that these inhibitors are responsible for venom resistance. Thus, the evolution of these inhibitors appears to have been selected for due to the diverse toxic actions of venom PLA_2s, including anticoagulant, myotoxic, and neurotoxic activities. Our paper provides a catalyst for future work investigating the role of inhibitors in the ecological dynamics of chemical arms races. Future work should examine if this trait has evolved in other snakes occurring sympatrically with coral snakes, particularly slow moving, vunerable types such as *Epicrates* species.

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Data Availability All data is included in the manuscript figures and raw data is present in Supplementary File 1.

Code Availability Not applicable.

Declarations

Ethics Approval Plasma work was done under the Australian Red Cross Research Agreement #18-03QLD-09; University of Queensland Human Ethics Committee Approval #2016000256. The BoaγPLI was purified under the Instituto Butantan Ethics Committee Approval # 6916110917.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interests The authors declare no conflict of interests.

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