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Compositional and functional investigation of individual and pooled venoms from long-term captive and recently wild-caught *Bothrops jararaca* snakes

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ABSTRACT

Intraspecific venom variability has been extensively reported in a number of species and is documented to be the result of several factors. However, current evidence for snake venom variability related to captivity maintenance is controversial. Here we report a compositional and functional investigation of individual and pooled venoms from long-term captive (LTC) and recently wild-caught (RWC) *B. jararaca* snakes. The composition of individual venoms showed a remarkable variability in terms of relative abundance of toxins (evidenced by 1-DE and RP-HPLC), enzymatic activities (proteolytic, PLA_2, and LAAO) and coagulant activity, even among captive specimens. Thus, no compositional and functional pattern could be established to assign each individual venom to a specific group. Conversely, pooled venom from LTC and RWC snakes showed no significant differences regarding protein composition (characterized by 1-DE and shotgun proteomics), enzymatic activities (proteolytic, PLA_2 and LAAO) and biological function (coagulant, hemorrhagic and lethal activities), except for edematogenic activity, which was more prominent in RWC venom pool. Additionally, both pooled venoms displayed similar immunoreactivity with the bothropic antivenom produced by Instituto Butantan. Taken together, our results highlight the complexity and the high intraspecific variation of *B. jararaca* venom, that is not influenced at a discernible extent by captivity maintenance.

Biological significance: Bothrops jararaca snakes are one of the main causes of snakebites in Southeastern Brazil. Due to its medical interest, the venom of this species is the most studied and characterized among Brazilian snakes and captive *B. jararaca* specimens are maintained for long periods of time in our venom production facility. However, knowledge on the influence of captivity maintenance on *B. jararaca* venom variability is scarce. In this report, we described a high compositional and functional variability of individual venoms from LTC and RWC *B. jararaca* snakes, which are not observed between LTC and RWC pooled venoms. This intraspecific variability is more likely to be due to genetic/populational differences rather than "captivity vs wild" conditions. In this regard, data generated by the present work support the use of venom from captive and wild snakes for antivenom production and scientific research. Moreover, the data generated by this study highlight the importance of analyzing individual venom samples in studies involving intraspecific venom variability.

1. Introduction

The snake *Bothrops jararaca* is one of the main responsible for envenomation cases recorded annually in Southeastern Brazil, due especially to its abundance, capacity to occupy anthropized habitats and broad range of geographic distribution [1]. This species is classified within Category 1 by the World Health Organization (WHO), which corresponds to species of highest medical importance, defined as 'highly venomous snakes which are common or widespread and cause numerous snake-bites, resulting in high levels of morbidity, disability or mortality' [2]. For these reasons, *B. jararaca* is considered the one of the most important snake species in Brazil, from medical standpoint, and its venom and isolated toxins are deeply studied and characterized [3].

The only effective therapy available for snakebite treatment is

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parenteral administration of animal-derived antivenoms. In Brazil, Instituto Butantan (http://www.butantan.gov.br) is one of the main producers of snake antivenoms [4], yielded by the immunization of horses with venoms of one or several snake species. Specifically, the Laboratory of Herpetology of Instituto Butantan keeps > 1000 snakes in captivity and prepares snake venoms not only for antivenom production, but also for scientific research. Besides keeping and breeding several species of snakes, the Laboratory of Herpetology also receives snakes recently collected from nature (usually brought by the population), which are also milked for the preparation of venom pools. Consequently, these venom pools are composed by venoms from long-term captive and recently wild caught snakes, in a variable proportion of each one.

This proportion depends on several factors, such as the number of snakes donated to Instituto Butantan. In this context, due to its abundance and capacity to occupy areas near human settlement [1, 3], most of the specimens received by this institution belongs to the species *B. jararaca*. However, according to the records of the Laboratory of Herpetology, the number of snakes donated to Instituto Butantan has been gradually decreasing during the last years [5]. For example, 1334 specimens of *B. jararaca* were received in 2007, but this number had dropped to 603 in 2017 – a reduction of ~55% in ten years (Sávio Stefanini Sant'Anna, personal communication).

Another important event that influences the proportion in which venoms from long-term captive and recently wild-caught snakes are mixed in venom pools produced by the Laboratory of Herpetology is the preparation of Brazilian Bothropic Reference Venom (BBRV), used as standard for the potency determination of antivenoms produced in Brazil [6]. This reference venom pool, BBRV, is composed exclusively by venoms obtained in the first milking of recently wild-caught *B. jararaca* snakes and is provided exclusively by the Laboratory of Herpetology of Instituto Butantan. Once the BBRV is requested by the National Institute for Quality Control in Health (INCQS) (www.incqs.fiocruz.br), which is responsible for accessing the quality of all antivenoms produced in Brazil, most of the venom milked from recently wild-caught snakes is destined to the production of this reference venom pool. Thus, during the preparation of BBRV, *B. jararaca* venom pools produced by the Laboratory of Herpetology of Instituto Butantan is composed mainly (but not exclusively) by venoms from captive snakes.

