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The spleen of *Physalaemus nattereri* (Amphibia: Anura): morphology, melanomacrophage pigment compounds and responses to α-melanocyte stimulating hormone

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Abstract
The spleen is a lymphoid organ associated with defense mechanisms in anurans, and also it has pigmented cells. Melanomacrophages (MMs) are melanin-containing cells, which originated from hematopoietic stem cells and are found in hematopoietic organs. Melanin has bactericide and cytoprotective actions against free radicals. Furthermore, the α-melanocyte stimulating hormone (MSH) induces dispersion of melanosomes in melanocytes, besides having an anti-inflammatory action. Here, we describe the spleen morphology of *Physalaemus nattereri* and the response of splenic melanomacrophages to the α-MSH hormone. Animals were treated with α-MSH for 2, 6, 12, 24 and 48 h. Then, we measured the amount of melanin, lipofuscin and hemosiderin in each treatment. The spleen of *P. nattereri* is ovoid, and a connective tissue capsule covers the organ externally. There are no septa and the stroma is reduced. The parenchyma has two regions slightly separated from each other: white and red pulps. MMs occur in the red pulp. The α-MSH increased the volume of lipofuscin and hemosiderin in MMs after 6 h, but not melanin. Thus, the α-MSH altered only metabolic substances in splenic melanomacrophages, but not melanin, which usually is responsive to this hormone in hepatic MMs. Also, the description of the spleen morphology can help future comparative morphological and evolutionary studies on spleen morphology of vertebrates.

Keywords: *Spleen, melanin, lipofuscin, hemosiderin, melanomacrophages*

Introduction

The spleen is a single structure, reddish, oval or spherical in syntopy with organs of the digestive and urogenital systems in vertebrates (Romer & Parsons 1986). The vertebrate spleen is a major lymphoid organ of the immune system. In fish and amphibians, the spleen has hematopoietic and hemocateretic functions, besides phagocytosis, storage and release of erythrocytes (Alvarez 1990; Nilsson 2012). The spleen of ectothermic animals also has pigmented cells called melanomacrophages (MMs).

MMs occur in hematopoietic organs and have phagocytic activity similar to macrophages (Agius 1980). These cells belong to the hematopoietic stem cell line (Sichel et al. 1997; Colombo et al. 2011), have melanin and often clump together forming melanomacrophage centers (MMCs, Agius 1981). These centers are part of the mononuclear phagocytic system and perform phagocytosis of catabolic cellular material (Ellis et al. 1976). This fact suggests that they are responsible for the detoxification and recycling of both endogenous and exogenous products (Herráez & Zapata 1986). In addition to melanin, lipofuscin and hemosiderin are also present in the cytoplasm of MMs. These pigments are by-products of phagocytic degradation (Agius & Agbede 1984; Herráez & Zapata 1991).

Hepatic MMs of *Pelophylax lessonae* respond to the α-melanocyte stimulating hormone (α-MSH) by increasing tyrosinase expression (Guida et al. 2004). Furthermore, this hormone disperses melanin granules on the skin of ectothermic animals, and in melanophores, xanthophores and erythrophores (Castrucci et al. 1984; Hadley et al. 1985; Bagnara & Matsumoto 2006). In mammals, α-MSH acts as a neuroimmunomodulatory peptide capable of inhibiting disease (Lipton & Catania 1997). However, the effects of this hormone on the spleen and splenic MMS are unknown.

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Here, we describe the spleen morphology of one species of anuran, since it is a highly variable organ in vertebrates. Also, we tested the response of splenic melanomacrophage to α-MSH hormone, which regulates cutaneous pigmentation in anurans. We hypothesized that splenic MMs of *P. nattereri* would respond to α-MSH by increasing the volume of melanin and catabolic substances (e.g., lipofuscin and hemosiderin). Our results increase the knowledge about spleen morphology of anurans and form the basis for further physiological studies.

