

José Roberto Machado Cunha da Silva  
Idércio Luis Sinhorini · Bernard Ernesto Jensch-Junior  
Laércio Ribeiro Porto-Neto  
Francisco Javier Hernandez-Blazquez  
Bruno Cossermelli Vellutini  
Leandro Nogueira Pressinotti  
Frederico Azevedo Costa Pinto · Edwin Lowell Cooper  
João Carlos Shimada Borges

## Kinetics of induced wound repair at 0°C in the Antarctic fish (Cabeçuda) *Notothenia coriiceps*

Received: 7 October 2003 / Revised: 22 February 2004 / Accepted: 23 February 2004 / Published online: 7 May 2004  
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**Abstract** *Notothenia coriiceps* were kept at  $0 \pm 2^\circ\text{C}$ . Following anaesthesia, a square excision ( $4 \text{ cm}^2$ ) was made on the dorsal lateral anterior regions on both sides. The process of wound healing was monitored after 0, 1, 2, 7, 15, 23, 60 and 90 days. The wounds were processed for scanning electron microscopy. Following surgery, haemorrhage was abundant; after 24–48 h, the wound was covered by mucous exudes. At 7–15 days, there was an epidermal migration towards the centre of the wound, while the borders became oedematous. During 23–30 days, all the wounds were closed like a sphincter and there was retraction of the borders. After 60–90 days, the wounds contracted and became black. No scales were seen in the wounded area. The regenerating epidermis migration speed was  $33.3\text{--}43.4 \mu\text{m}/\text{day}$ . This work shows for the first time the kinetics of wound repair at  $0^\circ\text{C}$ .

J. R. M. C. da Silva (✉) · B. E. Jensch-Junior  
L. R. Porto-Neto · B. C. Vellutini · L. N. Pressinotti  
J. C. S. Borges  
Department of Histology and Embryology,  
Institute of Biomedical Sciences, University of São Paulo,  
Av. Prof. Lineu Prestes, 1524, sala 409, 04601-000 São Paulo,  
Brazil  
E-mail: jrms@usp.br

I. L. Sinhorini · F. A. C. Pinto  
Department of Pathology, Faculty of Veterinary Medicine,  
University of São Paulo, São Paulo, Brazil

E. L. Cooper  
Laboratory of Comparative Neuroimmunology,  
Department of Neurobiology,  
David Geffen School of Medicine at UCLA,  
University of California, Los Angeles, CA  
190095-4763, USA

F. J. Hernandez-Blazquez  
Department of Surgery, Faculty of Veterinary Medicine,  
University of São Paulo, São Paulo, Brazil

### Introduction

The intensity and velocity of wound healing in ectothermic vertebrates varies according to seasonality and local temperature (Reddan and Rothstein 1965; Finn and Nielsen 1971; Grout and Morris 1987; Hardie et al. 1994). Tissue repair always seeks to close the wounded region by means of tissue regeneration, resulting usually in a scar, in order to recover the original function (Majno and Joris 1996).

Despite some studies on the influence of fish acclimatisation from tropical and temperate waters on the regenerating process (Mittal and Munshi 1974; Anderson and Roberts 1975; Phromsuthirak 1977; Bullock et al. 1978; Majno and Joris 1996; Quilhac and Sire 1998, 1999), there are no studies on wound repair under polar temperatures. However, inflammatory processes (Silva et al. 1998, 1999) and phagocytosis have already been described at  $0^\circ\text{C}$  (Silva and Peck 2000; Silva et al. 2001, 2002; Borges et al. 2002).

*Notothenia coriiceps* (Richardson 1844), also called “Cabeçuda”, is a shallow-water benthic, primarily predator species, and has widespread distribution, probably circum-Antarctic on the continental shelf. This Antarctic species lives at depth range of 0–550 m, at temperatures of  $-1.8$  to near  $0^\circ\text{C}$  (Gon and Heemstra 1990; Prisco et al. 1991).

During fieldwork, we collected one *N. coriiceps* with a conic-shaped scar that suggests a possible seal bite. Therefore, it appears that the skin is able to regenerate properly at low temperatures, close to  $0^\circ\text{C}$ . But, do these processes of wound healing fit well with those at higher temperatures? The present study analyses the kinetics after experimentally inducing wounds in *N. coriiceps* at  $0^\circ\text{C}$ , using scanning electron microscopy. The data from tissues and cells involved in the wound repair are being

processed, and will be published in another work. The process of wound healing is compared in the available literature on temperate and tropical teleost fishes.

