ARTICLE

Skin healing in the terrestrial toad *Melanophryniscus montevidensis* (Philippi, 1902): an experimental approach

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Abstract: We studied in this work the skin healing process in the terrestrial toad *Melanophryniscus montevidensis*. Wild specimens were acclimated in the laboratory, and an experimental skin wound of 1.5 mm diameter was made in the dorsum region under clove oil anesthesia, leaving the dermis exposed. Monitoring of the healing process by conventional histology was made up to 129 days. The epidermal protection of the wound was recovered after two days, and apparently, a complete recovery of dermal glandular structures was evident after 37 days. This feature includes the serous glands that play a relevant role in the defensive strategy of this species. No complications were recorded from the anesthetic procedure, not previously assessed in *Melanophryniscus*.

Keywords: amphibians, healing, histology, anesthesia, *Melanophryniscus*.

Reparação da pele no sapo terrestre *Melanophryniscus montevidensis* (Philippi, 1902): uma abordagem experimental

Resumo: Estudamos neste trabalho o processo de cicatrização da pele do sapo terrestre *Melanophryniscus montevidensis*. Espécimes selvagens foram aclimatados em laboratório e uma ferida cutânea experimental de 1,5 mm de diâmetro foi feita na região do dorso sob anestesia com óleo de cravo, deixando a derme exposta. O acompanhamento do processo cicatricial por histologia convencional foi feito até 129 dias. A proteção epidérmica da ferida foi recuperada após 2 dias, e aparentemente uma recuperação completa das estruturas glandulares dérmicas foi evidente após 37 dias. Esta característica inclui as glândulas serosas que desempenham um papel relevante na estratégia defensiva desta espécie. Não foram registradas complicações do procedimento anestésico, não avaliadas previamente em *Melanophryniscus*.

Palavras-chave: anfíbios, cicatrização, histología, anestesia, *Melanophryniscus*.

Introduction

The class Amphibia has successfully colonized diverse environments aided by the development of several relevant adaptations such as particular skin structure and functions (Duellman & Trueb, 1986). Some pioneer authors have characterized the structure and function of the amphibian skin in terms of its importance for water and electrolyte balance, and the contribution as a respiratory organ, among other features (Fox, 1994). The skin of the amphibians has a variable thickness, gland richness and functionality, features that are strongly associated to the environment occupied by the species involved (Barbeau & Lillywhite, 2005). As in other vertebrate groups, the epidermis bears a *stratum corneum* as the more external layer, composed of keratinocytes derived from the underlying layers, produced by the germinative one. The dermis is a conjunctive tissue that gives mechanical attachment and nutrition to the epidermis and usually bears some mucous and serous defensive glands (Fox, 1994).

Skin integrity, including associated glandular tissue, is crucial for survivorship during adverse environmental conditions, for instance, in dry periods (i.e. McClanahan et al., 1976). Besides, skin colouration patterns, aposematic colours and toxins are relevant features of the evolutionary strategies to avoid predation (Toledo & Haddad 2009). These features are exhibited by Neotropical toads of the genus *Melanophryniscus*. These toads are relatively poisonous prey because of the storage of defensive alkaloids in skin glands that are incorporated by dietary items (Hantak et al., 2013). This condition of toxic prey is advertised to visually oriented predators by the exhibition of a stereotyped behaviour called “unken-reflex” in which aposematic reddish colouration present mainly on their hands, feet and the pelvic patch is exposed (Toledo & Haddad, 2009). Learning of predators to avoid toxic anurans may damage prey skin during a predation attempt. Subsequent repair of this valuable organ may be crucial for survivorship.

In this work, we studied the healing process of the skin in *Melanophryniscus montevidensis* (Philippi, 1902), a diurnal species native from Uruguay and extreme south Brazil (Tedros et al., 2001). We made a follow-up of experimental skin wounds that simulated a predation attempt using conventional histology.