Material and methods

Anuran sampling

Male specimens of *Physalaemus nattereri* (Steindachner, 1863) were collected in wetlands and temporary ponds in São José do Rio Preto, São Paulo, southeastern Brazil (20°47′07.05″S, 49°02′42.09″W), during the rainy season (December 2011 to January 2012). Animals were kept in the laboratory for 7 days at room temperature and daylight regime (27 + 0.5°C and 12:12 h light:dark) for acclimatization before the experiments.

Effects of Nle^4, D-Phe^7-α-MSH (NDP-MSH) hormone

Specimens (n = 25) received a single intraperitoneal 2.5×10^{-7} μmol/10 g dose of the synthetic analogue Nle^4, D-Phe^7-α-MSH (Sigma-Aldrich, St. Louis, MO, USA), diluted in sterile physiological solution with osmolarity adjusted to amphibians (60% of the osmolarity of mammalian, adapted from Hadley et al. 1985). The synthetic analogue was used because it is non-biodegradable and more suitable for *in vivo* experiments, since the natural α-MSH hormone is rapidly metabolized. We divided the specimens into five experimental groups: MSH2h, MSH6h, MSH12h, MSH24h and MSH48, which received the hormone and were analyzed after 2, 6, 12, 24 and 48 h. The control group (CONT0h) consisted of five specimens analyzed after acclimation and did not receive injections, to compare whether injections with physiological solution changed splenic pigmentation. Another 15 animals received a physiological solution with osmolarity adjusted to amphibians and were analyzed after 6 (CONT6h), 12 (CONT12h) and 48 h (CONT48 h). These times were used to mimic treatments. The 6 h control was used because it is the shortest time in which we detected an increase in lipofuscin and hemosiderin in the treatments. The 48 h control was used to test whether there were any changes in metabolic substances due to handling after the experimental period. Moreover, the 12 h control was used to test whether there were changes in metabolic substances in intermediate times. Then, we ran a one-way analysis of variance (ANOVA) to test for a difference between control groups. Thus, we lumped the data of all control groups together and calculated the mean. For further analysis, we used this mean of control groups to compare it with treatments.

Histological processing

After experiments, all animals were anesthetized and euthanized with a lethal dose of benzocaine (1 g/L of water). Subsequently, they were dissected by a median incision from the cloaca to the pectoral girdle. The spleen was removed and weighed using an analytical balance to the nearest 0.005 g. The handling of animals and all experimental procedures were approved by the Committee on Ethics and Animal Experimentation of the São Paulo State University (CEUA-IBILCE/UNESP #038/2011) and followed the recommendations of the Guide for the Care and Use of Laboratory Animals (US National Research Council Committee).

For morphological analysis, the spleen was fixed in Karnovsky fixative solution (0.1 M Sörensën phosphate buffer, phosphate buffer pH 7.2 containing 5% paraformaldehyde and 2.5% glutaraldehyde) at 4°C, for 24 h. After that, samples were washed in water, dehydrated in alcohol series, and embedded in historesin (Leica-Historesin embedding kit). Sections of 2 μm were obtained with a microtome (RM 2265, Leica, Switzerland).

Morphological analysis

Sections were stained with hematoxylin-eosin for general description of the tissue and pigment quantification. Slides were observed under a microscope (Leica DM4000 B) equipped with an image capture system (Leica DFC 280). Spleen histological sections were incubated for 15 min in Schmorl solution containing 75 mL of 1% ferric chloride, 10 mL of potassium ferricyanide and 15 mL of distilled water, then immersed in aqueous 1% neutral red solution followed by 1% eosin to detect lipofuscin. Sections were also incubated in ferrocyanide acid solution, obtained by dissolving 2 g of potassium ferrocyanide in 100 mL of hydrochloric acid solution 0.75 mol/L, for the same period to detect hemosiderin, and immersed in aqueous 1% neutral red followed by aqueous 1% eosin.

