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Structural cutaneous adaptations for defense in toad (*Rhinella icterica*) parotoid macroglands

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ABSTRACT

Toads have a pair of glandular accumulations on each side of the dorsal region of the head known as parotoid macroglands. These macroglands consist of secretory units (granular glands), each one capped with an epithelial plug. When threatened, toads point one of the parotoids toward the aggressor, and if the aggressor squeezes the parotoid with its teeth, jets of poison will come out of the secretory units and hit the predator's oral mucosa, thereby causing poisoning. Our study focused on the mechanism of parotoid function by comparing parotoids from toads naturally attacked by dogs with those manually compressed. We verified that the process of glandular emptying in response to dog bites is very similar to that following manual compression. We observed that the structure of the plug plays an essential role in the release of the poison jets. Our results suggest that the parotoids may act as "bulletproof vests," reducing the impact of the force exerted by predator attacks, and thus may function as a passive anti-predator mechanism.

1. Introduction

Amphibians have cutaneous glands scattered throughout the body that play a key role in respiration, water balance and chemical defense against predators and microorganisms (Fox, 1986; Toledo and Jared, 1995; Duellman and Trueb, 1986; Clarke, 1997). These glands are of two types: mucous glands and poison (granular) glands. Particularly in toads, the poison glands form a pair of glandular accumulations located in the dorsal region just behind the eyes, one on each side of the body, known as parotoid macroglands (Toledo and Jared, 1995; Duellman and Trueb, 1986; Jared et al., 2009). When threatened, toads present a stereotypical defensive behavior, in which they inflate the lungs and point one of the parotoids towards the aggressor, drawing attack to the parotoid region (Jared et al., 2009; Mailho-Fontana et al., 2014).

Although lacking associated skeletal muscles, the parotoids, if pressed, can release poison rapidly in the form of jets (Jared et al., 2009, 2014; Antoniazzi et al., 2013). Therefore, if a predator bites the toad and squeezes the parotoid, poison jets hit the predator's oral mucosa, causing poisoning (Jared et al., 2009; Jared and Antoniazzi, 2009; Mailho-Fontana et al., 2014).

When horizontally cut, the parotoids expose an internal honeycomb-like cluster of glands, in which individual alveoli constitute secretory units (secretory alveoli), each one capped with an epithelial plug (Jared et al., 2009, 2014; Antoniazzi et al., 2013; Mailho-Fontana et al., 2014).

Comparing the morphology of manually compressed and uncompressed parotoids in the toad *Rhinella jimi*, Jared et al. (2009) showed that pressure exerted on the parotoids triggers a cascade of events that culminates in the abrupt release of poison jets. Using toads of the species *Rhinella icterica,* the present work aims to confirm and extend the findings of Jared et al. (2009) by comparing parotoids from toads naturally attacked by dogs with manually compressed parotoids.

2. Material and methods

Ten adult male toads *Rhinella icterica* Spix (Fig. 1) were collected in Cunha, State of São Paulo (Brazil). Fieldwork and collecting permits were furnished by the Brazilian Institute for the

