An Acad Bras Cienc (2023) 95(1): e20211581 DOI 10.1590/0001-3765202320211581
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

ANIMAL SCIENCE

Short-term effects of α-melanocyte-stimulating hormone in three distinct melanin-pigmented cell types of Anura

RODRIGO ZIERI, LILIAN FRANCO-BELUSSI & CLASSIUS DE OLIVEIRA

Abstract: Ectothermic animals present melanin-containing cells in their integument and viscera. Besides cutaneous melanophores, amphibians have melanomacrophages in the hepatic parenchyma and melanocytes in the viscera, which are also present in their testicular stroma. The native melanocyte stimulating hormone (α-MSH) is the main hormone that modulates the color change in melanophores. However, we still know too little about how the α-MSH acts *in vivo* on visceral melanin-containing cells. In this study, we collected 30 adult males of *Physalaemus nattereri* (Anura, Leptodactylidae) to evaluate the short-term effects of α-MSH on melanophores, melanocytes and melanomacrophages under light microscopy. For this, we injected 0.05 ml of a single intraperitoneal dose containing $2.5x10^{-7}$ mmol/10g of α-MSH, diluted in ringer solution, in five experimental groups with five individuals each one. The different groups were analyzed after 1, 3, 6, 12 and 24h. The control group with five other individuals received only 0.05 ml of ringer solution. The skin pigmentation increased quickly after animals received the hormone α-MSH with the consequent darkening of the body (body darkness). Melanophores, melanocytes and melanomacrophages responded similarly to the test, with an increase in the area containing melanin. However, melanophores and melanomacrophages reached their darkest pigmentation in a shorter period of time in comparison to the testicular melanocytes, probably due to specific metabolic characteristics of each organ. Thus, we verified that the three types of cells, although present in different organs, are responsive to the native hormone α-MSH, which enables us to treat them as a pigmentary system.

Key words: melanophores, melanocytes, melanomacrophages, α-MSH, *Physalaemus nattereri*.

INTRODUCTION

Chromatophores are cells specialized in producing and storing pigments in vertebrates (Wallin 2002). Melanophores are chromatophores with melanin in their cytoplasm and play an important role in rapid color change (Bagnara & Matsumoto 2006). They are located in the dermis of amphibians and other ectothermic animals, present a stellar appearance and occupy large areas. They can be specially observed in anuran larvae as these animals present a thin epithelium (Franco-Belussi et al. 2018).

Pigment cells are not restricted to the dermis. They often occur in the connective tissue and in vital internal organs, such as liver, kidneys, heart, thymus, gonads, oocytes, as well as in blood vessels, peritoneum and meninges of anurans (Oliveira & Zieri 2005, Franco-Belussi et al. 2011, Goldberg et al. 2020) and fish (Agius & Roberts 2003, Arciuli et al. 2012). Among these visceral pigment cells are the melanocytes,

which are similar to melanophores (Zuasti et al. 1998). Melanocytes are produced by the ectodermal neural crest (Colombo et al. 2011) and are able to produce and store melanin (Agius & Roberts 2003). *Physalaemus nattereri* (former *Eupemphix nattereri*) and other species from the Leptodactylidae family present a conspicuous visceral pigmentation, especially in the testes, which are highly pigmented (Zieri et al. 2007, Franco-Belussi et al. 2009).

In addition, ectothermic vertebrates, including amphibians, present melanomacrophages (MMs) in hematopoietic organs, such as the liver and the spleen. These cells are originated from hematopoietic stem cells and are similar to macrophages, present phagocytic activity and a high amount of melanin in their cytoplasm (Sichel et al. 1997) and often aggregate, producing melanomacrophages centers – MMCs (Stosik et al. 2019). The MMCs act on detoxification or recycling of endogenous and exogenous products (Zapata & Amemiya 2000), as well as in iron storage after erythrophagocytosis (Agius & Roberts 2003, Steinel & Bolnick 2017). Then MMs have catabolic substances in their cytoplasm, hemosiderin and lipofuscin beyond melanin (Herráez & Zapata 1991, Gutierre et al. 2017).