Quantitative analyses were performed using Image Pro-Plus software (Media Cybernetics Inc. v. 6.0), based on different staining intensity (Santos et al. 2014). Pigments were quantified in 25 histological

fields per animal. We used random sections and 30 spleen sections per slide, totalling four slides per animal for morphological description, pigment quantification and mast cells analyses. Afterwards, we stained one slide per animal using the histological techniques for analyses mentioned above. All slides of each technique were stained for the same time to ensure they had the same color intensity. Absolute volume of each pigmented substance was determined by multiplying the relative area by spleen weight, since 1 mg of tissue has approximately 1 mm^3 (Vilamaior et al. 2006).

Ten histological fields were stained with toluidine blue and borax to quantify mast cells in the spleen of each animal. The number of mast cells was expressed as cell frequency per histological section area.

Statistical analysis

The weight of the spleen and volume occupied by each pigment (melanin, hemosiderin and lipofuscin; response variables) was measured to determine whether it increased with time of exposure to α-MSH (predictor variables). Data were square-root transformed to meet assumptions of homogeneity of variance and normality. Then, we ran a one-way ANOVA to compare response variables between treatments, followed by a Tukey *post hoc* test. Analyses were performed in the R software v. 2.11.1 (R Core Team 2010).

Results

Morphological characterization

The spleen of *Physalaemus nattereri* has an ovoid shape and is located in the body cavity, supported by the peritoneum, in syntopy with organs of the digestive and urogenital systems (Figure 1A). A capsule of connective tissue covers the organ externally. There are no septa and the stroma is reduced (Figure 1B). The parenchyma has two regions slightly separated from each other: white and red pulps (Figure 1C). These pulps are spread throughout the spleen, with a few lymphoid tissue clusters (white pulp) interspersed with red pulp (Figure 1D). The red pulp consists of cords and splenic sinuses filled with erythrocytes. The red pulp contains neutrophils and lymphocytes (Figure 1E and F). There are two regions without boundaries in the red pulp: one with higher cell density in the marrow, and another one with lower cell density in the cortex (Figure 1C).

Splenic MMs are distributed throughout the spleen parenchyma in the red pulp. They are rounded and form melanomacrophage centers (Figure 2A and B). MMs have three pigments with distinct proportions (Figure 2C and D): melanin, lipofuscin and hemosiderin ($F_{(5,30)}$ = 37.182, $P < 0.0001$). Under normal physiological and metabolic conditions, the pigment composition inside MMs is 20% melanin, 35% lipofuscin and 45% hemosiderin. Mast cells occur in the red pulp (Figure 3). In animals treated with α-MSH, mast cell frequency did not change ($F_{(5,30)}$ = 1.57; P = 0.32).

Effects of α-MSH hormone in melanomacrophages

The proportional weight ($F_{(5,30)}$ = 38.54; P = 0.50) and volume ($F_{(5,30)}$ = 5.78, P = 0.21) occupied by MMs in the spleen did not change after α-MSH administration (Figure 4). However, catabolic pigments increased with hormone treatment. The volume of lipofuscin increased after 6 h of α-MSH hormone administration ($F_{(5,30)}$ = 7.94; $P < 0.0001$), but did not change after 2, 12, 24 and 48 h o ($F_{(5,30)}$ = 7.94; $P < 0.90$). Similarly, the volume of hemosiderin increased after 6 and 48 h ($F_{(5,30)}$ = 16.06; $P < 0.00001$; P = 0.01, respectively). Hemosiderin did not change after 2, 12 and 24 h ($F_{(5,30)}$ = 6.15, P = 0.15, P = 0.94, P = 0.48; Figure 4). The control group (CONT0 h) without injection did not differ from the control group injected with physiological solution for any of the analyzed substances after 6 h (CONT6 h), 12 (CONT12 h) and 48 h (CONT48 h; Melanin: $F_{(5,20)}$ = 5.88, P = 0.94; Lipofuscin: $F_{(5,20)}$ = 7.79, P = 0.47; Hemosiderin: $F_{(5,20)}$ = 6.75, P = 0.68; Table I).