## Materials and methods

### Fish

#### *Naturally occurring*

One *N. coriiceps* was found with a wounded scar on the posterior ventral lateral region. This fish was photographed and processed for scanning electron microscopy as described below.

#### *Experimental*

Twenty-five *N. coriiceps* (Richardson 1844) (according to Gon and Heemstra 1990), weight 523–1.163 g ( $843 \pm 320$  g) and total size 33.2–43.4 cm ( $38.3 \pm 5.1$  cm) were used in this experiment. They were collected in January-February 2000 ( $n=14$ ) and from December to April 2001 ( $n=11$ ) at Admiralty Bay, King George Island, in the South Shetland Islands ( $62^{\circ}06'S$  and  $58^{\circ}23'W$ ). The fish were kept in fibreglass tanks (2000 l) filled with seawater in an acclimatised room at  $0.0 \pm 1.0^{\circ}C$  and fed twice weekly. The experiments were carried out in the Biology Laboratories of the Brazilian Antarctic Station "Comandante Ferraz".

### Surgical lesions and anaesthesia

Surgical lesions were made, after anaesthesia with benzocaine 50.0 ppm (Silva et al. 2002), in the dorsal-lateral anterior region on both sides of experimental fish ( $n=22$ ). The scales, epidermis, dermis, and most of the perimysium were removed on  $4.0\text{ cm}^2$  (total of 44 lesions). Three more fishes were used as controls on normal skin in this region.

### Gross analysis of wound healing

After time spans of 0 (immediately after the wound), 1, 2, 7, 15, 23, 30, 45, 60 and 90 days, the fishes were killed by an overdose of benzocaine. For each period, photographs of the wound were taken (Canon eos-300 with macro lens-100 mm) on both sides (along with a scale in millimetres) until their sacrifice, in order to study macroscopically the kinetics of wound healing.

### Scanning electron microscopy

The tissue samples from the whole wound area after 15 days ( $n=2$ ), 30 days ( $n=3$ ), 60 days ( $n=4$ ) and 90 days ( $n=4$ ) were fixed in cold marine McDowell solution (McDowell and Trump 1976) for 24 h at  $0^{\circ}C$ ,

dehydrated in a graded series of ethanol, dried through the critical point method with  $CO_2$ , covered with a gold layer and examined in an SEM-JEOL (6100) and LEO (435VP).

### Quantification of regenerated tissue

The photographs of the left and right wounded regions of each fish were scanned using the software SigmaScan. The wounded area and the regenerated area were measured (in  $mm^2$ ) and the percentage of the regenerated area was calculated.

## Results

### Normal skin

In *N. coriiceps*, the skin is scaled and the outer epidermal surface is ornamented with fingerprints. There are also mucous cells whose pores are identified with scanning electron micrographs (SEM) (Fig. 1).

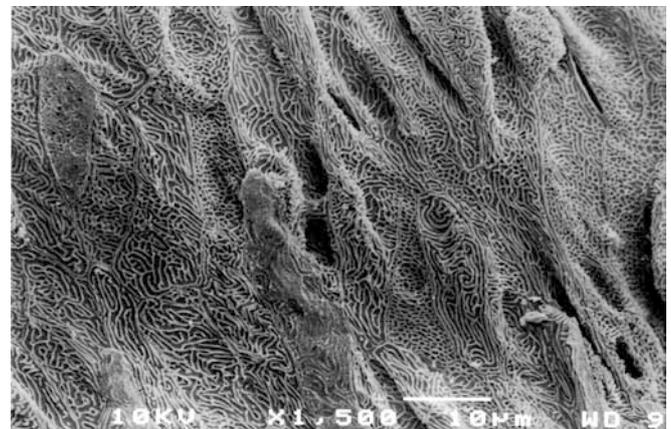
### Naturally found wound

We noticed the presence of a scar in one of the collected *N. coriiceps*. The scar was complete and the centre was smooth, bright and dark-brown to black. The wound presented smaller scales on the periphery, and irregular borders (Fig. 2a). With SEM, the majority of the scar border cells did not show the typical fingerprint surface while none of the wound centre epidermal cells had the fingerprint surface (Fig. 2b, c).

