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Occurrence of parotoid glands in tadpoles of the tropical frog, *Clinotarsus curtipes* and their role in predator deterrence

Sachin M. Gosavi [a], Prashant S. Gaikwad [b], Narahari P. Gramapurohit [a,*], Ameeta Ravi Kumar [b]

[a] Department of Zoology, University of Pune, Pune 411007, India
[b] Institute of Bioinformatics and Biotechnology, University of Pune, Pune 411007, India

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ABSTRACT

Tadpoles of the tropical bicolored frog, *Clinotarsus curtipes* are unique in having parotoid glands secreting a white viscous fluid and are structurally similar to granular glands from other amphibians. To ascertain the involvement of these glands and their secretion in predator deterrence, it was tested against a predatory fish, *Clarias gariepinus*, using a paired choice behavioral assay. The results showed that the fish avoid eating *C. curtipes* tadpoles when paired with tadpoles of a sympatric species, *Sylvirana temporalis*. While the fish fed on *C. curtipes* tadpoles whose parotoid glands were surgically removed, did not touch those with intact glands, suggesting a role for the parotoid gland secretion in predator deterrence. Histochemical and biochemical analyses of the gland secretion revealed the presence of high concentrations of proteins, lipids, and alkaloids. SDS-PAGE showed the presence of proteins with prominent bands at 17 and 50 kDa. The presence of other small molecules (950–2000 amu) as detected by LC–MS showed the presence of five major peaks. Peaks 1 and 2 are probably tetrodotoxin and/or its analogs. Peaks 3 and 5 are possibly bufalin and argininosuccinic acid, respectively while peak 4 remains unidentified. Thus, secretion of parotoid glands of larval *C. curtipes* contains chemicals which, either alone or in combination, might be responsible for deterring predators.

1. Introduction

Amphibian skin has evolved many multicellular alveolar and tubular glands that form either clusters at specific regions or spread all over the body (Duellman and Trueb, 1994; Toledo and Jared, 1995; De Almeida et al., 2007). The morphology and pattern of distribution of these cutaneous glands vary among species, sometimes, as a function of their ecology and natural history (Toledo and Jared, 1995). These glands are known to secrete fluids and are a source of diverse chemical compounds with roles in protection and defense (Clarke, 1997; Shepherd et al., 1998; Brito-Gitirana and Azevedo, 2005; Pukala et al., 2006; Heiss et al., 2009).

The skin of amphibians has three types of glands; mucous, serous (granular or poison), and lipid (Duellman and Trueb, 1994; Clarke, 1997). While poison or granular glands are larger than mucous glands, both are alveoliform and end in a circular pore that opens to the exterior. Mucous glands keep the integument moist in terrestrial environments while lubricating it in an aquatic medium. They secrete mucous with known antimicrobial activity (Pough et al., 2003) while in some cases, the mucous mechanically traps microbial and fungal pathogens (De Almeida et al., 2007). For example, Salamanders (Caudata: *Cryptobranchidae*) use a thick mucous coat as a defense and escape mechanism (Stebbins and Cohen, 1997). Granular glands are specialized to produce a sticky white secretion that is more toxic than the mucous, thereby providing protection against microbial infection as well as defense against different kinds of predators (De Almeida et al., 2007).

Additionally, salamanders, newts, and toads possess parotoid glands also referred to as warts containing high concentrations of toxins (Clarke, 1997; Brito-Gitirana and Azevedo, 2005; Wells, 2007). Parotoid glands are visible to varying degrees and are typically positioned between the otic region of the skull and scapular region (Duellman and Trueb, 1994; Toledo and Jared, 1995; Clarke, 1997). In some salamanders, parotoid glands are also present in dorsolateral rows along both sides of the spine (Duellman and Trueb, 1994; Clarke, 1997). Upon mechanical stimulation, some amphibians release the secretion from these glands onto the body surface while others spray it using muscle contractions (Brodie, 1983; Brodie and Smatresk, 1990). Frogs, belonging to the genera *Dendrobates*, *Phyllobates*, and *Epipedobates* (poison frogs) are highly toxic and on release of secretion can cause severe damage or even death of a predator (Brodie and Brodie, 1999). Although more than 100 bioactive peptides, 30 bioactive amines, and over 800 alkaloids are reported from adult amphibians (Heiss et al., 2009), information on the occurrence of such toxic chemicals used for defense in their larvae is less common (Clarke, 1997; Marion and Hay, 2011).