Discussion

The spleen morphology of *Physalaemus nattereri* is similar to that of amphibians and reptiles, with a thick conjunctive capsule of oval shape, and white and red pulps (Scalia et al. 2004; Kassab et al. 2009). However, the trabecular connective tissue of the capsule which enters into the organ, separating the red and white pulps, was not observed in *P. nattereri*, as in the turtle *Testudo graeca* (Kassab et al. 2009). The spleen parenchyma of *P. nattereri* has thick blood vessels, macrophages and immune cells similar to other vertebrates (Fänge & Nilsson 1985). There is no white pulp in caecilians (e.g., *Ichthyophis kohtaoensisin*) and *Rana perezi*, which instead have scattered lymphoid cells spread in red pulp (Cooper & Wright 1976; Zapata et al. 1982; Alvarez 1990). This pattern is called diffuse, in opposition to the follicular pattern of the spleen of *Bufo calamita* and *Bombina variegata*. The spleen of these species has two distinct portions: the white and red pulps separated by a septum (Cooper & Wright 1976; Barrutia et al. 1983; Dulak 1990). The

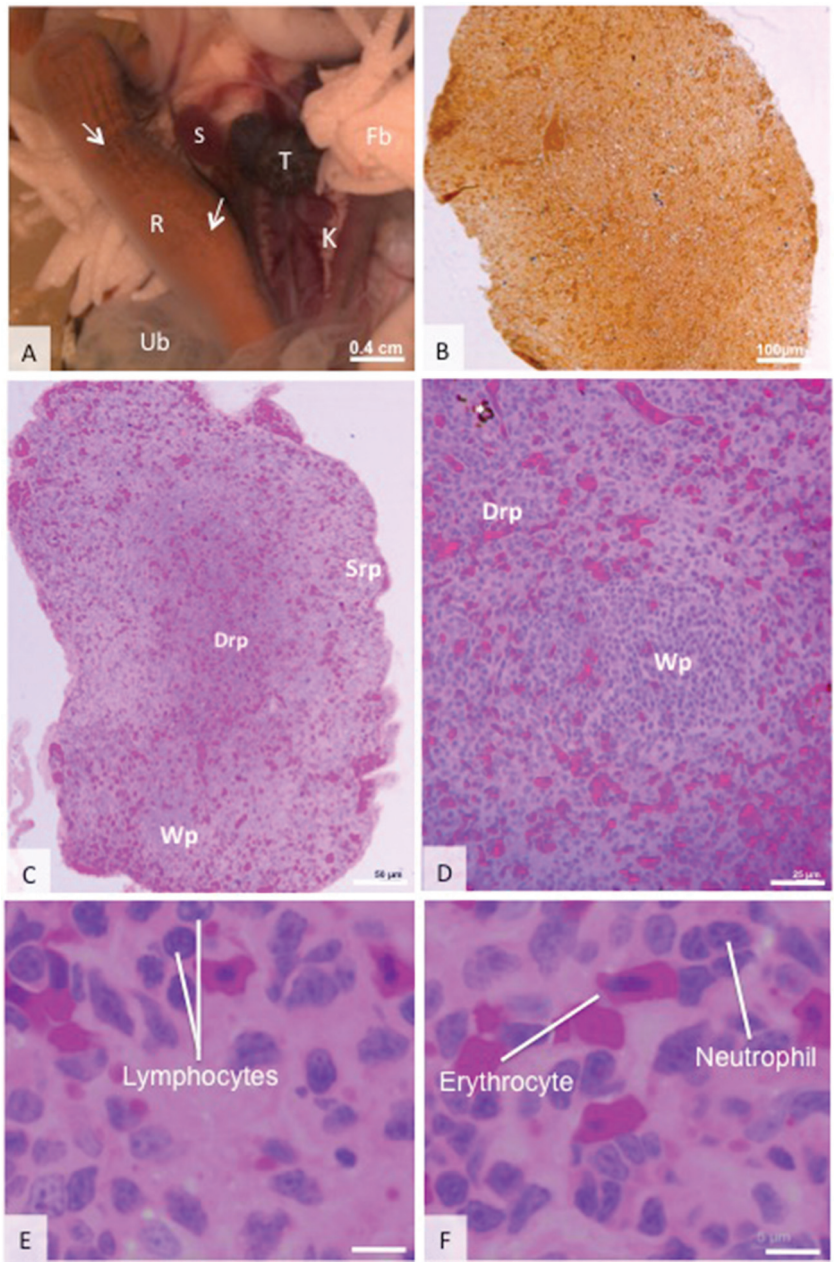