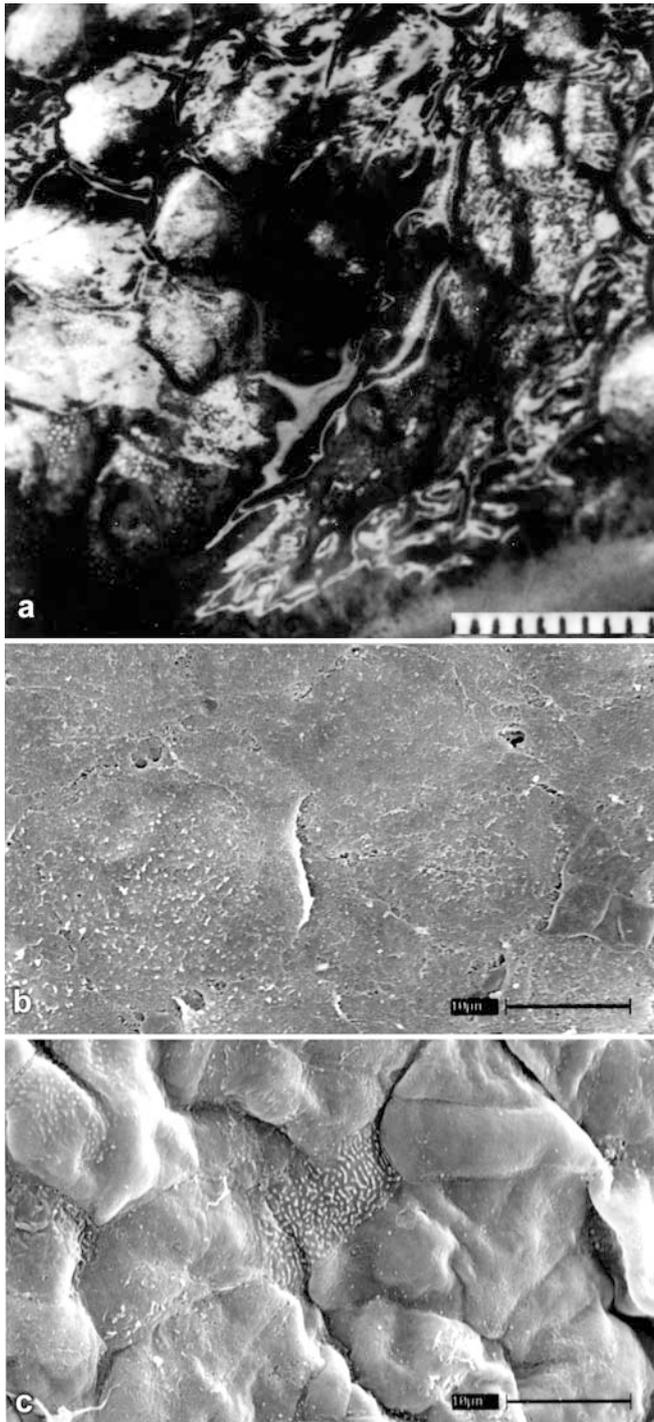
### Induced wounds at different time spans

#### *After the lesion*

Immediately after surgery, haemorrhage was visible mainly on the wound borders (but also in the central



**Fig. 1** Scanning electron micrograph of *N. coriiceps* skin surface, showing the flat epidermal cells with their typical fingerprints surface pattern



**Fig. 2** **a** Naturally found scar at the ventral lateral region of a *N. coriiceps*. The scales are absent and the central region is black. **b** Central region SEM of the same material, showing that epidermal cells do not have the fingerprint surface. **c** Wound borders SEM. Only a few epidermal cells present the typical fingerprint surface (compare to Fig. 1)

area). Small black spots can be observed all over the surface of the remaining perimysium that covers the muscular tissues. Figure 3 shows the sequence of wound healing on the same fish region after the different time spans.