Material and Methods

On 22 February 2018 we captured 31 adult and apparently sound specimens of *M. montevidensis* (Fig. 1A) in the surroundings of La Paloma (34°38'S; 54°11'W), Departamento de Rocha, Uruguay, during a massive explosive reproduction episode in temporary ponds. The toads were transported to the laboratory and conditioned in a terrarium following Kolenc (1988). The enclosure measured 70x30x30 cm, prepared with soil substrate mainly maintained dry except for a small humid area, and was provided and tree bark (Pinus sp.) for shelter. During the whole period they were fed ad libitum with termites (Isoptera). The specimens weighed 1.00±0.26 g (0.54-1.64) and measured 2.35±0.18 cm (2.14-2.80) of snout-vent-length.

After a week of acclimation, all toads were anesthetized during the same day (taken as day 0) to perform an experimental wound under general anaesthesia. Toads were placed individually in small enclosures of 10x5 cm with 1 cm column of anaesthetic solution. The anaesthetic was clove oil (Eugenol DIU®, Emilio Benzo S. A., Uruguay) 1:10,000 (1/1,000 of 1:10 solution in ethanol). The procedure was as follows: 1) bath in the clove oil at 23°C until reaching a deep anaesthetic plane, considered when toads were not responsive to the “toe pinch” stimulus (Mitchell et al., 2009), gently performed with a surgical clip in toe fingers; 2) removal of anaesthetic present in the skin, washed out by flowing tap water during 3 s; 3) perform of an experimental wound of 1.5 mm diameter on the anterior part of the dorsal area, done under a binocular microscope (NIKON C-LEDS®, Japan) using individual uterine biopsy pouches (Kai Medical BP-15F, Japan) that excised the whole skin, exposing the underlying muscle tissue (Leévesque et al., 2010). 4) recovery from anaesthesia by partial submersion of the abdomen and gular region in tap water, followed by a return to the terrarium once normal locomotion was noticed.

The specimens were kept at room temperature (roughly 17-25°C) and euthanized at regular intervals by percutaneous application of

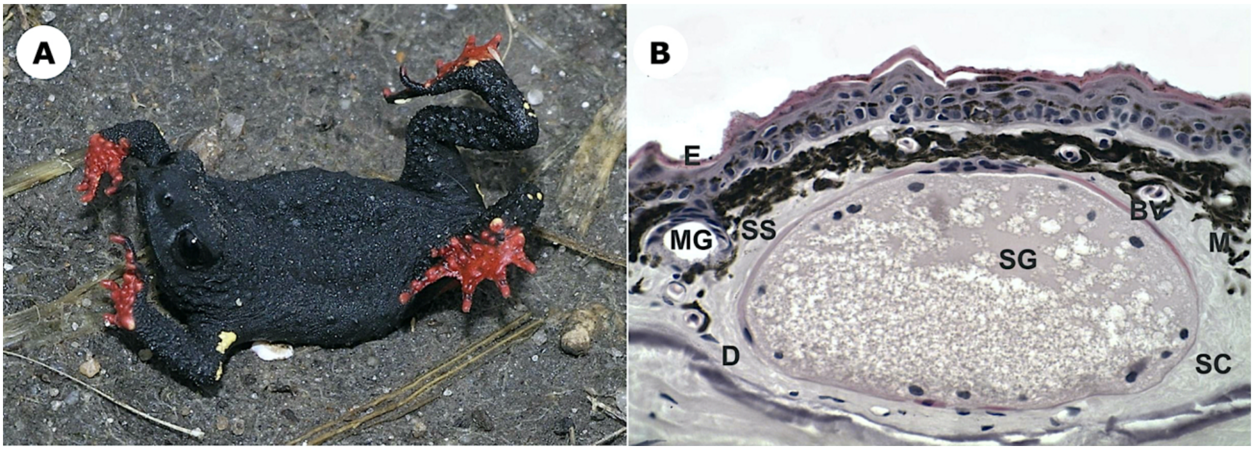

Fig. 1. Adult male of *Melanophryniscus montevidensis* (Philippi, 1902) from la Paloma, Rocha, Uruguay (snout-vent length 23 mm), displaying the defensive behaviour "unken-reflex", with exposure of aposematic coloration (A). Normal skin histology of dorsal skin in this species (B). Abbreviations: E: epidermis. BV: blood vessel. D: dermis. M: melanocytes. MG: mucous gland. SC: *stratum compactum*. SG: serous gland. SS: *stratum spongiosum*.

