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Pipa carvalhoi skin secretion profiling: Absence of peptides and identification of kynurenic acid as the major constitutive component

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ABSTRACT

The presence of peptides has been identified in all African pipid genera; nevertheless, little is known about skin secretion of South American frog genus *Pipa*. Skin secretion from captive and wild *Pipa carvalhoi* were obtained in the presence or absence of norepinephrine stimulation. The <10 kDa fraction was analyzed by liquid chromatography and mass spectrometry, searching for peptides. Chromatographic profiles show the presence of a major component in this secretion, regardless of the stimulation method (norepinephrine or mechanical stimulation) and the origin of the animal (captivity or wild), as well as in the absence of any stimulus. The general mass distribution profile in *P. carvalhoi* skin secretion shows numerous components below 800 Da. Moreover, no peptide could be identified, regardless of the chromatographic approach. The major component was purified and identified as kynurenic acid, an L-tryptophan derivative. *P. carvalhoi* does not secrete peptides as toxins in its skin. In addition, we here report that kynurenic acid is the main component of *P. carvalhoi* skin secretion. Although no biological activity was associated with kynurenic acid, we propose that this molecule is a pheromone that signals the presence of a co-specific in the shady environment in which this animal lives. In this study we demonstrate the absence of peptidic toxins in the skin secretion of *P. carvalhoi*, a break of paradigm in the pipid family.

1. Introduction

Amphibia were the first group of vertebrates to conquer the terrestrial environment and became dominant during the Carboniferous and Permian periods (Carroll et al., 1999; Schoch, 2009). The present amphibians lie in the subclass Lissamphibia that includes three orders: Anura, Caudata and Gymnophiona (Zardoya and Meyer, 2001; Frost, 2014). Anura is the most diverse group, containing circa 6200 species grouped in 54 families (Frost, 2014). Frogs of the family Pipidae comprise five genera, four present in Africa (*Hymenochirus*, *Pseudhymenochirus*, *Silurana* and *Xenopus*) and only the genus *Pipa* inhabiting South America (Frost, 2014). Pipids constitute the only exclusively aquatic anuran family and are characterized for being aglossa and having a dorsal-ventrally flattened body and hind limbs adapted to swimming (Pough et al., 2001; Fernandes et al., 2011).

The skin exerts important biological functions in all amphibians, such as gas-exchange, thermoregulation, and ionic and osmotic balance (Toledo and Jared, 1993, 1995; Amey and Grigg, 1995; Clarke, 1997; Larsen and Ramløv, 2013). In particular, *Pipa* possess a unique mode of parental care in which the fertilized eggs are placed by the male on the back of the female, where they are individually immersed inside the dorsal skin and develop. Depending on the species, the female releases either larvae, or fully metamorphosed froglets (Zippel, 2006; Greven and Richter, 2009; Fernandes et al., 2011).

The amphibian skin is rich in mucous and granular glands involved in physiological processes and protection (Toledo and Jared, 1993, 1995; Amey and Grigg, 1995; Clarke, 1997; Jared et al., 2009; Prates et al., 2012; Larsen and Ramløv, 2013; Mailho-Fontana et al., 2014). Granular glands are thought to accumulate several bioactive compounds, such as alkaloids, peptides, proteins and steroids that are used as an active (Mailho-Fontana et al., 2014) or passive chemical defense against predators or infections (Toledo and Jared, 1995; Jared et al., 2009; Conlon, 2011; Prates et al., 2012; Sciani et al., 2013a). Among these compounds, several biological activities have already been described, such as antimicrobial (Zasloff, 1987), antileishmanial and antitrypanosomal (Tempone et al., 2008), vasoactive (Conceição et al., 2009), cytotoxic (Sciani et al., 2013b) and neurotoxic effects (Toledo and Jared, 1995).

The first amphibian antimicrobial peptides (AMP) isolated and biochemically characterized were the magainins, molecules that present growth inhibitory activities against bacteria and fungi (Zasloff, 1987).