#### *Aspect of the wounded region at days 1–15*

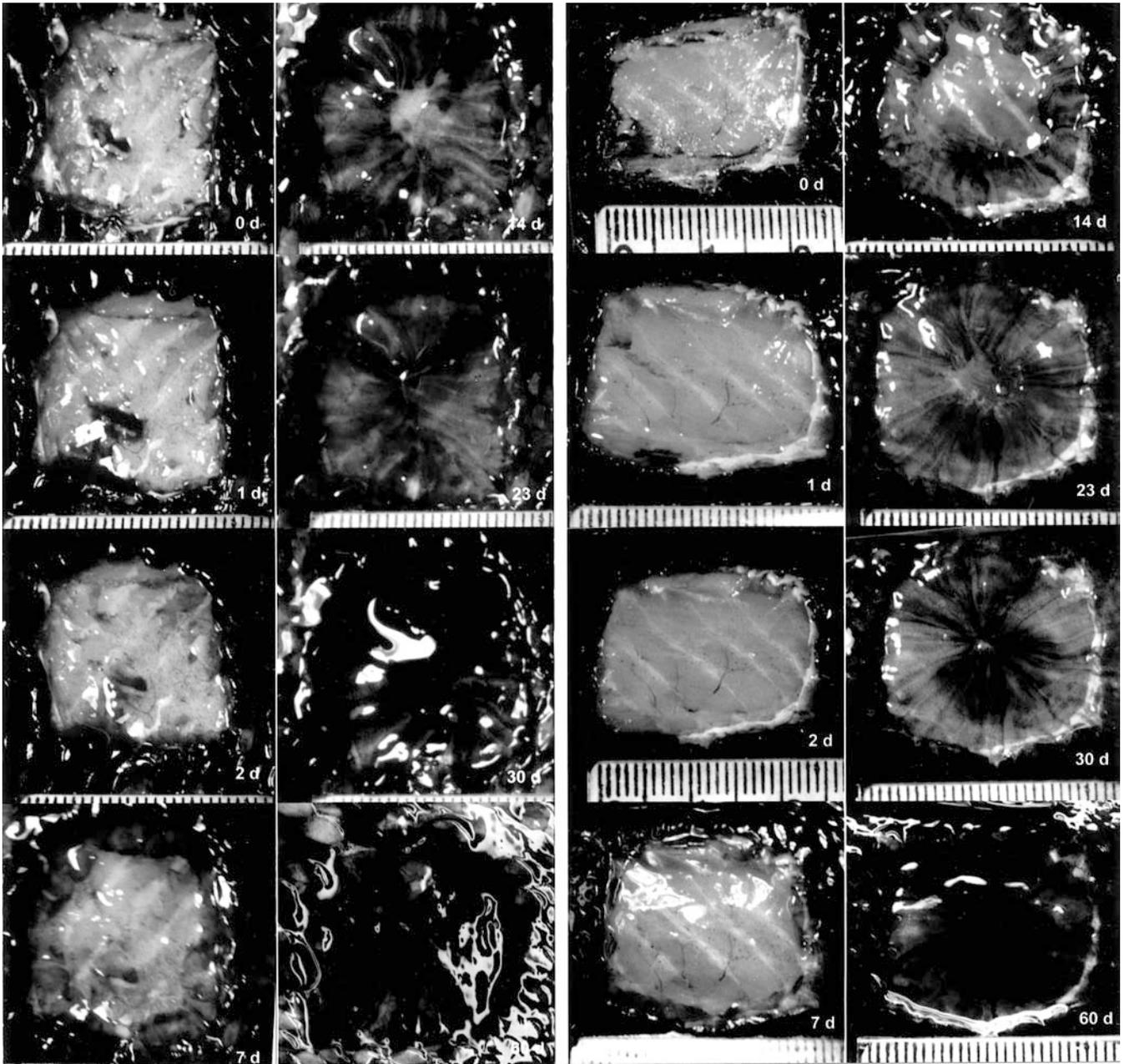
At day 1, the wounded region presented blood clots, mainly at the wound borders; large amount of mucus and exude can also be seen. After 2 days, observations were the same as on day 1, with the beginning of a discrete oedema on the wound borders (Fig. 3). On day 7, from the wound borders, a thin layer of migrating epidermis can be seen over the coagulated blood. This projection is about 1–3 mm in length all over the border with some pigmented areas (unpublished data). There is an increase of the wound border oedema, under the remaining scales (the original square wound became rounded). The centre of the wound is similar to day 1 (Fig. 3). On day 15, there is an increase of the wound border oedema. The regenerating epidermis covers almost half of the wound, and has a length of 4–8 mm; it is possible to see some pigmented radial black lines, appearing from the periphery to the central region (Fig. 3), composed of melanocytes (unpublished data). The epidermis border has an irregular surface (Fig. 4), with some different patterns of fingerprints (Fig. 5). The central wound region not covered by the epidermis (i.e. exposed to the seawater) presents a disorganised pattern, compared to the normal skin surface, with numerous filamentous structures under SEM, suggesting presence of bacteria (Fig. 6).

#### *Aspects of the wound region after 23 and 30 days*

The wound face is completely covered by the regenerating epidermis in the majority of the experimental fishes. The oedema is still present under the scales at the wound border. The central region is more pigmented. In some wounds, the centre of the regenerated epidermis cover is circular, but in others, it is a short straight line (Fig. 3). At day 30, the oedema under the scales near the border is still present and the wound has been completely closed. Thicker black radial lines, being almost homogeneously pigmented in the central region, form the pigmented area. Observed with SEM, the wound central area resembles a sphincter (Figs. 3,7). There are no longer epidermal borders, suggesting fusion.

