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Influence of low environmental temperature on inflammation in bullfrog (*Rana catesbeiana*): qualitative and quantitative evaluation

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Influência da baixa temperatura ambiental sobre a inflamação em rã-touro gigante (*Rana catesbeiana*): avaliações qualitativa e quantitativa

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SUMMARY

Objectives of this study were to investigate the influence of low environmental temperature on the experimentally induced inflammatory response in post-metamorphic *Rana catesbeiana* (bullfrogs). To accomplish these goals, 120 specimens of *Rana catesbeiana* were kept at 6°C and 24°C, and treated by transfixion of thigh muscular tissue with a 5-0 suture or IM carrageenan injection. Results obtained through qualitative and quantitative evaluations showed that the lower environmental temperature significantly modulates the inflammatory process development. The animals in both models that were kept at 6°C showed a significantly lower number of inflammatory cells in the lesion site than the one verified at 24°C, apart from the evolution time. On the other hand, any factor related to the host mechanism of defense ought not to be blocked by the temperature, since the area of reaction to the injury showed to be equivalent in most of the studied time.

UNITERMS: Inflammation; Temperature; Amphibia; *Rana catesbeiana*; Bullfrog.

INTRODUCTION

The inflammatory reaction in mammals and birds is a well investigated biological process and is thought to be a local response of vascularized connective tissue to injury[3,18]. However, in amphibians the amount of information available is scarce[1,2,6,7,11,16,22].

Ectothermic vertebrates (reptiles, amphibians and fish) are unable to produce endogenous heat to maintain their physiologic activities; consequently, the body temperature of cold blood vertebrates is regulated by their surrounding habitat[23]. Therefore, multiple important physiologic activities are influenced by the environmental temperature[4,19].

On a similar basis, the influence of environmental temperature on multiple host defense mechanisms has already been documented[14,17].

The modulation of inflammation by the environmental temperature in some species of ectothermic vertebrates has already been investigated. Finn; Nielsen[10] demonstrated that low environmental temperature delayed the evolution of experimentally induced inflammatory reaction in *Salmo irideus*, conferring a "slow motion" pattern to the process. Dias; Sinhorini[6,7] demonstrated that bullfrog tadpoles kept at 6°C inflamed and repaired the lesions more slowly than those kept at 24°C and 31°C. Penha *et al.*[20] recently showed that low environmental temperature decreased the phagocytic activity of bullfrog thrombocytes induced by the injection of colloidal carbon in the dorsal lymphatic sac. However, Smith; Barker[24], investigating the healing of cutaneous wounds in common garter snake (*Thamnophis sirtalis*) kept at 13.5°C, 21°C and 30°C, reported that the qualitative and quantitative evaluations of the inflammatory reaction were not regulated by the temperature.

The objectives of this study were to investigate the

influence of low environmental temperature on the experimentally induced inflammatory response in post-metamorphic bullfrogs.

MATERIAL AND METHOD

Animals

One hundred-twenty specimens of post-metamorphic *R. catesbeiana* were employed. All animals were adapted to the laboratory conditions for at least 30 days before the experiments, plus 7 days for the corresponding experimental temperature.

At the laboratory, the specimens were kept in standardized 25 x 12 x 20 cm glass aquaria, at a maximum population density of 6 animals\aquarium. The animals were fed neonate mice\rat twice a week. The light cycle established for all groups was 12 h light\ 12 h dark.

Experimental Design

The temperatures selected were 6°C and 24°C with an interval of confidence of ± 1°C. The temperature of 24°C was obtained keeping the aquaria in a thermal stable laboratory. The temperature of 6°C was achieved by housing the animals in a temperature-controlled cooler. The selected temperatures were controlled by thermostats calibrated with a biological precision thermometer daily in the morning and in the afternoon.

Two thermal stable methods were selected to induce the inflammatory reaction. All animals were previously anesthetized in a glass jar with diethyl ether. Inflammation was induced in a group of sixty specimens by transfixing the thigh muscular tissue with a 5-0 (Supramid^{R}, Cirumédica) sterile nylon suture (SUT) or by injecting 0.1 ml of 0.5% carrageenan (Sigma) diluted in buffered phosphate solution in the thigh muscular tissue in another group of sixty bullfrogs (CAR).