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These peptides were identified in the skin of the African clawed frog *Xenopus laevis* by Zasloff in 1987. Since the discovery of magainins, other AMPs, such as the peptides glycine–leucine-amide (PGLa), caerulein-precursor fragment (CPF) and xenopsin-precursor fragment have been described in the skin secretion of *Silurana* and *Xeponus* species after norepinephrine-stimulation (Conlon et al., 2010, 2012; Mechkarska et al., 2010). Mechkarska et al. (2012) and Conlon et al. (2013) also identified antimicrobial peptides in *Hymenochirus boettgeri* and *Pseudohymenochirus merlini*, respectively.

Besides the unusual parental care, very little is known about the biology of the different species of *Pipa* and about the biochemical and biological activity of their skin secretion. In this work, we have profiled the skin secretion collected from captivity and wildlife *P. carvalhoi* individuals, in the presence and absence of norepinephrine (NE) stimulation, aiming the biochemical characterization and, particularly, searching for peptides (magainin-like). With this study we intend to provide some insights about the evolutionary adaptations within *Pipa* genus that were isolated from other Pipidae from Africa during the continental drift.

2. Material and methods

2.1. Reagents

All the employed reagents were purchased from Sigma Co. (St. Louis, MO, USA), unless otherwise stated. Amicon Ultra-4 Centrifugal Filter Units were purchased from Millipore, USA.

2.2. Animals

Captive *P. carvalhoi* individuals were kept in 200 L water tanks at the animal facility of the Cellular Biology Laboratory (Zimmermann, 1995; Fernandes et al., 2011). Alternatively, wild *P. carvalhoi* were collected in Ilhéus (BA, Brazil) and introduced in the bioterium. All procedures were approved by the ethics committee under protocol number 892/12. Animal collecting was authorized by IBAMA (license # 15964-1-MMA) and ICMBio (license # 42500-1-CJ).

2.3. Skin secretion collection

Regardless of the method of collection, individual animal skin secretion solutions were pooled and lyophilized prior to subsequent analyses.

2.3.1. Norepinephrine-stimulated skin secretion collection

An adaptation of the protocol described by Ali et al. (2001) was employed. Briefly, *P. carvalhoi* were subcutaneously administrated with 1 nmol/g (body mass) norepinephrine hydrochloride and immersed in ammonium acetate (100 mL, 25 mM, pH 7.0) for 30 min, at room temperature. Alternatively, we tested the administration of a higher dose of norepinephrine hydrochloride (50 nmol/g) in *P. carvalhoi*, as described by Conlon et al. (2010) and Mechkarska et al. (2010), and follow the protocol described above.

2.3.2. Mechanically-stimulated skin secretion collection

An adaptation of the methodology employed by Sciani et al. (2013a) was used. Briefly, anurans were immersed in ammonium acetate (100 mL, 25 mM, pH 7.0), and gently compressed for 5 min at room temperature.

2.3.3. Absence of stimulation

P. carvalhoi were immersed in ammonium acetate (100 mL, 25 mM, pH 7.0) for 15 min, aiming to verify if anurans could release these skin secretions without the presence of any stimulation.

2.4. Sample preparation

Pooled lyophilized secretion was ressuspended in 5% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA), mechanically filtered on a 0.22 μm membrane (Millex-GV, Millipore) and then filtered in a 10 kDa cut-off centrifugal filter (Amicon Ultra-4, Millipore). Only the filtered material (<10 kDa) was further processed in this study.

2.5. Biochemical characterization

2.5.1. Reversed-phase liquid chromatography

Skin secretion solutions were analyzed by Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) using a binary HPLC system (20A Prominence, Shimadzu Co., Japan). Aliquots of each material were loaded in a C18 column (ACE C18, 5 μm; 100 Å, 250 mm × 4.6 mm) in a two solvent system: (A_1) TFA/water (1/999, v/v) and (B_1) ACN/water/TFA (900/99/1, v/v/v). The column was eluted at a constant flow rate of 1 mL/min with a 0 to 100% gradient of solvent B_1 over 20 min. The eluates were monitored by a Shimadzu SPD-M20A detector at 214 nm. After skin secretion profiles, fractions were collected manually.