#### *Aspects of the wound region after 45–60 days*

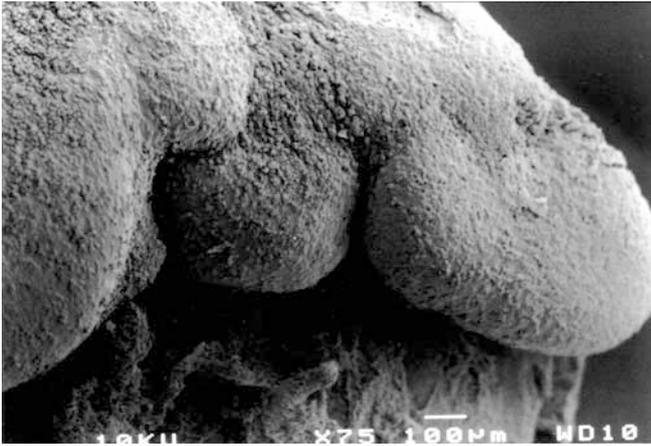
After 45 days, the oedema was still present and the wound area became more homogeneously pigmented in general, with many small black spots (Fig. 3). At day 60, the wound region is almost entirely black, it being no longer possible to identify the radial lines and wound centre. The surface became more homogeneously pigmented with many black spots, larger than in previous time spans and, consequently, closer to each other. The wound border oedema remains prominent under the scales. The wound borders have changed from angled to round (Fig. 3).



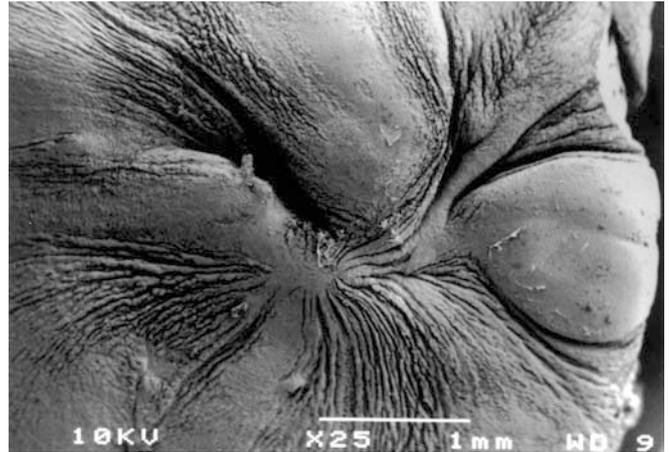
**Fig. 3** Photographs of the induced anterior dorsum lateral wounds of two different *N. coriiceps* after different time spans—0, 1, 2, 7, 14, 23, 30, 60 days. Haemorrhage occurred immediately after the lesion was inflicted, turning into blood clots between 0 and 2 days. The oedema that had begun to accumulate in the early days is more prominent at the borders after 7 days, changing the angulated corners of the wound to a rounded shape, and a thin epidermal layer from the borders to the centre is already present. After 15 days, there is an increase of the regenerating epidermis-covered area; at day 23 the wound was completely closed in the *left column*, forming a line, and after 30 days in the *right column*, both with pigmented radial *black lines*. After 30 days, thicker pigmented lines can be seen in the *left column* and after 60 days in the *right column*. After 60 days, the wound became homogeneously black. All photographs are at the same scale and the *bar* is in millimetres

#### *The condition at 90 days*

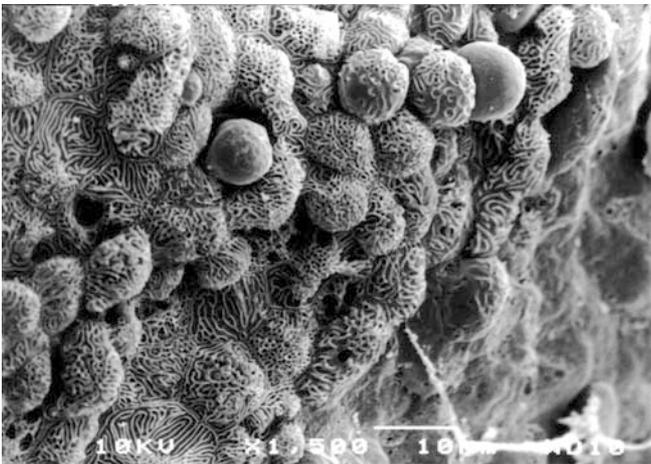
The wound border oedema under the scales has diminished but is still present, and the wound general aspect is darker and more rounded. A dark tissue (darker than the rest of the animal) covers the whole wounded region homogeneously. The original squared shape of the wound is no longer discernible. Only a rounded depression is visible (Fig. 8a). At the SEM level, in the wound centre, the cells are flat and most of them present the typical fingerprint surface (Fig. 8b). No regenerating scales were seen in the wounded region (Fig. 8).



