Low temperatures reduce skin healing in the Jacaré do Pantanal (Caiman yacare, Daudin 1802)

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Summary
Studies of skin wound healing in crocodilians are necessary given the frequent occurrence of cannibalism in intensive farming systems. Air temperature affects tissue recovery because crocodilians are ectothermic. Therefore, the kinetics of skin wound healing in Caiman yacare were examined at temperatures of 33°C and 23°C. Sixteen caiman were selected and divided into two groups of eight maintained at 23°C or 33°C. The studied individuals’ scars were photographed after 1, 2, 3, 7, 15 and 30 days of the experimental conditions, and samples were collected for histological processing after 3, 7, 15 and 30 days. Macroscopically, the blood clot (heterophilic granuloma) noticeably remained in place covering the wound longer for the caiman kept at 23°C. Microscopically, the temperature of 23°C slowed epidermal migration and skin repair. Comparatively, new blood vessels, labeled using von Willebrand factor (vWF) antibody staining, were more frequently found in the scars of the 33°C group. The collagen fibers in the dermis were denser in the 33°C treatment. Considering the delayed healing at 23°C, producers are recommended to keep wounded animals at 33°C, especially when tanks are cold, to enable rapid wound closure and better repair of collagen fibers because such lesions tend to compromise the use of their skin as leather.

Introduction
Crocodilian wound healing and tissue regeneration processes are still poorly studied. There are reports of the tail tip and lower jaw having a partial regenerative capacity. The regenerating vertebral segments are replaced by an endochondral ossification in the form of an unarticulated straight bat. The regenerating skin segment does not repair the osteoderms, therefore differing from the original skin and retaining only pigmentation similar to the lower layers of the skin and muscle layer and, finally, the environmental temperature (Alibardi, 2010). A study of the effect of temperature on the regeneration of the tails of Anolis carolinensis lizards indicated that the tail grew longer and faster at 32°C than at 21°C (Maderson and Licht, 1968). Wounds on Thamnophis sirtalis snakes healed better at 30°C than at 13.5°C or 21°C as long as the snakes’ nutritional demands are met (Smith et al., 1988). To our knowledge, there have been no reports on the effect of temperature on crocodilian wound healing.

Conversely, the effects of temperature on the crocodilian immune system are well studied. Low temperatures tend to decrease the bactericidal, amoebicidal and hemolytic effects of Alligator mississippiensis serum (Merchant et al., 2003; Merchant et al., 2004; Merchant et al., 2005); the effect of A. mississippiensis phospholipase A2 (Merchant et al., 2009); the hemolytic activity of Crocodylus porosus and Crocodylus johnstoni sera (Merchant and Britton, 2006) and the activity of dipeptidyl peptidase IV in Caiman yacare and Caiman latirostris...
(Siroski et al., 2012). Thus, changes are expected to occur in the wound healing and tissue repair processes of crocodilians subjected to different temperatures.

In other reptiles, including the gecko *Hemidactylus flaviviridis*, temperatures lower than 25˚C suppress the activity of phagocytes (Mondal and Rai, 2001). Conversely, in amphibians, the proliferative activity of the lymphocytes and complement system in *Rana pipiens* decrease at 5˚C, although there is an increase in the activity of the complement system, above that of the control group, when the frog is transferred from 5˚C back to 22˚C (Maniero and Carey, 1997). Such a “compensatory” increase was not found in the tail regeneration of *A. carolinensis* lizards when transferred from 21˚C back to 32˚C (Maderson and Licht, 1968). Following carrageenan challenge and suture, *Rana catesbeiana*, also an amphibian, exhibited a lower number of inflammatory cells in the wound at 6˚C than at 24˚C (Catão-Dias and Sinhorini, 1999).

Crocodilians’ skin provides protection from infection and has commercial value as leather (Woodward et al., 1993). The effect of temperature on skin repair must be examined to aid in avoiding epizootic outbreaks and enable skin repair to maximize its usability as leather, considering the mutilations that frequently occur under farming conditions.

### Materials and Methods

Sixteen *C. yacare* aged between 1 and 1.5 years, donated by the Cooperative of Caiman yacare farmers (Cooperativa de Criadores de Jacarés-do-Pantanal (COOCRIJAPAN)) located in the Cáceres municipality, Mato Grosso (MT), were used in this study. This research was approved by the Commission of Ethics in Animal Experimentation of the Biomedical Science Institute, University of São Paulo (USP), São Paulo (SP).