2.5.2. Mass spectrometry

Liquid chromatography–Mass Spectrometry (*LC–MS*). LC–MS analyses were performed using an Electrospray-Ion Trap-Time of Flight (ESI-IT-TOF) (Shimadzu Co., Japan) equipped with binary Ultra-Fast Liquid Chromatography system (UFLC) (20A Prominence, Shimadzu). Aliquots of both stimulations were dried, resuspended in water/formic acid (0.99/0.01, v/v) and loaded in a C18 column (Shimadzu-pack XR-ODS, 2.2 μm; 100 × 3 mm) in a binary solvent system: (A_2) water/formic acid (FA) (999/1, v/v) and (B_2) ACN/water/FA (900/99/1, v/v/v). The column was eluted at a constant flow rate of 0.2 $mL.min^{-1}$ with a 0 to 100% gradient of solvent B_2 over 20 min. The eluates were monitored by a Shimadzu SPD-M20A PDA detector before introduction into the mass spectrometer, in which the spray voltage was kept at 4.5 KV, the capillary voltage at 1.76 KV, at 200 °C. MS spectra were acquired under positive mode and collected in the 80–2000 *m/z* range. Instrument control, data acquisition, and data processing were performed with LabSolutions (LCMSsolution 3.60.361 version, Shimadzu).

Direct infusion mass spectrometry. Mass spectrometric analyses were performed in an ESI-IT-TOF as described above. Skin secretion fractions (obtained in 2.5.1 subsection) were dried and ressuspended in 0.1% FA for positive mode electrospray ionization (ESI+) or in methanol for negative mode ionization (ESI−). The fractions were manually injected in a Rheodyne injector, at a flow rate 50 μL/min, in 50% B_2 (ESI+) or 50% methanol (ESI−). Instrument control, data acquisition, and data processing were performed with LabSolutions (LCMSsolution 3.60.361 version, Shimadzu).

2.5.3. Nuclear magnetic resonance (NMR)

The identification of the purified compound was carried out by ^{1}H and ^{13}C and 2D (HMBC) NMR analysis (Bruker DRX 500 MHz) in $CDCl_3$ with TMS as internal standard.

3. Results

3.1. RP-HPLC

In order to evaluate the <10 kDa skin secretion composition of *P. carvalhoi*, UV-monitored RP-HPLC profiles were obtained and compared under different conditions: NE stimulation (1 or 50 nmol/g; Fig. 1A and B) versus mechanical stimulation (Fig. 1D and E) versus no stimulation (Fig. 1F) and captivity versus wildlife animals (Fig. 1B and C). The complete scheme of collection is summarized on Table 1. Fig. 1A–E also shows that regardless of the stimulation method and origin of the anuran (captivity or wildlife), there is one clear major