In melanin-containing cells, cellular organelles containing pigment, as the melanosomes, can disperse or aggregate depending on the stimulus received by the pigmented cell. Stimulus can result from direct innervation in fishes or hormonal control in both fishes and anurans (Fujii 2000, Aspengren et al. 2009a). Rapid color change in anurans is related to camouflage and social signaling (Sköld et al. 2012). The most important hormone involved in color change in vertebrate is the α-melanocyte-stimulating hormone or α-MSH (Aspengren et al. 2009b). This hormone acts on the melanin-containing cells dispersing the pigments (Bagnara & Matsumoto 2006) by to increase tyrosinase gene expression (Guida et al. 2004, Arciuli et al. 2012).

We still know little about the role of visceral pigmentation on lower vertebrates (Schartl et al. 2015). There are strong evidences that visceral pigment cells have functions related to pathologies (Agius & Roberts 2003, Silva et al. 2013), cytoprotection against free radicals (Mcgraw 2005) and detoxification against pollutants (Fenoglio et al. 2005, Manera et al. 2018, Fanali et al. 2018, Pérez-Iglesias et al. 2019). However, effects of hormonal modulation of melanin-containing in organs and tissues of anurans are scarce. This is especially the case of sexual hormones (Zieri et al. 2015) and the superpotent synthetic analog of α-MSH (Franco-Belussi et al. 2013).

For this reason, we evaluate here the short-term effects of the native hormone α-MSH on melanophores, melanocytes and melanomacrophages of the anuran *P. nattereri*, a generalist species with broad area of occurrence, including the northwestern region of São Paulo State (Santos et al. 2007) and, listed as last concern by IUCN (2021). *P. nattereri* also has provided important responses in laboratory experiments, such as changes in organ pigmentation under exposure to high temperatures, UV radiation, bacterial components, endocrine disruptors and xenobiotic agents. For this reason, this species can be considered as an important biological model for pigmented-containing cells studies.

In this study we compare the responses of these three pigment cell types present, respectively, in the skin, testes and liver of the individuals. We hypothesize that the α-MSH will act on dispersing the melanin granules in the three cell types, leading to the tissues' darkening.

MATERIALS AND METHODS

Collection of animals

We collected a total of 30 adult males of the anuran species *Physalaemus nattereri* (Steindachner, 1863) in temporary ponds located in an area with intense agropastoral activity in the vicinity of the municipality of São José do Rio Preto, São Paulo, Brazil, (20°45'47''S; 49°19'59''W), during the rainy season of January 2013, when individuals were in vocalization activity during the reproductive season. The capture of the specimens was authorized by the governmental environmental agency (Protocol #18573-1 – SISBIO/ICMbio).

The animals were kept in the Comparative Anatomy Laboratory (UNESP - IBILCE), at room temperature (27±0.5ºC) and natural photoperiod (14/10 light/dark), during six days for acclimation in individual terrariums, and fed with termites. Before the hormonal treatment, we moved the animals to white polyethylene aquaria with shallow water for 24h (adaptation to the white background) where they were kept until the end of the experiment, under the same conditions described above.

Effects of the native hormone α-MSH

We established six experimental groups with five individuals for each one, one control (CONT) and five treatments (MSH1h, MSH3h, MSH6h, MSH12h and MSH24h). For the individuals of the treatments, we injected 0.05 ml of a single intraperitoneal dose with $2.5x10^{-7}$ mmol/10g of the native hormone α-MSH (Sigma-Aldrich, St. Louis, MO, USA) based on comparative dose-response curve of α-MSH in skin bioassays of frogs (Castrucci et al. 1984a, Hadley et al. 1985), diluted in sterile physiological solution (ringer solution) with osmolarity adjusted to amphibians (60% of mammals' osmolarity, adapted from Hadley et al. 1985). The individuals of the control group received the same dose but only of ringer solution. Each treatment corresponded to a period of time (1, 3, 6, 12 and 24 hours) after the hormone administration that we waited to start our observations, based on the time of hormone degradation by the amphibian serum (Castrucci et al. 1984b).