lidocaine gel (20%) in the pelvic patch (Altig, 1980). This sampling of one specimen was made daily from day 1 to 9, every 3-4 days until day 55, and then every 7-10 days until day 129. The wound area was photographed under the microscope, and specimens were fixed in buffered formalin (10 %). The anterior dorsal portion of the body, a whole transverse section of ca. 5 mm wide) was dissected with the binocular microscope, embedded in paraffin and processed for routine histological examination (Sadeghipour & Babaheidarian, 2019), stained with haematoxylin-eosin (AFIP, 1949),and sectioned at 5 µm. Pictures of the preparations were taken on a binocular microscope (NIKON C-LEDS ®, Japan) with a digital camera (Micrometrics®, China). The protocol was approved by the ethics committee of Veterinary Faculty (CEUA-FVET, res. 632/2019).

Results

Maintenance and anaesthesia

During the whole experience, no spontaneous mortality was recorded. Besides, abnormal behaviours were not observed, and toads were fed continuously, including the day following the anaesthetic procedure. Induction and recovery time of clove oil anaesthesia are shown in Tab. 1.

Normal skin histology in *Melanophryniscus montevidensis*

The normal skin of the *dorsum* presents a thin epidermis with a small amount of keratin externally, and interspersed melanin granules over an intermediate layer of 2-3 cells that lie over the germinative layer. Melanin granules appear scattered throughout the epidermis. This layer shows characteristic projections that give the toad skin a rugose appearance at low magnification. The dermis of the anterior dorsal area is also thin and bears an abundant number of melanophores and well-differentiated mucous and serous glands. The firsts are smaller, rounded, and more superficial. Serous glands are relevant defensive structures, bigger than mucous glands, roughly oval and more variable in size. A representative image is shown in Fig. 1B.

Wound healing

Immediately after experimental wounds (EW) were done, the wound surface was haemorrhagic, leaving exposed the underlying dorsal muscles (Fig. 2A). Mayor subsequent

Tab. 1. Induction and recovery times (minutes) for general anaesthesia in the toad *Melanophryniscus montevidensis* (Philippi, 1902) using clove oil (1/10,000). Values are mean±SD (range).

Induction	Recovery	
Toe pinch unresponsiveness (n=29)	Head rise up (n=28)	Exploratory locomotion (n=29)
18,29±6,63 (2,00-34,56)	20,52± 13,04 (3,37-56,24)	61,00±23,05 (26,83-124,09)

changes in the healing process are as follows. By day 2, a flat thin layer of 2-3 epithelial cells completely covered the EW. Some of these elements were basophilic cells, while others presented granular melanin content (Fig. 2B). On day nine, the epidermis has fully recovered thickness, and epidermal projections appear (Fig. 2C). The presence of melanophores at this stage is still incipient, bringing the EW area a lighter appearance macroscopically. A young dermal stratum starts to develop, with small developing glands.

By day 13, the gross appearance of the epidermal layer in the EW is roughly flat com-