Each group (SUT; CAR) was then divided into two sub-groups of thirty animals, and maintained according to their established experimental temperature (6°C; 24°C). Six specimens per sub-group (CAR\6°C; CAR\24°C; SUT\6°C; SUT\24°C) were sacrificed on days 1, 3, 7, 14 and 21 after induction of inflammation, and thigh skeletal muscle tissue was collected, fixed in neutral 10% isotonic formaldehyde, embedded in paraffin, cut into 4-5 µm sections and stained with hematoxylin-eosin and Masson's trichromic.

Table 1
Qualitative evaluation of experimentally induced inflammatory reaction in *Rana catesbeiana*. São Paulo, 1992.

Treatment	Parameter	1 day	3 days	7 days	14 days	21 days
SUT 6ºC	NEC	+	+	+	+	+
	ICI	+	+	+	+	+
	FBP	-	-	-	-	-
SUT 24ºC	NEC	+	+	+	-	-
	ICI	+	+	+	+	+
	FBP	-	+	+	+	+
CAR 6ºC	NEC	+	+	+	+	+
	ICI	+	+	+	+	+
	FBP	-	-	-	-	-
CAR 24ºC	NEC	+	+	+	-	-
	ICI	+	+	+	+	+
	FBP	-	-	+	+	+

Models: SUT (transfixion of the thigh muscular tissue with a sterile nylon suture); CAR (injection of carrageenan in the thigh muscular tissue). Environmental temperature: 6°C and 24°C; Parameters: NEC: necrosis and degeneration; ICI: inflammatory cellular infiltration; FBP: fibroplasia. Parameter considered positive when present in, at least, (n/2 +1). n = 6.

Table 2
Quantitative evaluation of the Inflammatory and Repair Area (IRA) experimentally induced *Rana catesbeiana* by transfixing the thigh muscular tissue with a sterile nylon suture. São Paulo, 1992.

Temperature	1 day	3 days	7 days	14 days	21 days
6ºC	0.58+0.08	0.53+.010	0.63+0.18	0.75+0.09	1.07+0.31
24ºC	0.77+0.13	0.86+0.12*	0.76+0.15	1.26+0.24*	1.20+0.26

Results expressed as mean ± sd in 1.62 mm^2 of thigh muscular tissue.
* Significant value when compared with that obtained at 6°C. $p \leq 0.05$. n = 6.

Qualitative Evaluation (QLE)

The model used was that proposed by Finn; Nielsen[10], who consider the examined phenomenon positive (+) when it is present in (n\2 + 1) the studied animals. In the current investigation, since the number of bullfrog (n) was six per sub-group, a parameter was considered positive for a sub-group when present in, at least, four individuals. The analyzed parameters were necrosis and degeneration (NEC); inflammatory cellular infiltration (ICI) and fibroplasia (FBP).

Quantitative Evaluation (QTE)

QTE was accomplished by using a morphometric model established by Weibel *et al.*[25]. Sections of thigh skeletal muscle adjacent to the inflammatory agent were examined with a Zeiss microscope fitted with an integrated grid Zeiss eyepiece with variable point density. The patterns evaluated were the inflammatory and repair area (IRA) and the inflammatory cell infiltration (ICI). Degeneration and tissue necrosis, ICI cells and fibroplasia were considered to be IRA components.

The examined IRA was 1.62 mm²\animal, including the nylon suture (0.55 mm²), and the results were expressed as mm² of reaction area. The IRA was not examined in the sub-groups treated with carrageenan, due to the lack of uniformity of the area occupied by the inoculum.

The analyzed area for ICI was 80 µm²\animal, and all sub-groups (CAR\6°C; CAR\24°C; SUT\6°C; SUT\24°C) were studied. At the level of light microscopy, the inflammatory cells were divided in two subpopulations, according to their morphological features: mononuclear agranulocytic (AGN) and poli-mononuclear granulocytic (GRN). The results were expressed in number of inflammatory cells\80 µm² of tissue.

Statistical Analysis

Data obtained in the quantitative evaluation were examined statistically by analysis of variance followed by Duncan's multiple range test, with the level of significance established at $p \leq 0.05$.