Histological processing and analysis

After the experiments, all animals were anesthetized and euthanized with a lethal dose of benzocaine (1 g/l of water). Liver and testes were removed and tissue samples of these organs, as well as of the skin, were collected for biopsy. Handling of animals and all experimental procedures were approved by the Committee on Ethics and Animal Experimentation of the São Paulo State University (Protocol 70/07 CEEA) and followed the recommendations of the Guide for the Care and Use of Laboratory Animals (National Research Council (US) Committee).

For the morphological analysis, the tissues were 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 hours. Then, the samples were washed in water, dehydrated in alcohol series and embedded in historesin (Leica-Historesin embedding kit). Fragments of the mid lobe of the liver, central region of the testes and of the dorsal skin were submitted to histological routine.

For each group, we obtained five slides with 20 sections of 2 µm, which were stained with hematoxylin-eosin for tissue general description and pigment quantification. They were observed under a microscope (Leica DM4000 B) equipped with image capture system (Leica DFC 280).

Quantitative analyses were performed using the Image Pro-Plus software Media Cybernetics Inc. (version 6.0). This method was used to quantify the melanin-containing area based on

color difference observed in the parenchyma of liver and testes, according to a modified method initially described by Lehr et al. (1997). The pigments were quantified in 25 histological fields (pseudoreplicates) per animal (Santos et al. 2014). For skin quantification, we calculated the relative area, expressed as melanin area by analyzed tissue area.

Body darkness

For body darkness analysis, we took pictures of the whole animals under standardized light conditions and analyzed the images using Adobe Photoshop 6.0, following Franco-Belussi et al. (2016). Digital images were converted into CIE *L* * *a* * *b* *, where *L* * is the brightness parameter. The *L** channel was normalized by setting the total black image as *L**=0 and the total white image as *L**=255. Landmarks were defined to ensure that the same area was analyzed in all animals. We analyzed 2 cm^2 of the dorsal skin below the insertion of forelimbs using the rectangular marquee tool. The mean values of lightness of the selected areas were measured using the histogram tool. Darkness was calculated as the complement of lightness, and data were shown in percentage of darkness (black=100%). For each animal, two different images were analyzed and the average value was used.

Statistical analysis

We tested whether the melanin area in the liver, testes and skin (response variables) increased with time of exposure to the α-MSH hormone (predictor). To verify this hypothesis, we used a One-Criterion Variance Analysis (one-way ANOVA) followed by a Tukey test. The data were square root transformed and we tested model assumptions with diagnostic plots in R package *sjPlot* (Lüdecke 2016). Residuals had homogeneity of variance and normal distribution. To test the body darkness response, we fitted a generalized linear mixed-effects models (GLMM) with beta distribution, with treatment as categorical predictor (Franco-Belussi et al. 2018).

Analyses were performed using the R software version 3.3.2 (R Development Core Team 2016). In addition, we used a Pearson's correlation to test for an association between the responses of distinct responses. Specifically, all responses variables were correlated with melanin in testes, skin and liver, and body darkness. These analyses were performed in the *corrplot p*ackage (Wei & Simko 2017).

RESULTS

Melanin-containing cells were present in the skin, testes and liver of *P. nattereri* (Figs. 1 and 2). In the skin, these cells were arranged in the underlying layer of the epidermis, together with iridophores and xanthophores. Cutaneous melanophores were found in high quantities and right below these cell types (Figs. 1a and 1b). Melanocytes, as well as cutaneous melanophores, presented a dendritic shape and were present on the surface of the testes, as observed through the transparency of the testicular capsule, and in the parenchyma, around the seminiferous locules (Figs. 1c and 1d). In the liver, melanomacrophages presented a globular shape and were dispersed throughout the hepatic parenchyma (Figs. 1e and 1f).