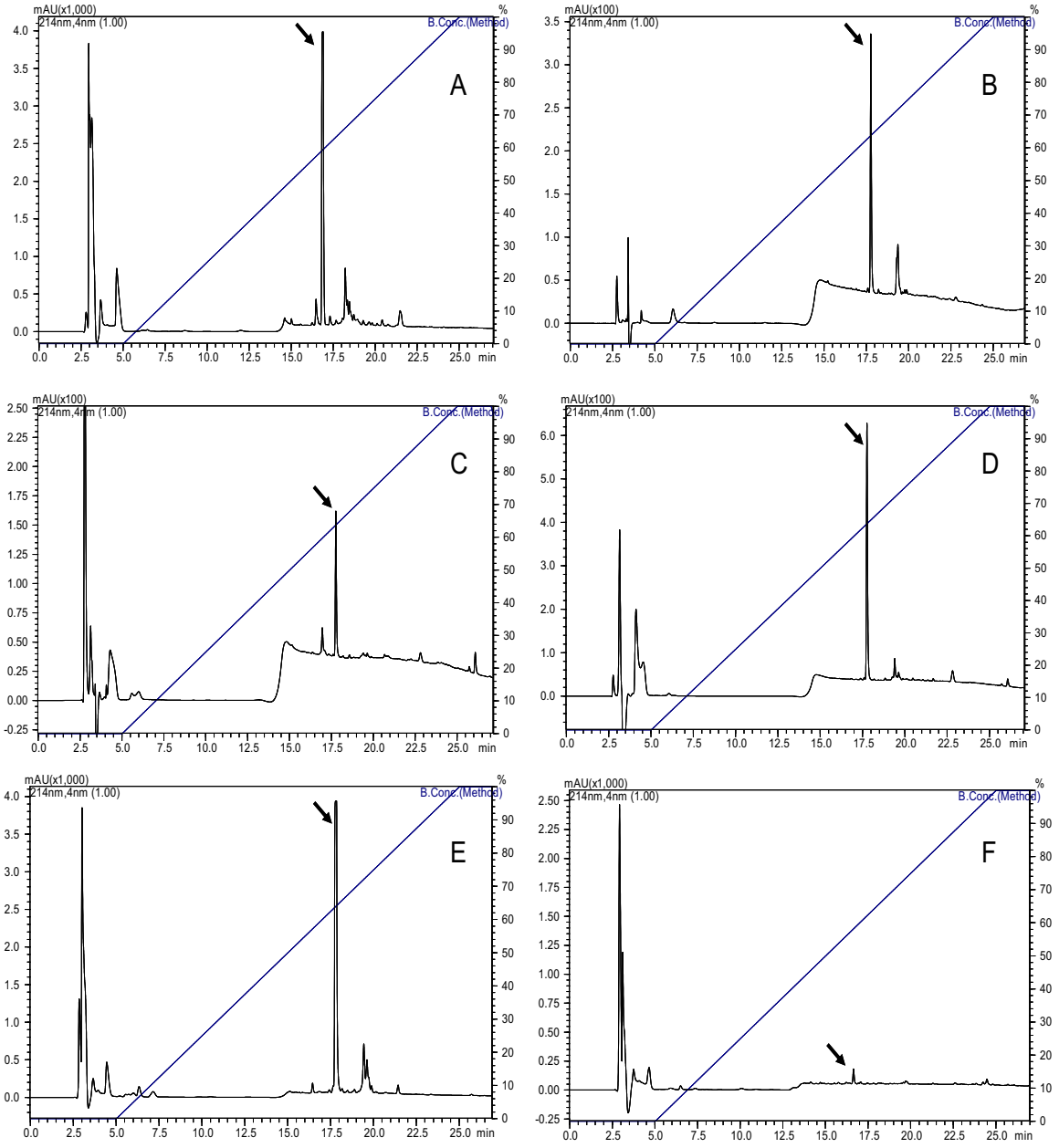

Fig. 1. RP-HPLC profile comparison of *Pipa carvalhoi* skin secretion under different stimuli. Captivity anurans were subcutaneously administrated with 1 (A) or 50 nmol/g (B) norepinephrine hydrochloride (NE) to stimulate skin secretion. Wildlife *P. carvalhoi* (immediately prior to bioterium introduction) under 50 nmol/g NE stimulation (C) or mechanical stimulation (D). Acclimatized *P. carvalhoi* (60 days), skin secretion obtained again by mechanical stimulation (E). *P. carvalhoi* spontaneous skin secretion (F). This scheme is summarized in Table 1.

Table 1
Pipa carvalhoi skin secretion collection scheme.