RESULTS

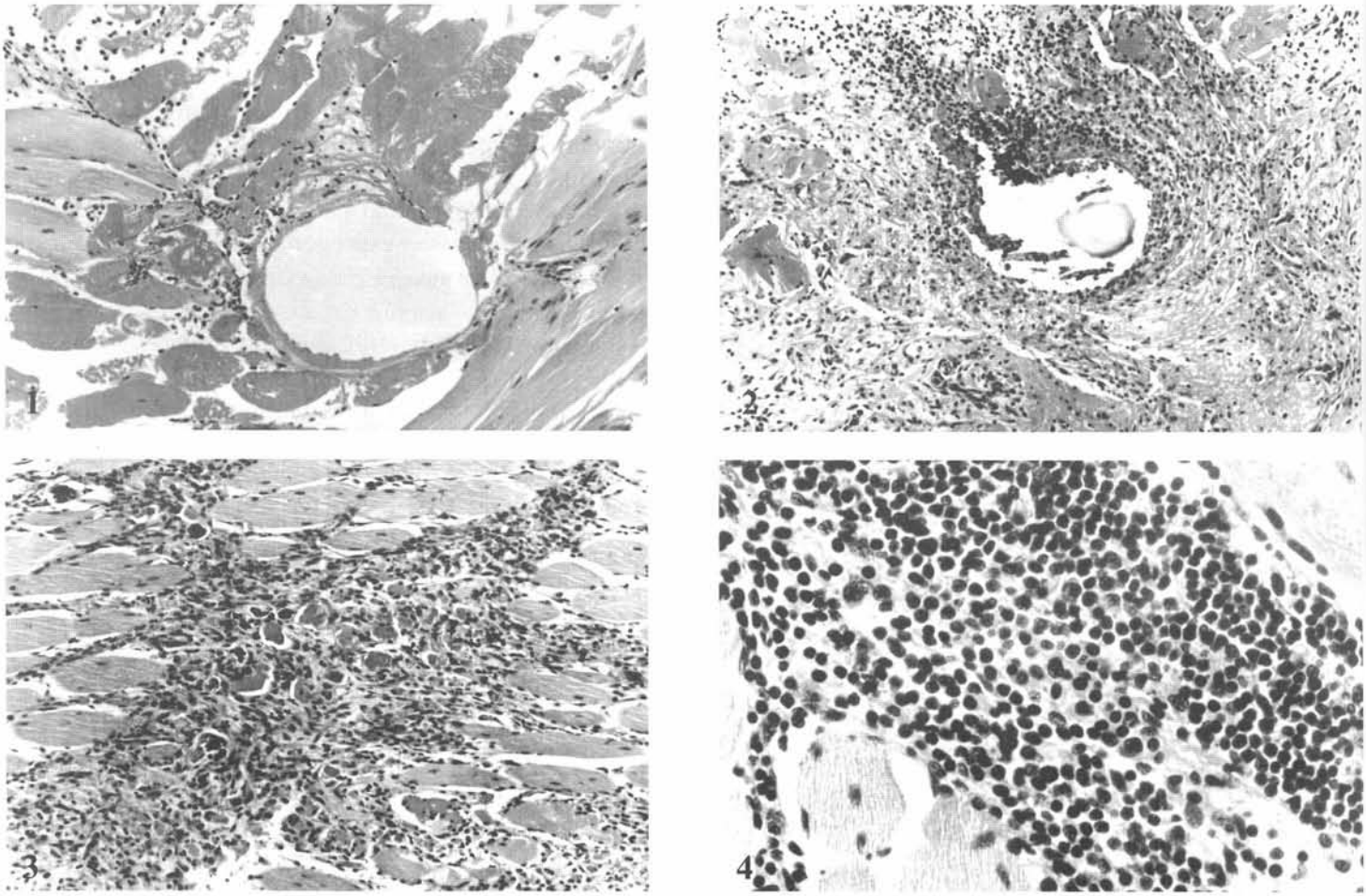
Qualitative evaluation

The results obtained for QLE are expressed in Tab. 1. The parameter NEC was considered positive when figures of necrosis and degeneration, including cellular and tissue debris, pyknosis and karyorrhexis, were present, mainly surrounding

Table 3
Quantitative evaluation of the Inflammatory Cell Infiltration (ICI) experimentally induced in *Rana catesbeiana*. São Paulo, 1992.