Figure 1. **A,** body cavity of *Physalaemus nattereri* showing the spleen (S) along with organs of the digestive and urogenital systems. Fb: adipose bodies. K: kidney. R: rectum. T: tests. Ub: urinary bladder. Arrows: pigmentation on the surface of the organs. **B–D,** histological spleen section showing the absence of septa in the organ and the reduced stroma. It is possible to observe the presence of the red pulp with two distinct regions, one with high cell density (Drp) and the other with more dispersed cells (Srp). It is also possible to see the white pulp (Wp) interspersed with the red pulp. Melanomacrophages (*) are seen in the red pulp. **E–F,** Details of cells in red pulp. Staining: Gomori Reticulin (B), hematoxylin-eosin (C–F). Scale bars: A = 0.4 cm; B = 100 µm; C = 50 µm; D = 20 µm; E and F = 5 µm.

difference between spleen arrangement and disposition of white pulp apparently has a phylogenetic pattern in anurans, with early diverging species having a diffuse pattern, whereas derived species have the folicular pattern (Cooper & Wright 1976). Therefore, *P. nattereri* seems to have a plesiomorphic arrangement of the spleen.

The spleen is an important organ that responds either directly or indirectly to toxicity and external stressors (Suttie 2006). Pigmented MMs occur in the spleen of fish and anurans (Agius 1980). Splenic MMs respond directly to an inflammatory stimulus by increasing the MMs area (Manrique et al. 2014). In the spleen of fish, MMs are very