After intravenously administering anesthesia with propofol at a dose of 5 to 10 mg/kg, animals were subjected to skin abscission, equivalent to the area of an osteoderm (mean area was 91.75±25.13 mm$^2$ at dorsal site and 74.80±14.16 mm$^2$ at ventral site), in the dorsal and ventral portions of the tail. Subsequently, the caiman were maintained in environments with regulated air temperatures in closed systems in order to avoid epizootic outbreaks and enable skin repair to maximize its commercial value as leather (Woodward et al., 1993). The effect of temperature on skin repair must be examined to aid in avoiding epizootic outbreaks and enable skin repair to maximize its usability as leather, considering the mutilations that frequently occur under farming conditions.

The wounds were photographed 0, 1, 2, 3, 7, 15 and 30 days after the abscission next to a metric scale enabling their measurement. At 3, 7, 15 and 30 days, full lengths of the wounds, until the muscle layer, were collected in two caiman each next to a metric scale enabling their measurement. At 3, 7, 15 and 30 days, full lengths of the wounds, until the muscle layer, were collected in two caiman each.

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The wound depth reached the muscle fascia at the site where the osteoderm was collected at day 3 of wound healing. The morphology of the epidermis in the most distant wound areas was similar to that of normal skin. Staining intensity per pixel is an inversely proportional value, that is, the brighter pixel was defined as the greatest pixel value ranging from round to elliptical, and were electron-dense under TEM. The light microscopy preparations were stained using the Rosenfeld method (Rosenfield, 1947). The other half of each specimen was fixed in McDowell’s fixative for 48 h under refrigeration (McDowell and Trump, 1976) for histochemistry and electron microscopy (TEM).

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eosinophilic mass of coagulated cells with several visible cell nuclei. Heterophils were the most common clot-forming cells (heterophilic granuloma), and they have preserved morphology in the most basal layers of the clot. Cross sections of muscle fibers were often observed embedded in the heterophilic granuloma.

Rows of multinucleated giant cells forming a palisade layer, delimiting the clot base from the other tissues, were noted below the clot on wounds healing for 3 and 7 days. Ultrastructural analysis confirmed the multinucleated pattern of those cells, with two predominant cell types: giant electron-dense cells and giant electron-lucent cells. The giant electron-dense cells have cell-shaped nuclei, and their outline tends to appear triangular. The cytoplasm has vesicles filled with amorphous material, electron-lucent circular structures and numerous filopodia surrounding the cell (Fig. 3A). The giant electron-lucent cells have round nuclei, predominantly with euchromatin; cytoplasm filled with amorphous membranous compartments, which gives a foamy appearance to cells; and granulation filled with heterogeneous electron-dense content (Fig. 3B). The number of such multinucleated giant cells decreased as the epidermis began to cover the wound. The ultrastructure of macrophages showed nonspecific small granules and few filopodia on the cell surface.

The muscle tissue exhibited intercellular edema and intracellular swelling, identifiable by the distance between myofibrils. It is noteworthy that the nucleus was observed in the center of muscle cells and not at the periphery. The characteristics of inflammation gradually decreased in both the dermis and muscle tissue moving away from the center of the wound until they were undetectable.

Also at day 7, the beginning of an epithelial projection could be observed below the clot. The migrating epidermis had (3–4) few layers, flattened keratinocytes, the basal layer had no striation in basal lamina region, lacked keratin, was thinner than the original and exhibited intercellular edema. The clot increased in thickness and tissue necrosis in the underlying layer was more conspicuous. A greater number of dilated vessels lined with endothelial cells with round nuclei filled with euchromatin were observed in the more distal wound areas.

After day 7, there was a wide variation in morphology between the two temperatures. Notably, new epidermis covered the wound for the individuals maintained at 33°C. For this treatment, there was a clear decrease in the amount of necrosis in the center of the wound. The repaired tissue was basically composed of disorganized thin fibers, fibroblasts and a few inflammatory cells that were still found on the wound. Sometimes, macrophages focus on the formation of giant cells that are able to encircle and remove foreign bodies and newly formed tissue remnants, including fragments of heterophilic granuloma and cellular debris.

**Temperature effect on the microscopic morphology of the wound healing process in *Caiman yacare***

At day 3, the skin wounds of animals maintained at 23°C exhibited a higher detachment of muscle fibers trapped amid the heterophilic granuloma than 33°C. The vascular endothelial growth factor (VEGF) labeling was more frequent in muscle fibers at 23°C than at 33°C (Fig. 4A–D). The muscle fibers under
dermis were VEGF positive and frequently had loss their typical form or present edema among myofibrils. Furthermore, the muscle fibers trapped amid the heterophilic granuloma were always VEGF negative. All the immunohistochemistry performed had no labeling at control (suppression of primary Ig) skin sections.