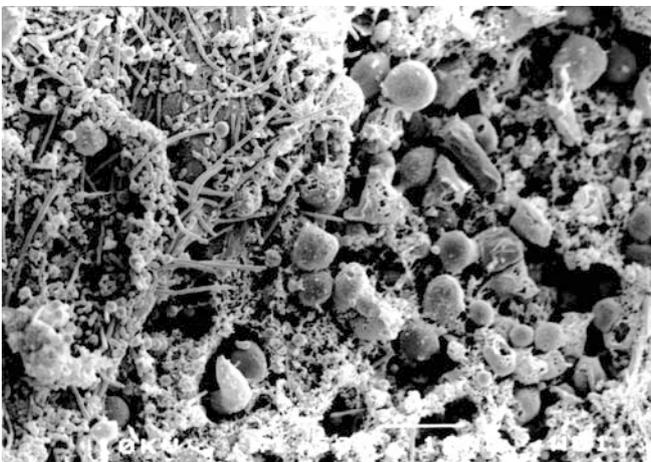
**Fig. 4** SEM. The wound surface after 15 days. Migration front of the regenerating epidermis. The tongue tip can be seen (*bottom* of the figure)



**Fig. 7** SEM. Wound surface after 30 days. The wound surface is completely closed and the regenerated epidermis presents many radial lines converging towards the wound centre in a sphincter-like fashion



**Fig. 5** SEM. Wound surface after 15 days. Higher magnification of the regenerating epidermis surface at the wound border. The epidermal cells are rounded with different and incipient patterns of fingerprints



**Fig. 6** SEM. Wound central region, after 15 days, is covered by cell debris and filaments

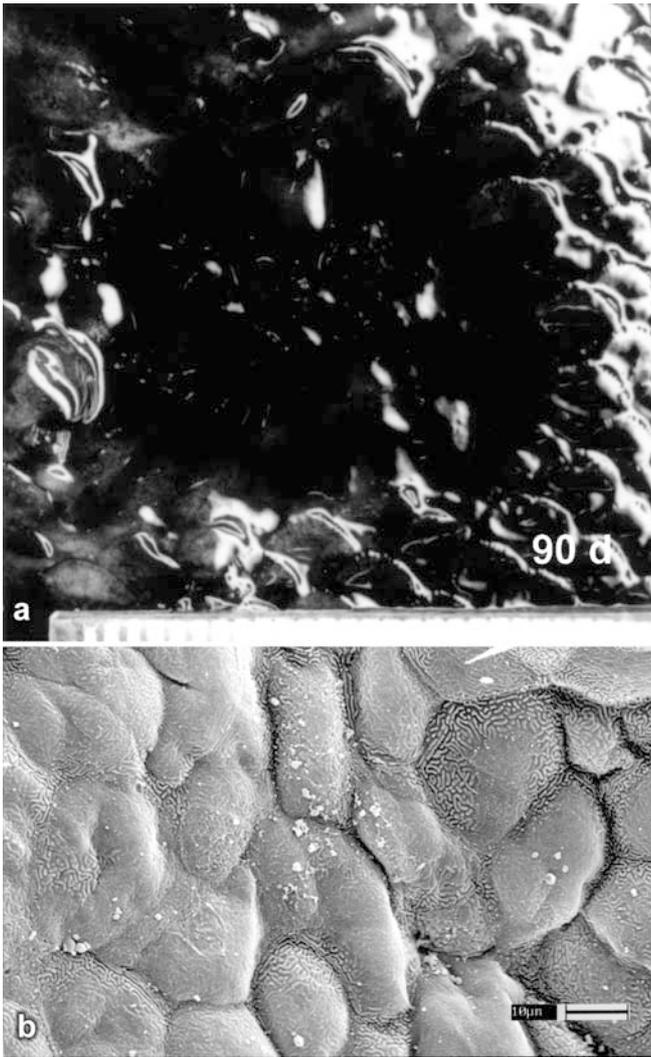
### Kinetic studies

The number of wounds and associated data and regenerating epidermis surface are presented in Table 1 and Fig. 9. The kinetics of the wound healing process can be seen in Fig. 9. The percentages of the regenerated epidermis compared to the induced wound surface after different time spans were calculated.