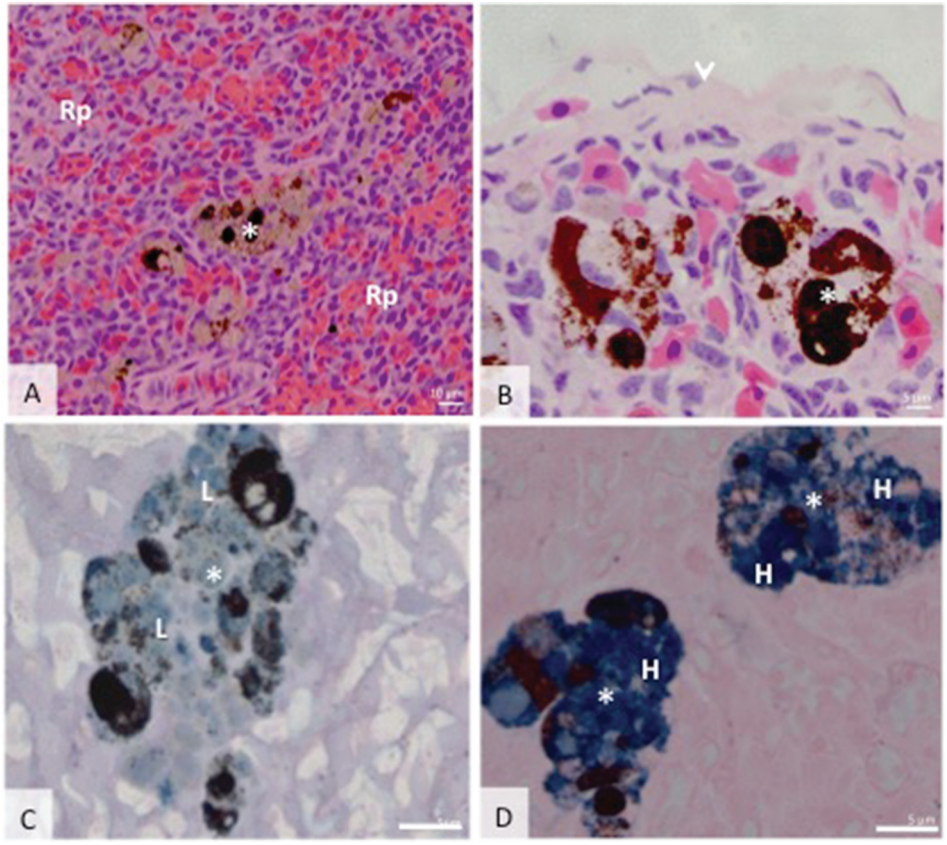

Figure 2. Photomicrographs of *Physalaemus nattereri* spleen. **A–B,** Distribution of melanomacrophages (*) in the red pulp (Rp). A capsule of connective tissue encases the organ (arrowhead). Staining: hematoxylin-eosin. **C,** Presence of lipofuscin (L) in melanomacrophages (*). Staining: Schmorl solution against neutral red and eosin stain. **D,** Presence of hemosiderin (H) in melanomacrophages (*). Staining: ferrocyanide acid solution against neutral red and eosin stain. Scale bars: A = 10 μm; B–D = 5 μm.

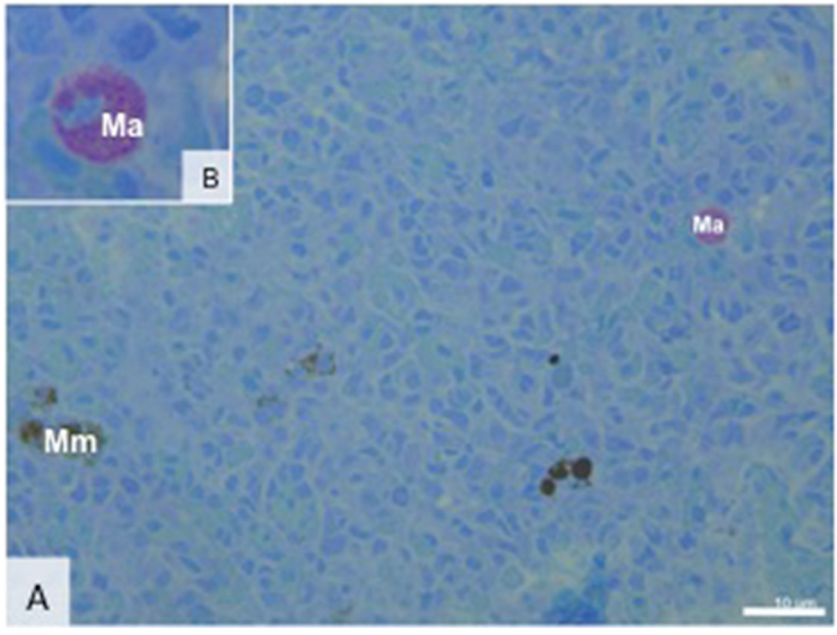

Figure 3. Mast cells (Ma) in the spleen (**A–B**) of *P. nattereri*. Mm: melanomacrophages. Staining: Toluidine blue. Scale bar: A = 10 μm.