At day 7, discontinuity points of the skin fibers for wounds at 23°C could be noted in all slide sections, this observations were based on the detachment of epidermis from the dermis (Fig. 4E), whereas no detachment were observed at animals healing at 33°C (Fig. 4F). The VEGF labels more muscle fibers at 23°C than at 33°C.

At day 15, the epidermal migration protrusions still remained detached from the dermis at 23°C, whereas the epidermis at 33°C was observed to be fully repaired. Furthermore, skins of animals healing at 23°C continued to exhibit abundant necrotic material, muscle tissue edema and inflammatory infiltrate, whereas, at 33°C, there were deposition of collagen fibers and the presence of fibroblasts and vessels. The vWF labeling was positive for muscle sections, giant cells, epidermis and endothelial cells and the labeling indicated newly formed vessels in the dermis at 33°C; most of those vessels were congested with erythrocytes. Accumulations of erythrocytes were also found embedded in the matrix but not enveloped by endothelial cells (Fig. 5A,B). The VEGF labels were slight higher in epidermis at 23°C than 33°C, mainly at basal layer of epidermis, and the muscular VEGF labels kept the same differences described above.

At day 30 at 23°C, epidermis still did not cover the wound and the mass of necrotic cells and signs of inflammation could still be observed (Fig. 5C). At 33°C dermis was repaired, with a decreased number of vessels congested by erythrocytes, increased blastema and densification of collagen fibers (Fig. 5D). The vWF labeling at 23°C indicated the formation of new skin vessels in an early stage of repair that were frequently congested by erythrocytes, also including accumulations of erythrocytes embedded in the matrix but not enveloped by endothelial cells. At 33°C the skin vessels were thinner, perpendicular to the muscle and epidermal tissues (Fig. 5E–H).

Picrosirius staining showed that the density of regenerating skin fibers at 33°C was higher than at 23°C (Fig. 6C,D), as well as the epidermis at 23°C. VEGF labels more muscle fibers at 23°C (Fig. 6C,D), as well as the epidermis at 23°C.

The proliferating cell nuclear antigen (PCNA) labeling revealed no difference between the two temperatures regarding the distribution of labeled cells; the labeling of nuclei of multinucleated giant cells, blastema cells, muscle fibers and epidermal cells in the basal layer and a few rows above is especially noteworthy. Clusters of PCNA-positive lymphocytes were often observed at 15 and 30 days in the scars maintained at 33°C but not those at 23°C.

Orcein staining did not indicate the presence of elastic fibers in the regenerating tissue, and elastic fibers were identified only in intact tissue located in distal wound areas. PAS staining labeled the basal lamina region only in the outer wound areas, and no staining was found in the basal lamina region using the PAS method, even in regenerated epidermis after 30 days at 33°C.
Finally, the muscle tissue with frequent loss of form was PAS-positive in all treatments.

Discussion

Reptiles are the only ectothermic amniotes and are therefore strategically useful for elucidating the effect of temperature on the immune system (Zimmerman et al., 2010). The results reported herein describe the differences in the histological characteristics of wound healing of *C. yacare* individuals maintained at 23˚C and 33˚C, revealing a decrease in wound healing ability at the lower temperature.

Macroscopically, there was no pattern of expansion or contraction of the wound edge, and the clear delay in the tissue repair process at 23˚C, evidenced by the delay in the shedding of the coagulated mass sealing the wound, is especially noteworthy. This result is consistent with the delay in the regeneration of *A. carolinensis* (Maderson and Licht, 1968), *Podarcis sicula*, *Podarcis muralis*, *Lampropholis delicata*, *Leiolopisma nigriplantare* and *Hoplodactylus maculatum* (Alibardi, 2010) lizard tails and the delay in wound healing of *Thamnophis sirtalis* (Smith et al., 1988) snakes maintained at different temperatures; in all of these instances, a lower temperature was linked to slower regeneration.

Crocodilians with inflammatory processes appear to seek warmer environments, as observed in Lipopolysaccharide (LPS)-challenged *A. mississippiensis*, who engage in search behavior for microenvironments that ensure higher body temperatures (Merchant et al., 2007), temporarily modifying the body temperature comfort zone.