Treatment	Parameter	1 day	3 days	7 days	14 days	21 days
SUT 6ºC	AGN	10.6+3.1[b]	12.1+2.8[b]	15.5+5.0[b]	23.7+6.9	39.2+14.3
	GRN	0.8+0.7	0.5+0.8[b]	1.5+1.6	1.4+1.4	0.8+1.1
	TOT	11.4+3.7	12.6+3.6	17.0+5.2	25.1+6.6	40.0+13.5
SUT 24ºC	AGN	46.0+6.6[a]	104.0+18.6[a]	143.3+31.0[a]	235.3+49.3[ab]	178.8+39.2[ab]
	GRN	2.1+1.0	2.1+2.1	6.3+1.9[ab]	5.8+3.3[ab]	1.8+2.1
	TOT	48.1+6.9	106.0+19.9	149.6+30.1	240.8+51.2	180.6+40.8
CAR 6ºC	AGN	21.3+4.0	30.8+7.8	36.3+9.8	9.8+5.8	32.5+7.4
	GRN	2.0+2.2	2.8+2.5	2.1+1.2	0.8+0.7	1.8+2.6
	TOT	23.3+4.5	33.6+7.3	38.4+10.0	30.6+5.9	34.3+8.2
CAR 24ºC	AGN	40.6+4.0	114.8+16.9[a]	144.8+31.2[a]	319.5+53.4[a]	322.6+56.1[a]
	GRN	1.5+1.2	1.5+0.8	1.0+1.0	1.7+2.4	0.0+0.0
	TOT	42.1+4.2	116.3+17.4	145.8+31.9	321.2+55.7	322.6+56.1

Models: SUT (transfixion of the thigh muscular tissue with a sterile nylon suture); CAR (injection of carrageenan in the thigh muscular tissue). Environmental temperature: 6°C and 24°C; Parameters: AGN: mononuclear agranulocytic cells; GRN: granulocytic cells; TOT: total. a: significant value when compared with that obtained by the same model at 6°C; b: significant value when compared with that obtained by the other model at the same temperature. Results expressed as mean ± sd of cells\80 µm² of muscular tissue surrounding the inflammatory agent. $p \leq 0.05$. n=6.



Figures 1-4

Photomicrographs of histological sections of *Rana catesbeiana* muscular tissue after transfixion with a foreign body (suture or carrageenan). Hematoxylin-eosin. **1:** Suture-6ºC after 21 days. Notice the presence of few inflammatory cells associated to necrotic area. 165X; **2:** Suture-24ºC after 14 days. Note the fibroplasia and extensive inflammatory reaction surrounding the foreign body. 165X; **3:** Carrageenan-24ºC after 14 days. Observe the organized inflammatory cell infiltrate. 165X. **4:** Suture-24ºC after 21 days. Verify the pattern of the inflammatory cellular infiltrate, mainly composed by agranulocytic mononuclear cells. 660X.

the inflammatory agent (Fig.1). NEC was reported in all groups kept at 6ºC and at 1 day, 3 days and 7 days of those maintained at 24ºC.

The parameter ICI was considered as positive when inflammatory cells were present in the reaction area, and this phenomenon was seen in all experimental groups, regardless the selected temperature or the inflammatory agent (Fig.1-4).

The analysis of FBP was achieved by studying sections stained with Masson's trichromic method. The parameter was considered positive when fibers or/and bundles of connective fibrous tissue, displaying a blue to greenish discoloration, were reported within the reaction area (Fig.2). The phenomenon was described in all sub-groups kept at 24ºC, but not in those maintained at 6ºC.

Different patterns of FBP were seen among the positive animals. A delicate, usually concentric frame of connective was observed at the intervals of 3 days and 7 days. In contrast, a dense net of fibrous tissue surrounding the foreign body was noted in animals maintained for 21 days.

Quantitative Evaluation

Inflammatory and repair area (IRA)

The results obtained for IRA may be observed in Tab.2. Specimens kept at 6ºC had, at 3 days and 14 days, a statistically significant lower IRA than those maintained at 24ºC.