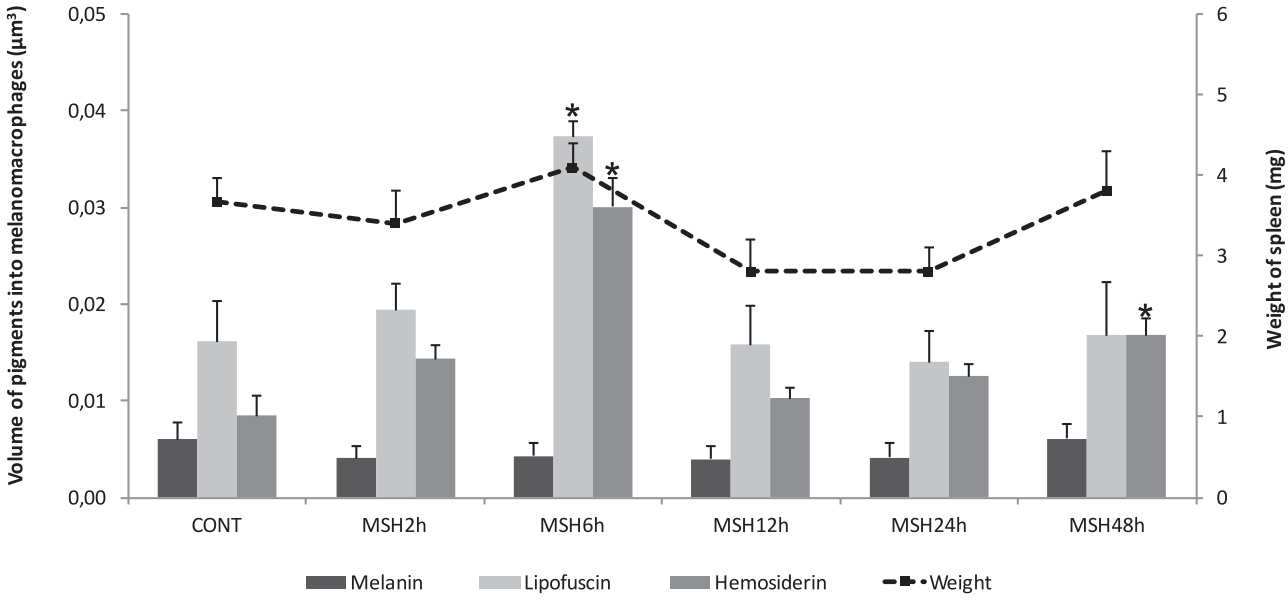

Figure 4. Melanin-, lipofuscin- and hemosiderin-occupied volume inside the melanomacrophages, and weight of spleen of *Physalaemus nattereri* treated with the alfa-melanocyte stimulating hormone (alfa-MSH). CONT12 h and 48 h: Animals injected with saline solution and analyzed after 12 and 48 h; MSH 2 h, 6 h, 12 h, 24 h and 48 h, animals analyzed 2, 6, 12, 24 and 48 h after the α-MSH treatment. There are no differences between CONT12 h and CONT 48 h. (*) Shows statistical differences between experimental times and the control group for the same substance.

Table I. Volume of pigment substances in splenic melanomacrophages of animals of control groups. CONT0 h: Animals before experiments. Mean ± standard error. CONT6 h, 12 h and 48 h: Animals injected with physiological solution and analyzed after 6, 12 and 48 h. No statistical difference was observed between all analyzed controls groups.

	CONT0 h	CONT6 h	CONT12 h	CONT48 h
Melanin	0.006 ± 0.001	0.005 ± 0.001	0.007 ± 0.002	0.005 ± 0.005
Lipofuscin	0.015 ± 0.004	0.016 ± 0.003	0.014 ± 0.002	0.018 ± 0.006
Hemosiderin	0.008 ± 0.002	0.007 ± 0.002	0.008 ± 0.001	0.009 ± 0.003

close to the lymphoid tissue, showing that MMs are involved in immune responses (Agius 1980; Fänge & Nilsson 1985). Additionally, MMCs are similar to the primitive germinal center of mammals (Agius 1980). Therefore, immune response is the primary function of MMs in the spleen. Also, splenic MMs increase with decreasing temperature (Balamurugan et al. 2012). Thus, these cells can potentially be used as a bioindicator of environmental alterations (Balamurugan et al. 2012).