The delay in epidermal migration is microscopically evident at 23˚C, a phenomenon previously reported in fish keratinocytes challenged at different temperatures (Ream et al., 2003). The delay in epidermal migration at 23˚C resulted in maintaining the heterophilic granuloma on the lesion even after 30 days. The delay in the formation of new epidermis enables an environment that is conducive to infection. In combination, it is worth noting that the structure of the tanks and the maintenance of caiman with a change of water every 24 hours were not adequate to keep the water free of food debris or waste, making that environment conducive to bacterial proliferation. Furthermore, several mechanisms of the crocodilians’ innate immune system are known to decrease in effectiveness with a decrease in temperature (Merchant et al., 2003; Merchant et al., 2004; Merchant et al., 2005; Merchant and Britton, 2006; Merchant et al., 2009; Siroski et al., 2012). At the lower temperature, the wound is exposed for a longer period, increasing the difficulties in keeping the water fully clean, and the immune system activity decreases. All of those factors represent a
dangerous combination for farming conditions because they promote infections and decrease the animals’ immune defense ability. However, little is known about the variations in C. yacare’s immune system with temperature variation (Merchant et al., 2006a; Siroski et al., 2012).

Heterophils act in the re-absorption of residual cellular debris during phagocytosis and defense (Montali, 1988) and are the most abundant crocodilian cells reacting against inflammatory challenges (Merchant et al., 2006b). The presence of extracellular pathogens stimulates the formation of heterophilic granuloma (Montali, 1988). The heterophilic granuloma may contribute to microbicidal activity (Alibardi, 2010), which is corroborated by the lack of infections even after 30 days at 23˚C without epidermal cover.

The heterophilic granuloma is frequently encircled by giant macrophage cells, as noted in our findings beginning at 3 days of wound healing, at which point the palisade layers of multinucleated macrophages were observed; corroborating findings have been reported for turpentine-challenged A. mississippiensis (Mateo et al., 1984). Rosenfeld staining enabled the recognition of pigments in macrophage vesicles including remnants of heterophils (eosinophilic pigments) and melanin pigments of dark brown color, primarily in dorsal wounds where there is a higher number of pigmented cells, in addition to cell debris.

Vascular endothelial growth factor (VEGF) is part of a family of cytokines that acts in paracrine signaling, enabling healthy angiogenesis and the recovery of tissue functions; their imbalance can result in the formation of granulation tissue and the deregulation of regeneration (Werner and Grose, 2003). The high VEGF immunodetection noted in sections of muscle fibers at 23˚C may be related to the persistent inflammatory condition observed that causes the muscular layer to continue to secrete VEGF even after 30 days, as opposed to the lack of VEGF labeling at the muscle fiber trapped amid the heterophilic granuloma, that will be released by the animal. The muscle fiber under the dermis labeled with VEGF indicates that this is actively participating to the inflammatory and proliferative process of wound repair. Furthermore were demonstrated that VEGF is common to be produced by muscle cells during suffering (Rissanen et al., 2002), VEGF diminishes apoptosis in skeletal muscle by autocrine regulation, and participate in proliferation and regeneration of this tissue (Germani et al., 2003; Arsic et al., 2004). Unfortunately the nucleus position was no informative of regeneration in contrast to mammals, once the muscle fibers can have nucleus inside (Claypole, 1897), even in central position, as detected in this work.

Failure to close the epidermis prevents the targeting of new blood vessels because, in addition to perpetuating the inflammatory process, the formation of a VEGF concentration gradient is impaired without an epidermis (Eilken and Adams, 2010). This is in accordance with what was noted at days 15 and 30 at 33˚C, wherein epidermis repaired and the formation of vessels through muscle tissue up to the epidermis were observed, otherwise the epidermis at day 15 at 23˚C presented a high VEGF positive labeling at basal layer of migrating epidermis, suggesting communication between dermis and epidermis.

The detachment of epidermis at 23˚C reported for days 7 and 15 suggests problems regarding skin repair. Those observations are corroborated by the findings from the use of the picrosirius dye, which stains dermal collagen fibers red. That staining revealed that partial skin repair was only observed after 30 days at 23˚C, whereas skin repair was noted beginning at day 15 at 33˚C. The repaired skin fibers at 23˚C did not correspond to the same pattern of organization of collagen fibers found in the original tissue or in the repaired skin at 33˚C. The differences are best evidenced in Table 1, wherein collagen density is significantly different between animals healed at both temperatures and the intact skin control: the smallest marked area and brightest intensity of red pixels, indicating fewer collagen fibers, were found at 23˚C, significantly increasing at 33˚C and in intact skin. Therefore, the lower temperature not only prevented the wound from closing but also changed the skin’s density.