Body pigmentation increased quickly after animals received the dose of the native hormone α-MSH. After 1 hour of the hormone administration, it was possible to observe the hormone effect on the organism through the body darkness (Figs. 2 and 3), promoted by the increase of the area occupied by melanin (dispersion) into the melanophores (P<0.0001) (Fig. 4). This large area occupied by melanin in the skin was still observed after 3 and 6 hours (P<0.0001), but the values were lower than those

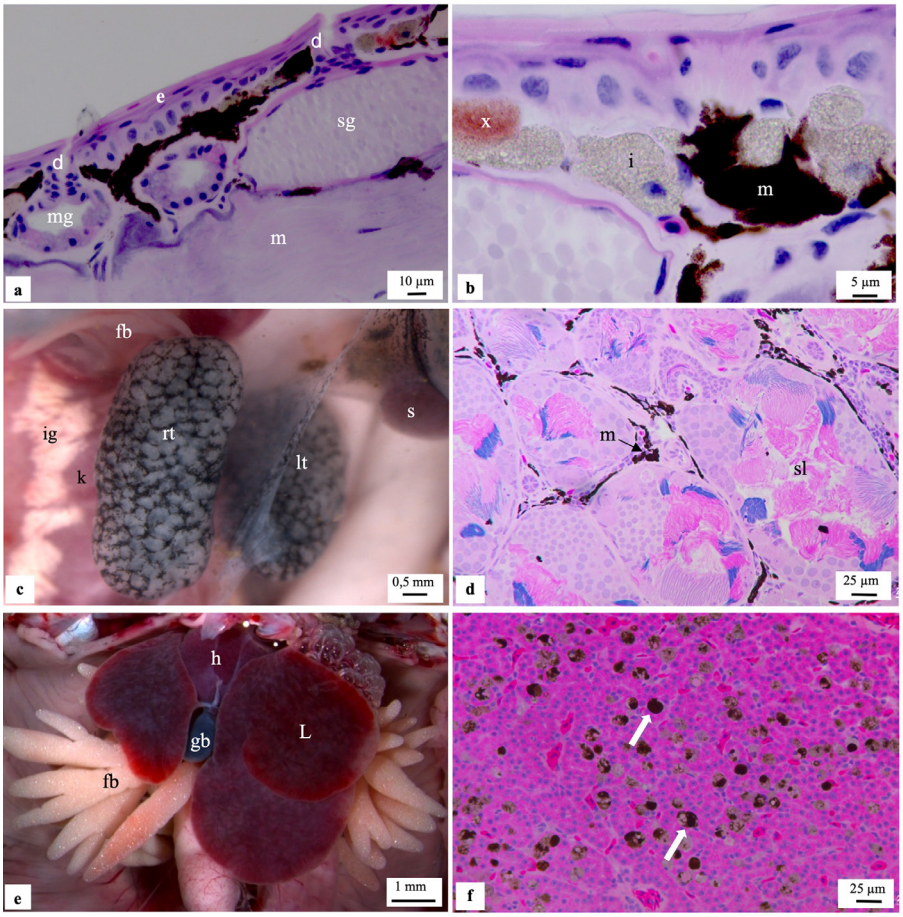

Figure 1. Histological sections of dorsal skin and morphological aspects of testes and liver of *Physalaemus nattereri*. a = General view showing the layer of dermal cromatophores, near to mucosal (mg) and serous gland (sg) (e=epidermis; d=ducts; m=muscle). b= Cromatophores in detail, showing melanophores (m), iridophores (i) and xantophores (x). c = Testicular melanocytes observed by transparency of the tunica albuginea (rt=right testis; lt=left testis; fb=faty body; s=spleen; k=kidney, ig= interrenal gland). d = Testicular section showing melanocytes in the interstitial region (m) around seminiferous locules (sl). e = Body cavity in general view (L=liver; gb=gallbladder; h=heart; fb=fatty body). f = Melanomacrophages (arrows) in the liver parenchyma.

registered after one hour of exposure to the hormone. After 12 hours there was no difference in the area occupied by melanin in the skin (P=0.94) in comparison to the initial group (Fig. 4).