Event	Origin	Number of pooled animals	Stimulation
A	Captive[1]	10	1 nmol/g NE[3]
B	Captive[1]	1	50 nmol/g NE
C	Wild	3	50 nmol/g NE
D	Wild	3	Mechanical
E	Captive[2]	20	Mechanical
F	Captive[2]	1	–[4]

[1] Long term acclimatized animals.
[2] Corresponding to recently introduced animals.
[3] Norepinephrine hydrochloride.
[4] No stimulation.

component, eluting at retention time (RT) ~17.5′ (arrow). Moreover, the basic peak distribution along the profile was virtually the same, with very few hydrophilic molecules and a second set of intense peaks at RT ~19′. Curiously, without any stimulation, *P. carvalhoi* apparently release a basal level of its skin secretion (Fig. 1F), specially the major peak (RT ~17.5′).

3.2. Mass spectrometry and nuclear magnetic resonance analyses

The <10 kDa fraction of *P. carvalhoi* skin secretion solution was also analyzed by LC–MS and LC–MS/MS (positive ionization mode) (data not shown). Peak integration and manual chromatogram processing showed only the presence of low molecular mass molecules (<800 Da). The automated MS/MS processing as well as manual

interpretation of the >500 Da fragmented molecules did not match any peptide fragmentation pattern. No maganin-like peptide could be identified in the skin secretion solution. A thorough m/z values list is present (supplemental material 3) to illustrate this feature.

Due to the absence of peptides, the major peak of *P. carvalhoi* skin secretion was purified and analyzed by mass spectrometry, both in positive and negative ionization modes. Fig. 2 presents the MS profiles of the purified molecule in (A) negative (ESI−) and (B) positive (ESI+) modes (189.040 ± 0,072 Da molecule), in which ions are much more (8×) evident under ESI−. Supplemental material 1AB shows the formula predictor report of the major constituent of *P. carvalhoi* skin secretion.

This molecule was purified by RP-HPLC in the same conditions described above and submitted to structural characterization, by Nuclear Magnetic Resonance (NMR). The ^{1}H NMR data indicated the presence of 5 aromatic signals as shown in the Table 2. Combining these with ^{13}C NMR and 2D (HMBC) NMR data, the major component in *P. carvalhoi* skin secretion was determined as kynurenic acid, a known tryptophan derivative (Fig. 3).

Table 2
^{1}H and ^{13}C NMR spectroscopic data for kynurenic acid.

Position	δ_H (m, J/Hz)	δ_C	HMBC
2	–	140.00	–
3	6.99 (s)	110.45	C4, C10
4	–	164.00	–
5	7.86 (d, 8.2)	120.00	C6, C10
6	7.45 (t, 8.2)	124.54	C5, C10
7	7.76 (t, 8.2)	133.63	C6, C9
8	8.26 (d, 8.2)	125.00	C7, C9, C11
9	–	140.05	–
10	–	125.6	–
11	–	177.5	–

4. Discussion

The presence of peptides is described in skin secretions of all African pipids: *H. boettgeri* (Mechkarska et al., 2012), *P. merlini* (Conlon et al., 2013), *Silurana epitropicalis* (Conlon et al., 2012) and *Xenopus sp.* (Zasloff, 1987; Conlon et al., 2010; Mechkarska et al., 2010). Therefore, the lack of peptides in *P. carvalhoi* skin secretion is noteworthy. Our results agree with those reported by Conlon (Conlon et al., 2009; Conlon, 2011; Conlon and Mechkarska, 2014) who mentions (but does not demonstrate) that the presence of multiple cationic α-helical peptides (or cytotoxic peptides) were not detected in *Pipa parva* or *Pipa pipa* skin secretion, indicating that the absence of peptides is possibly a common characteristic (perhaps a synapomorphy) to the whole genus. Using more sensitive techniques, there is still a possibility of revealing the presence of peptides in *P. carvalhoi*. However, for interpretation of this possible finding, it is important to take into consideration the case of *Rhinella marina* (Rash et al., 2011) in which the found peptides revealed to be debris (due to cell lyses) or cellular remains present in the skin.