In this regard, it is important to point out that the Laboratory of Herpetology of Instituto Butantan is committed to maintaining the snake collection as diverse as possible in terms of locality, and both juvenile and adult specimens, from both sexes, are milked to compose the venom pools, since ontogenetic, geographical and sex-related variation have been documented in *B. jararaca* venom [7–12].

Intraspecific venom variation has been extensively reported in a number of genera and species and is documented to be the result of several factors, including ontogenetic development [13–16], gender [17, 18], geographic origin [19, 20] and diet [21–23]. Additionally, long-term captivity maintenance has been pointed as a factor that could possibly contribute to the intraspecific variability of snake venom composition [24, 25].

Current evidence for snake venom variability related to captivity maintenance is mixed and even controversial [24–28]. Despite the relevance of this subject for understanding the mechanisms that drive snake venom variability and for rational design of venom pools for antivenom production, only few studies assessing the effects of long-term captivity on snake venom composition have been carried out [24, 26–28]. Moreover, these previous studies, in spite of their important contribution to the field, compared different captive populations, without using wild specimens as reference [24]; or compared two venom pools composed by a different number of specimens [25–27] from distinct localities [26, 28]. Besides, some of them are based only on the analysis of venom protein composition, with no evaluation of their toxic activities [24, 28].

In the present work, we describe a comparative compositional and functional analysis of individual and pooled venoms from long-term captive and recently wild-caught *B. jararaca* snakes. Herein, we have selected the specimens aiming to compose two comparable groups in terms of number of specimens, sex, size and locality.

2. Materials and methods

2.1. Snakes and venoms

For this study, we have selected 10 long-term captive *B. jararaca* snakes (5 males and 5 females, 109.5 ± 8 cm in snout-vent length) (LTC group), born or kept in captivity for, at least, 5 years at the Laboratory of Herpetology, Instituto Butantan (Sao Paulo, Brazil). These animals are maintained under controlled light/dark cycles (12:12) and temperature and fed on rodents (*Mus musculus* and/or *Rattus novergicus*) once a month. Snakes that compose LTC group (or their progenitors in the case of specimens born in captivity) came mostly from Sao Paulo State, Brazil, except two individuals (one from Santa Catarina state and another one from Rio de Janeiro state, Brazil). We have also selected 10 newly arrived *B. jararaca* snakes (5 males and 5 females, 110.3 ± 4.5 cm in snout-vent length) (recently wild caught group – RWC) from Sao Paulo State, Brazil. We defined as newly arrived snakes kept in captivity for a period inferior than 20 days. Geographic origin of the snakes selected for this work is shown in Supplementary Fig. 1 and Supplementary Table 1. Snout-vent length did not vary significantly between groups ($p = .08$, $F = 3.32$). Specimens from each group were numbered from 1 to 10, being that individuals 1 to 5 are females and individuals 6 to 10 are males.

After individual venom extraction, samples were centrifuged for 15 min at 1700*g* to remove mucus and cellular debris, lyophilized and stored at −20 °C until use.

Venom pools are composed by equal amounts of lyophilized venoms from the 10 selected specimens of LTC or RWC group. Information regarding the snakes that compose each group is available in Supplementary Table 1.

2.2. Protein quantification

Protein concentration was assayed on individual and pooled venoms according to the method described by Bradford [29], using the Bio-Rad Protein Assay reagent and bovine serum albumin (BSA) as standard. All samples were assayed in triplicate. Data were expressed as mean ± SDM.

2.3. Compositional analysis

2.3.1. One-dimensional gel electrophoresis (1-DE)

Twenty micrograms of venom samples were homogenized with sample buffer in the presence or absence of 2-mercaptoethanol. One-DE was carried out in 15% gels [30], and then gels were stained with Coomassie G250 according to manufacturer's recommendations (GE Healthcare).

2.3.2. Reversed-phase high performance liquid chromatography (RP-HPLC)

Five hundred micrograms of lyophilized individual and pooled venoms were dissolved in 200 µL of 0.1% trifluoroacetic acid (TFA; solution A), centrifuged at 13,000*g* for 15 min, and separated by RP-HPLC using a Teknokroma Europa Protein 300 C18 column (0.46 cm × 25 cm, 5 mm particle size, 300 Å pore size) and an Äkta Purifier system (GE Healthcare). Elution was carried out at 1 mL/min by applying a gradient toward solution B (95% acetonitrile containing 0.1% TFA), according to Gay et al. [31] with some modifications: 5% B for 5 min, 5–25% B for 10 min, 25–45% B for 60 min, 45–70% B for 10 min, 70–100% B for 10 min, and 100% B for 10 min.