The volume of catabolic pigments in the spleen increased with α-MSH treatment. However, spleen weight of animals treated with α-MSH did not change. The volumes of lipofuscin and hemosiderin increased with α-MSH treatment, but melanin did not change. The melanin of hepatic MMs did not change with α-MSH treatment, but increased with lipopolysaccharide (Franco-Belussi et al. 2013). However, lipofuscin and hemosiderin in the spleen increased after 6 h of α-MSH treatment. This rapid increase in the volume of catabolic substances in the spleen contrasts the late increase observed in liver MMs (e.g., 24 and 48 h; Franco-Belussi et al. 2013). This result may be related to metabolic differences between cell types of the liver and spleen (Ribeiro et al. 2011).

Splenic MMs of *P. nattereri* are located in the red pulp, similar to *Rana esculenta* L. (Gallone et al. 2002). These cell types produce melanin in organelles called melanosomes. However, the mechanisms by which melanin is synthesized in these cells are distinct from cutaneous melanocytes in vertebrates (Gallone et al. 2002). Melanin is a complex polymer synthesized endogenously in vertebrates and invertebrates (Césarini 1996). This substance absorbs and neutralizes free radicals, cations and other potentially toxic agents derived from the degradation of phagocytosed cellular material (Zuasti et al. 1989). Melanin is important against bacterial components in ectothermic animals due to the action of hydrogen peroxidase and its precursor quinones, acting as a bactericide, favoring enzymatic

activities, which can be limited at low temperatures (Wolke et al. 1985). The MMs of amphibians can phagocytose and also produce melanin (Scalia et al. 2004). MMs are dynamic cells responsive to various environmental stimuli. There is increased cell proliferation and melanosynthesis in MMs in the liver of *Pelophylax lessonae* before estivation, while pigmentation is reduced after estivation (Barni et al. 2002). In salmon, melanogenesis in the extracutaneous pigmentary system is related to the immune system (Arciuli et al. 2012). Although both splenic and hepatic MMs contain melanin, hemosiderin and lipofuscin, the relative quantities of these substances in splenic and hepatic MMs may vary, reflecting the activity of each cell type (Ribeiro et al. 2011).

The melanin in MMs of both spleen and liver (Franco-Belussi et al. 2013) did not change with α-MSH treatment. On the other hand, α-MSH increased the levels of both tyrosinase gene and dopa-oxidase activity in cultured liver MMs of *Pelophylax bergeri* (Guida et al. 2004). Thus, it seems that α-MSH increases levels of tyrosinase in *P. nattereri* (Guida et al. 2004), but does not induce high melanin production in its spleen.

The volume of hemosiderin increased after 6 h of treatment. Hemosiderin is an iron and protein compound derived from the degradation of hemoglobin in erythrocytes. Consequently, hemosiderin is an intermediate metabolic that is produced during iron recycling in erythropoiesis (Kranz 1989). Thus, high levels of hemosiderin indicate that MMs are associated with iron metabolism and also with phagocytic activity (Kranz 1989; Ribeiro et al. 2011). Therefore, it is possible to infer that the phagocytic and metabolic activities resulting from iron catabolism in these pigment cells increased after 6 h of α-MSH administration. The decrease of hemosiderin in splenic MMs suggests a decline in the phagocytic activity (Bucke et al. 1992). Furthermore, hemosiderin increases in liver MMs only after 24 and 48 h of hormone administration (Franco-Belussi et al. 2013), showing a delay compared to spleen MMs.

We found that lipofuscin increased after 6 and 48 h of α-MSH treatment. In contrast, the amount of lipofuscin in the liver does not change after α-MSH treatment (Franco-Belussi et al. 2013). Lipofuscin results from oxidative polymerization of polyunsaturated fatty acids and is associated with lipid peroxidation of membranous organelles (Agius & Roberts 2003), and is also indicative of phagocytic activity of macrophages.

The characterization of the morphology of spleen and MMs in *P. nattereri* can support future comparative and evolutionary studies about this organ in ectothermic vertebrates. Only metabolic substances responded to α-MSH in splenic MMs. Therefore, melanin in splenic MMs does not respond to α-MSH in a similar way to hepatic MMs. The α-MSH has also an anti-inflammatory role (Lipton & Catania 1997), but the frequency of mast cells did not change after treatment with this hormone.

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