Inflammatory cell infiltration - Mononuclear agranulocytic cells (ICI-AGN)

The data for ICI are illustrated in Tab. 3. The AGN were morphologically characterized by showing small to medium size, round to slightly indented nuclei. The cytoplasm had a homogeneous pale basophilic appearance; however, the main cytoplasmic feature was the absence of granules (Fig. 4). Some cells showed a distinct spindle shape, due to a bipolar cytoplasmic deposition.

For both experimental models, the number of cells migrating to the inflammatory site was significantly higher for animals kept at 24°C.

The model established to induce the inflammatory reaction also modulated the ICI-AGN. Animals kept at 24°C and treated with CAR showed, at 14 days and 21 days, a significantly higher ICI-AGN than those observed for SUT. On a similar basis, CAR induced a higher ICI-AGN in bullfrogs maintained at 6°C at the early (1 day, 3 days, 7 days) experimental stages.

For all sub-groups except CAR\6°C, the number of AGN was significantly higher at the late experimental times (14d, 21d).

Inflammatory cell infiltration - Granulocytic cells (ICI-GRN)

The results for ICI-GRN are shown in Tab. 3. The GRN were morphologically identified as variably sized, round to irregular-shaped cells, displaying heterogeneous nuclei ranging from round and small to slightly lobulated or markedly segmented. The cytoplasm was characterized by the presence of granules, which varied from very fine to small, round-shaped, pale eosinophilic to large, round to oval-shaped brightly eosinophilic.

Animals kept at 24°C and treated by SUT, had a significantly higher ICI-GRN at 7 days and 14 days than those maintained at 6°C. In contrast, the temperature did not influence the ICI-GRN in the CAR groups.

Bullfrogs kept at 24°C and treated by SUT showed, at 7 days and 14 days, a higher ICI-GRN than specimens treated by CAR at the same temperature. At 6°C, the experimental models failed to show significant variation.

The results obtained for SUT\24°C displayed a significantly higher ICI-GRN at 7 days and 14 days, when compared with results within the same subgroup. In contrast, ICI-GRN did not show significant variation within all other subgroups.

DISCUSSION

The present study describes qualitatively and quantitatively the effects of low environmental temperature on experimentally induced inflammation in post-metamorphic bullfrogs.

The inflammatory cells were divided in two major sub-populations, according to their morphologic characteristics, AGN and GRN. The main feature distinguishing both populations was the presence or absence of cytoplasmic granules. Despite the fact that amphibian leukocytes are, among non-mammal vertebrates, the ones that morphologically most resemble mammal white blood cells[18], attempts to identify accurately, at the light microscopy level, the cellular types were unsuccessful. However, previous ultrastructural analyses in pre-metamorphic were effective to demonstrate lymphocytes, macrophages and plasma cells as the major AGN components. Otherwise, GRN were considered to include neutrophils and eosinophils[5].

Lymphocytes seem to play an important role in inflammation and healing phenomena in amphibians. Pfeiffer *et al.*[21] based on peripheral white cell counts following tail amputation in Japanese newt (*Cyanops pyrrhogaster*) reported a moderate lymphocytosis at the early experimental stages. In bullfrog tadpoles, mononuclear cells were the predominant component of the inflammatory cellular exudate induced by foreign body[7].

The higher number of AGN observed can be partially explained by the nature of inflammatory agent selected. Both SUT and CAR are well-recognized inert models for the study of inflammation[6,7,9]. One important attribute of both models is their thermal stability. Biologically active models, like inoculation of bacteria or fungi, were not suitable for the proposed study, since the temperature could modulate the model itself. On the other hand, biological protocols might achieve a distinct inflammatory pattern. This hypothesis is supported by the fact that granulocytes, mainly neutrophils, are commonly reported during the acute phases of infectious diseases in amphibians[8].

The results obtained by the QLE showed that low environmental temperature slowed down the rhythm of the inflammatory process. NEC and ICI were present during all experiments in animals kept at 6°C, in contrast with FBP, which were not reported in such sub-groups. Otherwise, sub-groups at 24°C displayed FBP simultaneously to NEC disappearance (SUT: 3d; CAR: 7d).