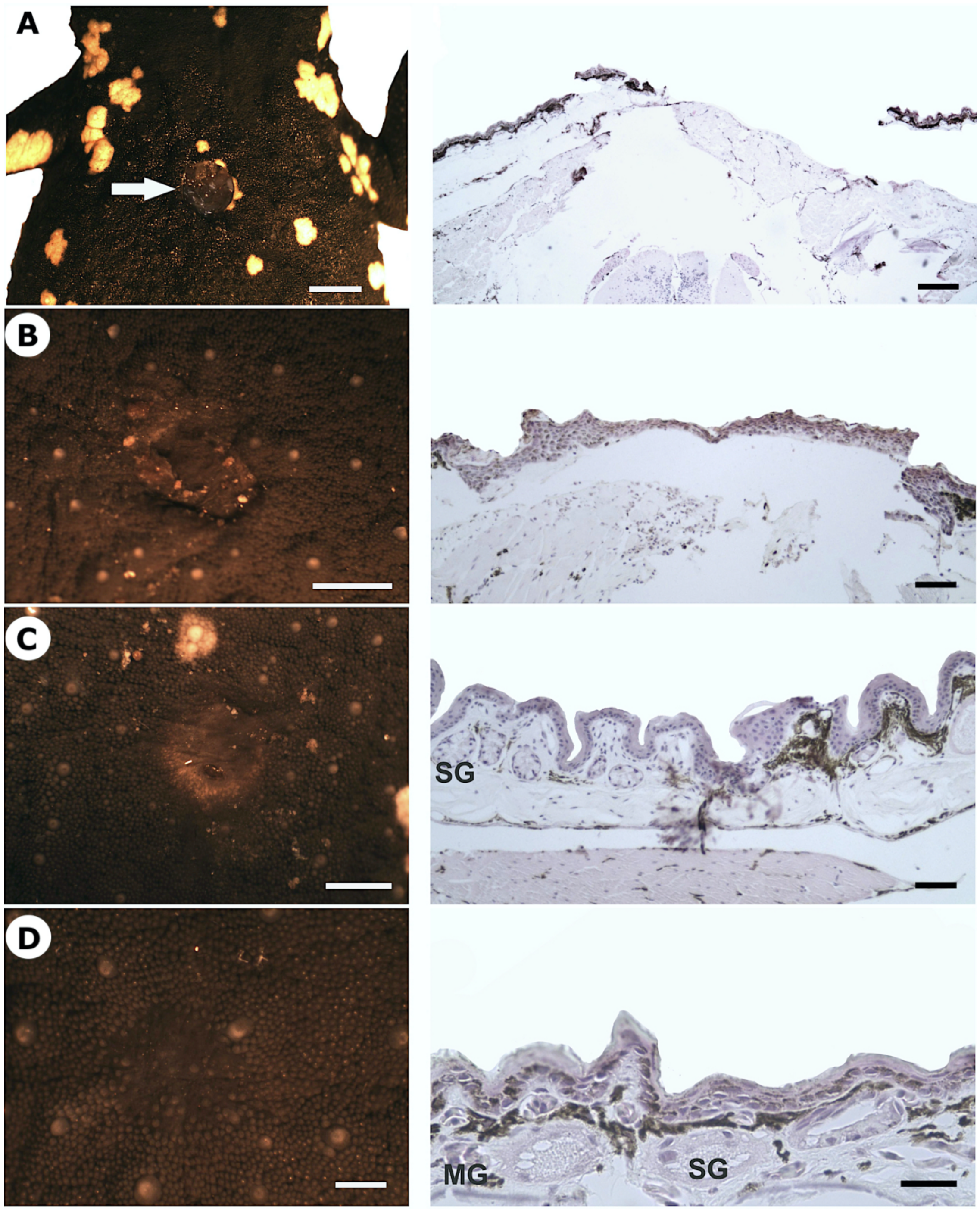

Fig. 2. Healing process of experimental wound in the dorsal skin of *Melanophryniscus montevidensis* (Philippi, 1902), macroscopically (left), and histologically (right; hematoxylin-eosin staining). A-D correspond to days 0, 2, 9, and 37 of the healing process respectively (see text). Scale bars of each macroscopic/histologic view are: A, 1.5 mm/200 μm; B, 1 mm/50 μm; C, 1 mm/50 μm; D, 0.5 mm/20 μm.

pared to the surrounding skin, while in the dermis, there is a marked proliferation of fibroblasts and collagen production. However, epidermal projections start to be evident at microscopic examination. Still, mostly undifferentiated glands become evident in the *stratum spongiosum* of the dermis. A week later (day 20), glands are more numerous, and the epidermis starts to change into a more rugose surface. These changes are associated to a noticeable presence of melanophores at the boundaries of the EW and the dermal layer. These cells surround the now more numerous serous skin glands, which are still underrepresented than in normal surrounding skin.