On the other hand, the absence of peptides in skin secretion of *P. carvalhoi* could be due to a divergent evolution event. Irisarri et al. (2011) support the monophyly of Pipidae family, but phylogenetic analysis within the family is still unresolved. These authors place the genus *Pipa* as a sister group to all other pipid genera. Nevertheless, Bewick et al. (2012) support the idea of *Pipa* as a sister group of the genus *Hymenochirus*. In addition, the authors hypothesize that pipid cladogenesis was influenced by continental drift, and divergence between *Pipa* and African pipids may have been triggered after South America and Africa rifting (Bewick et al., 2012).

The major constituent of this secretion was identified as kynurenic acid (KYN), an endogenous metabolite of L-tryptophan amino acid (Stone and Darlington, 2002). This molecule was also identified in human brain tissue (Turski et al., 1988) and in diverse animal and vegetal products, such as fish, pork, tomato or potato (Turski et al., 2009), but never in amphibian skin secretion. We have produced a KYN subtracted skin secretion, in which everything but KYN that elutes from the whole chromatographic separation is pooled and reanalyzed under the same conditions. This procedure greatly increased the relative concentration of the minor components. However, no peptide could be detected (Supplemental material 2). Despite the absence of peptides, mass spectrometry analyses show a great diversity of low molecular mass molecules in *P. carvalhoi* skin secretion (Supplemental material 3).

The constitutive secretion of KYN, observed in *P. carvalhoi* skin, poses an intriguing question regarding its biological effect. We have assayed KYN as a possible antibiotic, hemolytic, antifungal and/or larvicidal molecule but no biological effect could be observed under the tested standard conditions (data not shown). These assays were chosen in function of the possible challenges this species may face in the environment they live.

It is noteworthy to mention that regardless of the stimulation method, specimen origin and, ultimately, diet (when comparing freshly captured animals with long maintained ones), not only in relation to KYN, but the whole chromatographic profile remained virtually identical, except for the intensity of the peaks. This was an interesting observation regarding norepinephrine stimulation, since this method is largely employed for skin secretion obtainment, mainly in Pipidae family (Conlon et al., 2010; Mechkarska et al., 2010, 2012; Conlon et al., 2012, 2013). However, little is known about the biological effects on the release of the glandular content in the skin via myoepithelial cell contraction mediated by local α-adrenergic nerve terminals NE stimulation (Benson and Hadley, 1969; Gammill et al., 2012), specifically about

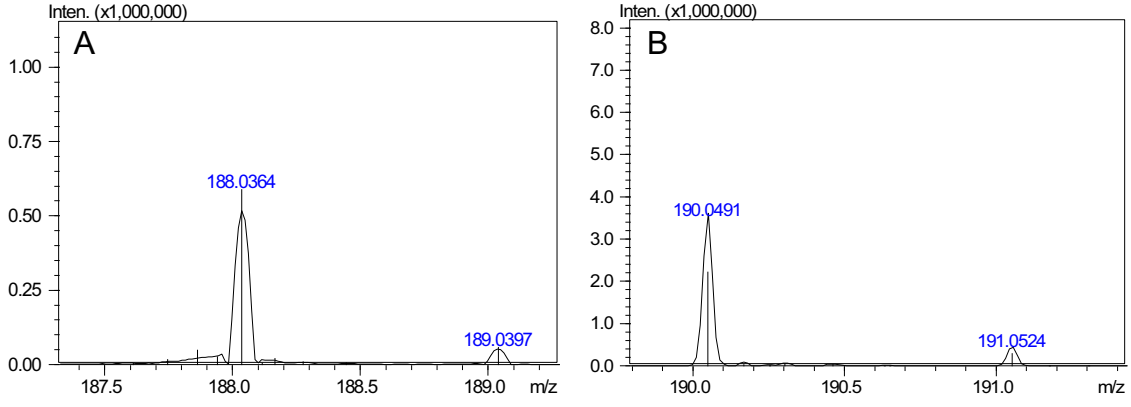

Fig. 2. Mass spectrometry analysis of major constituent of *P. carvalhoi* skin secretion. ESI-IT-TOF/MS profile of the purified molecule in negative (A) and positive (B) ionization modes.