### Discussion

This study is the first reporting on the wound repair kinetics in fish integument at 0°C. Only a few studies are concerned with the influence of the acclimatisation of fishes from tropical and temperate fresh waters on skin regeneration. This includes wound repair in a tropical cyprinid *Tanichthys albonubes* at 10–30°C and the Atlantic salmonid *Salmo salar* (temperate fish) at 5–23°C. Results suggest that the rate of wound healing in both species was directly correlated with environmental temperature, while temperature stress had little effect on healing rates (Anderson and Roberts 1975). The wound repair also depends on other factors, such as size of the lesion, blood and nerve supply, and contamination among others (Majno and Joris 1996).

Bullock et al. (1978), using *Pleuronectes platessa* reared at different temperatures (5, 10 and 15°C), observed that a lesion of 5×1 mm was completely closed after 9 h at 10°C, and after 12 h at 5°C, by Langerhans-type cell migration of the epidermis. The authors pointed out that under low temperatures, such as 5°C, the main mechanism of epidermal wound repair in teleost fishes is cellular migration, an epidermal layer sliding through the injury. In *N. coriiceps*, there was no quick epidermal sliding after the first hours wound repair. The regeneration process was affected by the epidermis sliding slowly from the wound borders, moving towards the



**Fig. 8** **a** Anterior dorsum lateral induced wound of *N. coriiceps* after 90 days (top) with a visible retraction of the borders. No scales can be seen in wounded area. **b** Wound surface SEM, after 90 days, where the majority of the epidermal cells present fingerprint surface

centre ( $4 \text{ cm}^2$ ). This process required 23–30 days in order to cover the whole area.

The healing speed studied by Quilhac and Sire (1999) on the tropical teleost *Hemichromis bimaculatus* was  $500 \mu\text{m/h}$ , after excision of the scales and part of the epidermis on a  $1\text{-cm}^2$  area. The re-epithelisation speed of the tropical fish *Prochilodus scrofa* ( $20 \pm 2^\circ\text{C}$ ), using the same methodology as in the present work except for smaller lesion ( $1.0 \text{ cm}^2$ ), was on average  $6.9 \mu\text{m/h}$  (personal unpublished data). In *N. coriiceps*, 23–30 days were necessary to cover  $1 \text{ cm}^2$  with a speed of regeneration ranging from  $1.4$  to  $1.8 \mu\text{m/h}$ , i.e. about 300 times slower than the data found by Quilhac and Sire (1999) and about 4 times slower than observed for *Prochilodus scrofa* (personal data).

The differences in wound healing speed found between *H. bimaculatus* and *Prochilodus scrofa* when

compared to *N. coriiceps* are relative to the temperature ( $25$ ,  $20$ , and  $0^\circ\text{C}$ ) and to the wound size ( $1.0$ ,  $1.0$  and  $2.0 \text{ cm}^2$ ). The difference observed between the wound healing speed of *H. bimaculatus*, described in the studies of Quilhac and Sire (1998), and *Prochilodus scrofa* (unpublished data), both with  $1.0\text{-cm}^2$  wound, is probably the permanence of connective tissue in the wound. Those authors removed only the scales and part of the epidermis, whilst we removed scales, dermis, hypoderm and most of the perimysium, probably complicating the sliding of the regenerating epidermis.

There is a faster epidermal closing mechanism of the wound by fishes living at higher temperatures, in order to reduce the contamination risk (Bullock et al. 1978). The supporting basis for cell migration is composed of matrix, membrane debris and fibrils. The literature indicates a *leap-frog* type re-epithelisation, due to the facility of cell adhesion to the substrate (Quilhac and Sire 1999). The migrating epidermis central layer slides between other layers and after adhesion and differentiation of the basal layer, a new substrate is formed to give continuity to the sliding process until fusion, making a stronger barrier to the external environment. *N. coriiceps* were able to deal with this problem due to the cellular debris and tissue (Fig. 7) which possibly isolates muscle cells from the hyperosmotic environment. After 2 days, *N. coriiceps* seems to adopt a similar strategy, i.e. of sliding the epidermis over this necrotic area, but much more slowly, with the tip region of the tongue detached from the underlying substrate as described by Quilhac and Sire (1999).

Quilhac and Sire (1999), analysing wound healing in *H. bimaculatus*, observed that the migrating epidermis borders made a fold over the wound after one side reached the opposite border epidermis, suggesting that the migration inhibitory mechanism does not occur immediately. This does not seem to be the strategy adopted by *N. coriiceps*, where there is immediate fusion of the migrating epidermis after contact.