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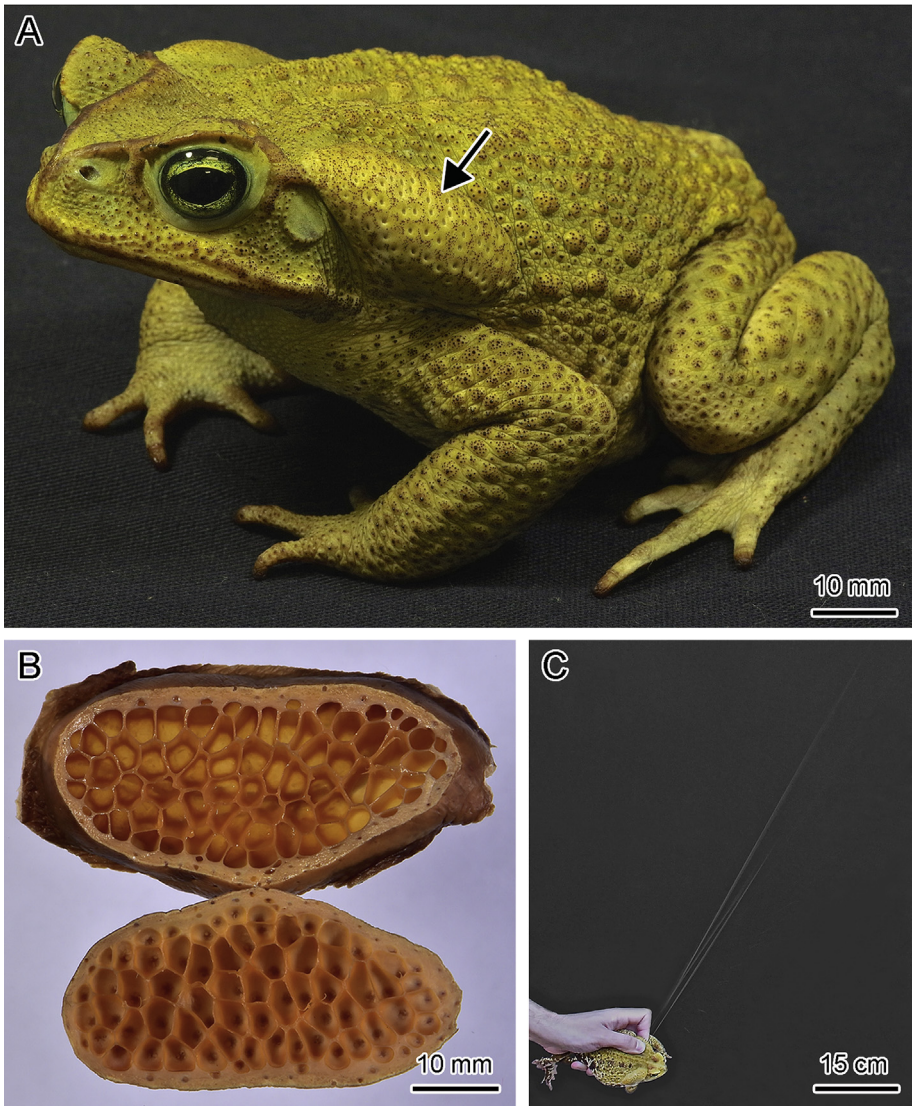

Fig. 1. (A) Adult male of *Rhinella icterica*. The arrow indicates pores on the surface of the parotoid macrogland, from where the poison is squirted. (B) Longitudinal section of a parotoid revealing the honeycomb-like organization of the juxtaposed alveoli. (C) Manual compression of the parotoid provoking the poison jets.

Environment and Renewing Natural Resources (IBAMA/SISBio, permit # 48080-1). All experimental procedures followed the norms of the Brazilian Society of Science in Laboratory Animals and were approved by the Committee of Ethics in the Use of Animals of the Butantan Institute (CEUAIB, protocol # 1359/15).

Six specimens were used for the study of parotoid morphology before and after poison release from manual compression. Three animals had one of the parotoids manually compressed, and three others were kept with the parotoids intact to serve as controls. Immediately after the compression the animals were euthanized with a lethal dose of Thiopental (50 mg/kg) and had the parotoids removed and fixed in 4% Bouin fixative or 10% buffered paraformaldehyde (pH 7.2).

For the behavioral data, the ten specimens were gently stimulated in their natural environment using a stick, following the protocol of Toledo et al. (2011). Four animals were used to establish the minimum pressure needed to elicit the poison jets before being euthanized for morphological studies and alveolar counts. Each specimen had one of the parotoids compressed with the aid of a manual dynamometer, following the method employed by Mailho-Fontana et al. (2014). The other parotoids were horizontally sectioned and (after careful removal of the poison) alveoli were counted. Results are presented as mean ± standard error of mean. Next, the alveolar plugs were extracted microsurgically under a stereomicroscope.

The pairs of parotoids of three toads attacked and killed by adult dogs (a pittbull, a rotweiller and a mongrel) were also examined after fixation in 10% formalin solution. These toads were made available after the dogs were referred to the veterinary emergency room with clear poisoning symptoms after biting the toads.

Pieces of parotoid macroglands and the entire epithelial plugs were embedded in transversal and longitudinal orientations in glycol methacrylate (Leica Historesin), sectioned 0.5–4 μm thick, and stained with toluidine blue-fuchsine. Other parotoid pieces were embedded in paraffin in the same orientations; sections 4–6 μm thick were stained with Hematoxylin and Eosin (HE) for general examination or picrosirius (posteriorly examined under polarized microscopy) for identification of collagen fibers (Junqueira et al., 1979). Light micrographs were taken with an Olympus BX51 light microscope equipped with a digital camera, using the software Image-Pro Express (Media Cybernetics).

For scanning electron microscopy, fixed samples of parotoid and