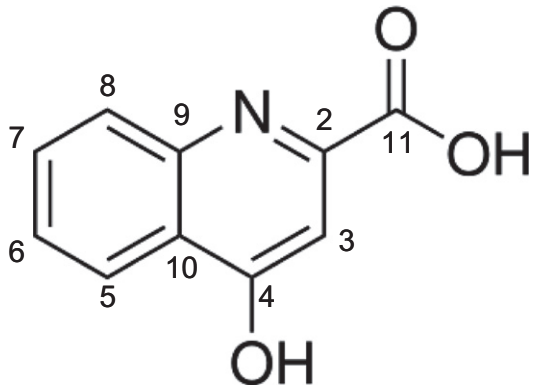

Fig. 3. NMR resolved structure of the major component of *P. carvalhoi* skin secretion: 4-hydroxyquinoline-2-carboxylic acid (IUPAC), or kynurenic acid.

the possible physiological effects on the animal skin. Gammill et al. (2012) reported that repeated high doses of NE were responsible for vasodilatation, apparent edema and some mucus gland depletion in the skin in NE-stimulated *X. laevis*. Therefore, mechanical stimulation was preferred over NE. Mechanical stimulation has been successfully employed by our group to obtain anurans skin secretion, such as in *Ameerega picta* and *Leptodactylus lineatus* (Prates et al., 2012), *Phyllomedusa nordestina* (Conceição et al., 2009), and *Rhinella* sp. (Sciani et al., 2013a).

Moreover, it was already reported that KYN act as a neuroprotective agent by antagonizing both N-methyl-D-aspartate and α7-nicotinic receptors (Parsons et al., 1997; Hilmas et al., 2001; Stone and Darlington, 2002). Due to the central nervous system action, associated to its poor solubility in water (0.9% at 100 °C) and taking into account that *P. carvalhoi* is aglossal, we chose to analyze KYN as a possible pheromone that could be slowly, but constitutively, secreted by this animal that lives in turbid waters and has poor vision.

Ellis-Steinborner et al. (2011) identified the presence of L-kynurenine, another tryptophan metabolite, in a tryptophyllin (opioid peptide), an analogue peptide isolated from the skin secretion of *Litoria rubella*. L-kynurenine itself has been identified as a chemical signalizing molecule by Yambe et al. (2006), who identified it in the urine of reproductively mature salmon (*Oncorhynchus masou*) females. Performing behavioral experiments, these authors showed that the release of this molecule in water acts as a sex pheromone, attracting male salmon to reproduction (Yambe et al., 2006).

Taking into consideration all existing data, we hypothesized that a poorly water soluble molecule as KYN, present in the skin glands, would form an aquatic gradient of diffusion/dilution which would signal to other animals the presence of co-specifics, acting as a pheromone. This idea has been preliminarily confirmed by a simple experiment in which cotton balls soaked with KYN (versus water soaked cotton balls as control) were put into water tanks containing one individual of *P. carvalhoi*. After a few minutes (1–2 min), agitation and other behaviors indicative of excitation could be observed (data not shown). Further studies are necessary for a more consistent characterization of these events.

In conclusion, we here report that *P. carvalhoi* does not possess peptides as toxins in its skin secretion and that the skin secretion profile does not change according to the nature of the stimulation (NE X mechanical), diet or anuran origins (captive versus wild). The other major finding reported here is that kynurenic acid is the main constitutive component of the skin secretion of *P. carvalhoi*. However, this tryptophan derivative does not act as a protective agent against any tested microorganism. Apparently it is a pheromone that signals the presence of a co-specific in the shady environment in which this animal lives.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpc.2014.08.001.

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