No regenerating scales were seen on *N. coriiceps* wound sections after 90 days (unpublished data), pointing to the importance of retraction to cover or minimise old wounds, like the naturally found wound observed with scales around the scar. Despite the long period needed to close the wound (23–30 days) in all studied fishes, there were no symptoms of contamination by bacteria or fungus retarding the process. This finding suggests either a very effective skin-mucus antiseptic action or the low pathogenic capacity of the Antarctic seawater germs, or both. This study indicates wound healing adaptation to the Antarctic temperatures ( $0^\circ\text{C}$ ) despite the differences with the other teleost species studied. *N. coriiceps* is able to deal with large extension lesions ( $4 \text{ cm}^2$ ) without death or contamination, demonstrating the high efficiency of the wound healing process in these animals from the Antarctic environment.

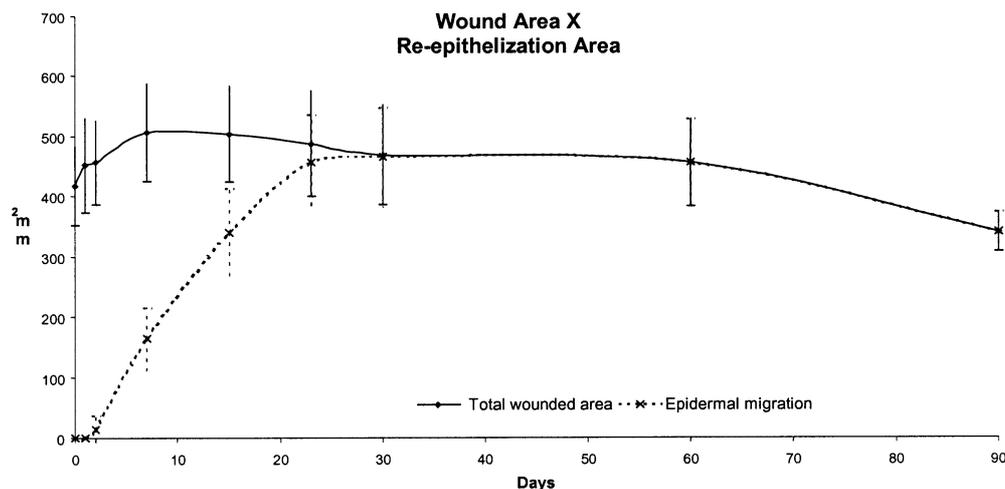
**Table 1** Number of wounded regions examined in the kinetics studies after different time spans, showing the wound surface, the regenerated surface and the percentage of regenerated surface

Days after injury	0	1	2	7	15	23	30	60	90
No. of wounds measured ( <i>n</i> )	43 <sup>a</sup>	43	43	43	41	40	38	20	4
Wound surface (mm <sup>2</sup> )									
Average	417.62	451.62	456.32	506.98	503.91	487.88	469.58	456.70	341.24
Standard deviation	65.45	78.47	69.42	80.93	79.71	87.10	83.69	73.12	32.07
Maximum value	544.32	604.42	645.50	719.81	674.52	767.14	677.76	672.58	375.30
Minimum value	288.40	319.73	330.68	357.08	372.15	340.37	336.37	360.02	299.69
Regenerated surface (mm <sup>2</sup> )									
Average	0.00	0.00	13.70	164.95	340.82	456.41	465.98	456.70	341.24
Standard deviation	0.00	0.00	22.18	50.89	72.61	79.68	81.93	73.12	32.07
Maximum value	0.00	0.00	75.21	261.71	476.96	731.06	677.76	672.58	375.30
Minimum value	0.00	0.00	0.00	79.60	223.70	318.79	336.67	360.02	299.69
Percentage of regenerated surface <sup>b</sup>									
Average	0.00	0.00	3.07	32.65	68.15	94.05	99.30	100.00	100.00
Standard deviation	0.00	0.00	4.86	9.10	12.51	8.17	2.40	0.00	0.00
Maximum value	0.00	0.00	15.26	49.38	96.65	100.00	100.09	100.00	100.00
Minimum value	0.00	0.00	0.00	15.22	41.40	67.81	86.39	100.00	100.00

<sup>a</sup>One wound was damaged due to mechanical shock in the tank and was not included in this study

<sup>b</sup>All the percent values of regenerated epidermis were significantly different (Turkey-Kramer-multiple comparison test)—GRAPHPAD INSTAT

**Fig. 9** Area of the regenerated epidermis and the wound area of *N. coriiceps* after different time spans. The wound borders start to retract after 7 days and this process persists until the longest period studied



**Acknowledgements** Financial support was given by CNPq (68.0047/00-0 and 48.0262/00-4 grants), PROANTAR, SECIRM, and logistic support was given by the Brazilian Navy. We also thank Edson R. de Oliveira for the photographs, Dr. M.A. Miglino from FMVZ-USP for the use of the SEM, and Yara Shimada Brotto for the English revision of the paper.

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