Such results suggest that animals kept at low temperatures would delay the development of fibroplastic reaction and, accordingly, the wound healing. Similar data were achieved by Finn; Nielsen[10] in *S. irideus* and by Dias; Sinhorini[7] in bullfrog larvae, indicating that the phenomena were not modulated by the metamorphosis. Nonetheless, the related mechanisms involved in such delays have not been established yet.

The quantitative analysis of IRA showed significantly higher reaction for bullfrogs kept at 24°C at the times of 3

days and 14 days but not for 1 day, 7 days and 21 days, when compared with those maintained at 6°C. These results sustain the possibility that factor(s) involved with the host's defense mechanisms and responsible for necrosis and degeneration would not be obstructed by low environmental temperature. This hypothesis is supported by the fact that in amphibians the complement system is activated and/or not blocked by low environmental temperature[15].

In contrast with IRA, the quantitative results for ICI-AGN showed that the environmental temperature modulates significantly the major cellular aspects of the inflammatory process. Regardless the type of inflammatory agent and for all experimental times but CAR-1d, ICI-AGN was significantly higher for animals kept at 24°C.

These data supports previous studies which indicated that environmental temperature plays a fundamental role in ectothermic vertebrates homeostasis, including antibody formation, immune response[14] and inflammation[6,7,10].

Finally, the results achieved in the present study, with regard to the influence of the environmental temperature on inflammation and types of cells involved, are very similar to those previously described in bullfrog tadpoles suggesting that metamorphosis, a fundamental physiological step in amphibians, does not interfere with some cellular aspects of inflammation[7]. In addition, they coincide with earlier works which demonstrated that immunological features were not disturbed by metamorphosis[12,13].

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RESUMO

O presente trabalho teve por objetivo investigar a modulação exercida pela temperatura ambiental sobre a cinética celular inflamatória experimentalmente induzida em *Rana catesbeiana*, rã-touro gigante. Para tanto, 120 espécimes pós-metamórficos foram mantidos a 6°C e 24°C e tratados pela transfixação do tecido muscular da coxa por fio de sutura ou injeção intramuscular de carragenina. Os resultados obtidos através de avaliações qualitativa e quantitativa do foco lesional mostraram que a baixa temperatura ambiental modula significativamente a evolução do processo inflamatório. Animais mantidos a 6°C, em ambos os modelos, apresentaram números de células inflamatórias significativamente menores que os verificados a 24°C, independentemente do tempo de avaliação. Por outro lado, algum fator pertencente aos mecanismos de defesa do hospedeiro não deve ter sido bloqueado pela temperatura, visto que a área de reação à injúria mostrou-se equivalente na maioria dos tempos pesquisados.

UNITERMOS: Inflamação; Temperatura; Amphibia; Rana catesbeiana; Rã-Touro Gigante.

REFERENCES

1- CLARK, H.F.; SHEPARD, C.C. Effect of environmental temperature on infection with *Mycobacterium marinum* (Balnei) of mice and a number of poikilothermic species. **Journal of Bacteriology**, v.86, p.1057-69, 1963.

2- CLOUGH, G.; MICHELL, C.C.; PHILLIPS, M.E. Inflammatory changes in permeability and ultrastructure of single vessels in the frog mesenteric microcirculation. **Journal of Physiology**, v.395, p.99-114, 1988.

3- COTRAN, R.S.; KUMAR, V.; ROBBINS, S.L. **Pathologic Basis of Disease**. 5.ed. Philadelphia : W.B. Saunders. 1994. 1400p.

4- DEEMING, D.C. Effects of incubation temperatures on alligators. **International Hatchery Practice**, v.4, n.8, p.13-7, 1990.

5- DIAS, J.L.C.; SINHORINI, I.L. Aspectos ultra-estruturais de células inflamatórias de girinos de *Rana catesbeiana* (rã-touro gigante). **Ciência & Cultura**, v.44(sup), p.730, 1992.

6- DIAS, J.L.C.; SINHORINI, I.L. Influence of high environmental temperature on inflammation and repair induced by a foreign body in bullfrog (*Rana catesbeiana*) tadpoles. **Brazilian Journal of Medical and Biological Research**, v.24, p.583-6, 1991a.

7- DIAS, J.L.C.; SINHORINI, I.L. Qualitative evaluation of the inflammatory response modulated by temperature in tadpoles of *Rana catesbeiana*. **Ciência & Cultura**, v.43, p.304-6, 1991b.