A few days after the first month (day 34) the glandular tissue is well developed, and areas with a significant presence of epidermal projections alternate with flat areas. Soon afterwards (day 37; Fig. 2C), the gross aspect of the EW is almost the same as the surrounding skin, except for a slightly less marked presence of epidermal projections that gives a flattened appearance. Melanophores are abundant, and the skin return to exhibit the usual black color. Microscopically, serous glands are well differentiated, still less abundant than in normal skin but growing in number. Mucous glands are now distinguishable and abundant (Fig. 2D). By day 51, noticeable changes are the higher number and size of epidermal projections, at this point more like normal skin, and a growing number of mucous glands. From this stage and up to day 129, there were no evident histological nor macroscopic remarkable features.

Discussion

Regarding the characterization of normal skin structure, only a few species of the whole amphibian diversity worldwide (ca. 8500 species; Frost, 2022) have been studied so far (Fox, 1994). Similarly, available reports about skin histology in Neotropical amphibians are scarce and mainly address the characteristics of skin appendages and secretions (i.e. Brunetti et al., 2016; Ferraro et al., 2013; Luna et al., 2018). Histological information regarding the genus *Melanophryniscus* was previously published in a few works that focused on normal histology (Delfino et al., 1998; Mangione & Alcaide, 1994; Naya et al., 2004). The skin structure and glands observed herein for *M. montevidensis* were the same as previously described by these authors. However, the present work is to our knowledge the first to address the study of wound healing in *Melanophryniscus* and more generally in Neotropical anurans.

During the second half of the 20th century, the skin of amphibians was studied with a focus on its capacity to repair without scarring (Yokoyama et al., 2011). Understanding of the underlying mechanisms was viewed as a promissory baseline information when seeking strategies for scar reduction in mammal skin wounds (Kawasumi et al., 2013; Otsuka-Yamaguchi et al., 2017; Yokohama et al., 2018).

Interspecific comparisons against *M. montevidensis* can be made with phylogenetically distant taxa like the Mexican axolotl *Ambystoma mexicanum* (Lévesque et al., 2010) and the African clawed frog *Xenopus laevis* (Daudin, 1802) (Otsuka-Yamaguchi et al., 2017; Yokohama et al., 2011). The skin healing process in this frog is apparently faster, maybe due to its aquatic habits with the need for skin repair to diminish exposure to potentially pathogenic microorganisms. Epithelization of similar EW in *X. laevis* is completed during the first day (Otsuka-Yamaguchi et al., 2017), with complete restoration of dermal structures by day 10 (Yokoyama et al., 2011). In *M. montevidensis*, the epidermal and dermal healing was also fast, although they lasted 2 and 13 days, respectively. However, an almost complete healing process of skin wounds is not generalized in anurans. For instance, the aquatic American bullfrog *Lithobates catesbeianus* (Shaw, 1802) loss dermal structures during skin repair (Yannas et al., 1996). More information is needed to assess the occurrence of distinct pathways of skin repair in different anuran lineages.

The EW we provoked in *M. montevidensis* simulated a wound produced by visually oriented predators like a small bird, lizard or snake. Under natural conditions, the rapid healing following a predation attempt would help not to diminish survivorship. Besides, the recovery of serous glands is a relevant feature as defensive secretions take part in the predator deterrence mechanisms of these toads.

Finally, the maintenance of specimens of *M. montevidensis* under laboratory conditions was only previously addressed briefly by Kolenc (1988). Similar captive management was successful, and the anaesthetic protocol for which we had no previous information regarding the genus *Melanophryniscus*. Although we did not record complications associated with clove oil anaesthesia, some authors have indicated the relatively high risk of toxicity of this agent in amphibians, with complications such as long recovery periods, stomach prolapse, respiratory distress and death (Hernández et al., 2012; Mitchell et al., 2009). In our case, induction and recovery times were acceptable for a rapid experimental procedure and allowed careful manipulation of specimens. Furthermore, recovery using combinations of more modern drug agents like ketamine and diazepam could be noticeably longer (Hernández et al., 2012).

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