The formation of the extracellular matrix and endothelial cell migration must occur between different stages for angiogenesis to occur (Eilken and Adams, 2010). The difficulty in forming new vessels at 23˚C is also related to the lack of an extracellular matrix, as shown by the picrosirius staining. Therefore, temperature affects the extracellular matrix components and the action of blastema cells.

Clusters of lymphocytes infiltrating the skin were observed only in the 33˚C group beginning at day 15, always associated with blood vessels. Studies in Mauremys caspica turtles showed an increased proliferation ability of lymphocytes in spring, that is, seasons affect lymphocyte proliferation (Muñoz and de la Fuente, 2001). Measurements of thymocyte proliferation in mice were used to estimate the levels of IL-1 expression in LPS-challenged H. flaviviridis at different temperatures, finding an optimal temperature of 25˚C with a decrease in thymocyte proliferation at temperatures below and above that optimum (Mondal and Rai, 2001). Accordingly, the results of the seasonal effect on lymphocyte proliferation in M. caspica may be related to the mean optimal temperature during spring. There are also reports of a decreased lymphocyte proliferative capacity of R. pipiens amphibians when maintained at 5˚C (Maniero and Carey, 1997), in addition to the aforementioned studies in reptiles. The lacks of clusters of lymphocytes at 23˚C is an evidence of the delay in adaptive immune reaction.

The lack of basal lamina, evidenced by PAS technique, under the regenerated epidermis suggests that the barrier between the...
epidermis and dermis is not complete, allowing a greater exchange of cytokines including VEGF from the epidermis-to-dermis direction. This result is corroborated by the findings of basal lamina discontinuity in A. carolinensis visible under TEM (Alibardi, 2010).

The importance of the palisade layer of multinucleated giant cells, muscle fibers and epidermis for regeneration is noteworthy because all of those cells are positively immunolabeled with vWF, VEGF and PCNA, and muscle fibers that are swollen or exhibit a loss of morphological integrity were also stained by PAS. Therefore, the functional plasticity of those tissues deserves further study.

The perpetuation of clusters of erythrocytes embedded in the skin after 30 days at 23°C and the delayed angiogenesis at 23°C may be related to the difficulty of maintaining efficient circulation in the wounded skin, healing at lower temperatures. This possibility is corroborated by observations in Thamnophis sirtalis snakes, in which the blood-flow rate tends to decrease and cephalic circulation to increase when maintained at low temperatures because such vascular changes provide greater protection to vital nerve structures when at low temperatures (Amiel et al., 2011). Therefore, low temperatures may compromise not only the rate of reactions but also the intake of cells and molecules required for tail tissue repair and vascular drainage.

The temperature of 23°C not only compromised the set of activities required for tissue repair; it also decreased the animal’s appetite, as found in our experiments (data not shown) and reported in studies with snakes (Smith et al., 1988).

Considering the results of this study, it is recommended that when farming C. yacare for commercial purposes, wounded caiman should be maintained at 33°C, especially if tanks are cold, enabling a faster epidermal closure and better immune system performance with regard to maintaining the skin integrity (Mitchell and Díaz-Figueroa, 2004). No osteoderm were detected at the repaired skins, and even at 33°C, C. yacare skins exhibited a fiber pattern that was significantly different from that of the intact skin, forming scars that may preclude the use of skin as leather; nevertheless, wound healing at 33°C is better recommended than 23°C in terms of skin fiber density.

It is noteworthy that the C. yacare farming method in Brazil is a ranching system that includes the regulation of egg collection from wild populations and is authorized by Ordinance no. 126 (Brasil Ministério do Meio Ambiente, 1990). The opportunity for the transfer of infectious agents from wild to captive animals is enhanced at each collection of eggs and/or hatchlings from the wild. Accordingly, optimal conditions enabling organisms to tolerate the entry of those microorganisms must be ensured in the farm at each period of egg collection.

This study is the first to elucidate the effect of temperature on the wound healing ability of C. yacare. Furthermore, it supports the conclusion that the temperature of 33°C, the preferred optimal temperature, is best for wound healing. Such findings should be included in discussions regarding the species’ management in addition to elucidating histological aspects of the tissue repair process in crocodilians.

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Competing Interests
The authors have no competing interests to declare.

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