8- DIAS, J.L.C.; COUTINHO, S.D.; COSTA, E.O.; CARVALHO, V.M.; MATUSHIMA, E.R.; SINHORINI, I.L.; GAVIOLLE, M.C. Surto de saprolegniose em girinos (*Rana catesbeiana*). **Revista Brasileira de Microbiologia**, v.19, n.4, p.432-7, 1988.

9- DOHERTY, N.S.; ROBISON, B.V. The inflammatory response to carrageenan. **Journal of Pharmacy and Pharmacology**, v.27, p.701-3, 1975.

10- FINN, J.P.; NIELSEN, N.O. The effect of temperature variation on the inflammatory response of rainbow trout. **Journal of Pathology**, v.105, p.257-68, 1971.

11- GAMMIE, A.E.; RUBEN, L.N. The phylogeny of macrophage function: antigen uptake and degradation by peritoneal exudate cells of two amphibian species and CAF_1 mice. **Cellular Immunology**, v.100, p.577-83, 1986.

12- GECZY, C.L.; GREEN, P.C.; STEINER, L.A. Immunoglobulins in the developing amphibian *Rana catesbeiana*. **Journal of Immunology**, v.111, p.1261-7, 1973.

13- KAMALI, D.D.; RUBEN, L.N.; GREGG, M.T. The development of inducer and effector immune suppressor cell function in *Xenopus laevis*, the South African clawed toad: the effect of metamorphosis. **Cell Differentiation**, v.18, p.225-31, 1986.

14- KLESIUS, P.H. Effect of size and temperature on the quantity of immunoglobulin in channel catfish, *Ictalurus punctatus*. **Veterinary Immunology and Immunopathology**, v.24, p.187-95, 1990.

15- KOPPENHEFFER, T.L. Serum complement systems of ectothermic vertebrates. **Developmental and Comparative Immunology**, v.11, p.279-86, 1987.

16- KORDYLEWSKI, L.; PLYTYCZ, B. Anuran lymphocyte-macrophage culture "in vivo": a simple method. **Immunologia Polska**, v.8, p.109-12.

17- MONAGAS, W.R.; GATENN Jr., R.E. Behavioural fever in turtles *Terrapene carolina* and *Chryemys picta*. **Journal of Thermical Biology**, v.8, n.3, p.285-8, 1983.

18- MONTALI, R.J. Comparative pathology of inflammation in the higher vertebrates (reptiles, birds and mammals). **Journal of Comparative Pathology**, v.99, p.1-26, 1988.

19- PAULSON, B.K.; HUTCHISON, V.H. Blood changes in *Bufo cognatus* following acute heat stress. **Comparative Biochemistry and Physiology**, v.87, p.461-6, 1987.

20- PENHA, M.L.; DIAS, J.L.C.; MALUCELLI, B.E. Influence of low environmental temperature on the phagocytic activity of bullfrog (*Rana catesbeiana*) thrombocytes. **Brazilian Journal of Veterinary Research and Animal Science**, v.33, p.15-8, 1996.

21- PFEIFFER, C.J.; HAYWOOD, P.; ASASHIMA, M. Blood cell morphology and counts in the Japanese newt (*Cynops pyrrhogaster*). **Journal of Zoo and Wildlife Medicine**, v.21, n.1, p.56-64, 1990.

22- PLYTYCZ, B.; BRAUN, R.; SELJELID, R. "in vitro" culture of frog peritoneal macrophages. **Folia Biologica** (Krakow), v.34, p.217-20, 1986.

23- POUGH, F.H. Recommendations for the care of amphibians and reptiles in academic institutions. **Institute of Laboratory Animal Research News**, v.33, n.4, S1-S2, 1991.

24- SMITH, D.A.; BARKER, I.K. The effect of ambient temperature and type of wound on healing of cutaneous wounds in the common garter snake (*Thamnophis sirtalis*). **Canadian Journal of Veterinary Research**, v.52, p.120-8, 1988b.

25- WEIBEL, E.R; KISTLER, G.S.; SCHERLE, W.F. Practical stereology methods for morphometric cytology. **Journal of Cell Biology**, v.30, p.23-39, 1966.

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