* Corresponding author. Tel.: +91 20 25601436, +91 20 25601437; fax: +91 20 25690617.
E-mail addresses: schn.gosavi@gmail.com (S.M. Gosavi), psgaikawad@hotmail.com (P.S. Gaikwad), naraharipg@unipune.ac.in (N.P. Gramapurohit), ameeta@unipune.ac.in (A.R. Kumar).

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Moreover, information on the existence of cutaneous glands in anuran tadpoles and their ecological significance are scarce (Gunzburger and Travis, 2005).

The bicolored frog, *Clinotarsus curtipes*, is endemic to the Western Ghats of peninsular India inhabiting the forest floors and shelters under the leaf litter (Dutta, 1997). The frog breeds in semi-permanent and permanent water bodies including streams and rivers (Hiragond et al., 2001). Breeding season coincides with the onset of the Southwest monsoon rains (June–September) and continues up to October. The tadpoles are golden yellow (early development) to black (older individuals) in color and are believed to be the largest larval frogs inhabiting the Western Ghats (Daniels, 2005). The tadpoles have a long larval period and can be seen almost throughout the year. They generally move slowly in shoals along the streams and rivers (Hiragond et al., 2001; Saidapur et al., 2003) that harbor many predators known to feed on these amphibian larvae. These tadpoles, with their long larval period seem to have evolved novel strategies to counteract predation pressure. *Clinotarsus curtipes* tadpoles have a specialized physiological suite in the form of cutaneous glands; two reddish-brown parotoid glands on the dorsal side of the body behind the eyes and a third supra-caudal gland at the base of the tail (Hiragond et al., 2001). Additionally, small glandular cells containing secretory substances are distributed almost all along the dorsal tail fin. A milky white viscous substance oozes out of these glands when the tadpoles are handled (personal observation). Neither the nature of chemicals present in the parotoid glands nor their ecological significance in the tadpoles of this tropical frog is known. In this study, we demonstrate for the first time their role in predator deterrence as well as biochemical characterization of their secretion.

2. Materials and methods

2.1. Materials

Ethanol, xylene, paraffin wax, glutaraldehyde, cacodylate buffer, hematoxylin–eosin, and Ninhydrin reagent were procured from Merck Specialties Pvt. Ltd., India. Alcian blue, amylase, trypsin and Tricane methane sulfonate (MS-222) were procured from Hi Media Laboratories, India. Bromophenol blue and hexamethyldisilazane (HMDS) were from SRL Pvt. Ltd., India. Schiff's reagent and Sudan black B stains were procured from s d Fine-chemicals Ltd., India. All chemicals were of either AR grade or HPLC grade and 99% pure according to the manufacturer.

2.2. Ethical statement

The study was carried out following the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals, (No. 538/CPCSEA). All procedures followed for collection of the required materials were non-invasive and all the animals after experimentation were released back to their natural habitat.

2.3. Animal collection, rearing, and collection of secretion

Tadpoles of *C. curtipes* were collected from a stream in the Western Ghats near Anamod, (15° 4′N and 74° 33′E) Karnataka, India during November–March, 2011 and transported quickly to the laboratory where they were maintained in large glass aquaria with aged tap water. The tadpoles were fed with boiled spinach leaves *ad libitum* and the water was changed every alternate day. Tadpoles of stage 30–35, identified as per Gosner (1960), were mechanically stimulated in the region of parotoid glands and the secreted milky white substance was collected using a pipette, dissolved in distilled water and stored at −20 °C. After the collection of secretion, the tadpoles were kept under observation for a few days and then released back into their natural habitats with no mortality being observed.

2.4. Paired choice predation bioassay

To assess palatability of *C. curtipes* tadpoles to coexisting fish predators, we paired them with *Sylvirana temporalis* tadpoles, a sympatric species, in a paired choice predation laboratory bioassay. A large carnivorous catfish (*Clarias gariepinus*) obtained from a commercial supplier was used as a predator in the bioassay. The fish ($n = 20$; body length, $\overline{X} \pm \mathrm{SD} = 26.87 \pm 2.89$ cm) were acclimated to laboratory conditions for five days during which they were fed with pieces of meat and the water renewed every other day. After acclimation, the fish were housed individually in glass aquaria (60 cm × 45 cm × 15 cm) with 10 L aged tap water and simultaneously offered four similar sized tadpoles (two each of *C. curtipes* and *S. temporalis*). Initially, the experiment was conducted for 24 h and the number of tadpoles of each species consumed recorded. Later, the experiment was extended up to 96 h to determine whether hunger could overcome any distastefulness. A total of twenty trials were conducted in which each fish was used only once. Differences in the number of tadpoles of each species consumed were analyzed as given in Section 2.7 under statistical analysis.

To ascertain if parotoid glands render larval *C. curtipes* unpalatable, their glands were surgically removed. If parotoid glands play a role in unpalatability as hypothesized, then their removal should render the tadpoles palatable. Hence, in a paired choice predation bioassay, predatory fish ($n = 20$; $\overline{X} \pm \mathrm{SD} = 24.87 \pm 2.14$ cm) were simultaneously offered four *C. curtipes* tadpoles; two with intact glands (controls) and two with their glands removed. The parotoid glands were surgically removed after deep anesthesia using MS-222. The control tadpoles were also similarly anesthetized but their glands were kept intact. Tadpoles of both the groups were thoroughly washed in distilled water to remove traces of MS-222. Again, twenty trials were conducted in which each fish was used only once. The number of tadpoles eaten with and without parotoid glands was recorded after 24 h and statistically analyzed as mentioned in Section 2.7.

2.5. Histomorphology and histochemical studies

To study histomorphology and histochemistry, the tadpoles ($n = 10$; stage 30–35) were anesthetized using MS-222, the parotoid glands were dissected out using a stereoscope (Carl Zeiss, Stemi 2000C) and the size was measured using digital callipers.

For scanning electron microscopy (SEM), freshly dissected parotoid gland tissues were immersed in physiological saline and immediately transferred to a solution containing 1% (v/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7 for 4 h at 4 °C (Sabatini et al., 1963). After prefixation, the tissue pieces were washed with distilled water and dehydrated in graded series of ethanol. Dehydrated tissues were immediately placed in Hexamethyldisilazane (HMDS) for 15 min, air dried in desiccators under low vacuum, coated with platinum and analyzed using JEOL analytical scanning electron microscope (JEOL JSM-6360A).

For light microscopic studies, the glands were fixed in Bouin's fluid for 24 h, washed, dehydrated using graded series of ethanol, and embedded in the paraffin wax. Transverse sections of the tissue cut at 6 μm using rotary microtome (Leica, RM 2235) were stained with haematoxylin–eosin (HE), and observed using a bright field microscope (Carl Zeiss, Axioscope — A1), equipped with a digital camera.

To study the gross chemical composition of the granular gland secretion, histochemical staining was carried out in tissue sections; Bouin's fixed tissue sections were used for the detection of carbohydrates and proteins while fresh frozen tissue sections were used for lipid detection. Frozen sections were obtained by quickly embedding freshly dissected parotoid glands into the cryomatrix at −20 °C and cutting 6 μm thin sections using a freezing microtome (Leica, CM 1510 S). Periodic Acid Schiff (PAS) and Alcian blue (AB) methods were used for detecting neutral, sulfated, and carboxylated mucopolysaccharides, respectively while mercury bromophenol blue (Hg-BPB) and ninhydrin-Schiff methods were used for protein detection. The Sudan black B method