In the testes, melanocytes increased their area in the parenchyma after one hour of exposure to the hormone, but the maximum area occupied by the melanin was reached after three hours (P<0.0001). After 6 and 12 hours, treatments were still more pigmented than the control group. However, after 24 hours the effects of the hormone were no longer observed on the testicles' pigmentation (P=0.97) (Figs. 5 and 7a-b).

In the liver, the response of the melanomacrophages was similar to those of melanocytes and melanophores. However, in this organ, as well as in the skin, the highest increase in area containing melanin was registered after one hour of exposure to the hormone (P<0.0001). After three hours (P=0.10), there were no effects of the hormone on the liver pigmentation (Figs. 6 and 7 c-d).

Combined effects of the α-MSH

We observed a positive correlation between the body darkness and the area occupied by melanin into the melanophores (skin melanin) after one hour of the hormone administration (Fig. 8). In this treatment (MSH1h), the dispersion of melanin into the melanophores increased, leading to an increase in the area containing melanin in the skin and, consequently, body darkness (P=0.04). On the other hand, we observed a decrease in the area occupied by melanophores after 24

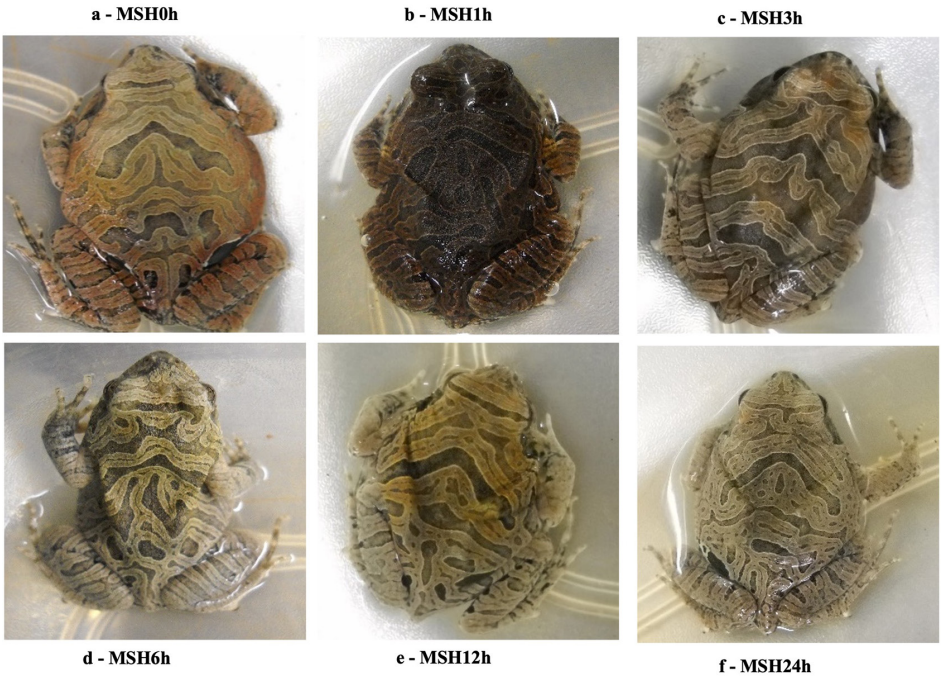

Figure 2. Physiological color change of the skin of *P. nattereri* after exposure to α-melanocyte-stimulating hormone. The peak of darkening was observed in the experimental time of 1h and the return to the initial condition occurred gradually at the following times. MSH0h: animal exposure to ringer solution. MSH1h, MSH3h, MSH6h, MSH12h and MSH24h: animals' exposure to α-MSH hormone by 1, 3, 6, 12 and 24 hours respectively.

hours and the consequent lightening of the skin (*P*=0.03) (Figs. 3 and 4).

We also observed a positive correlation between the darkening of the skin and the testicular pigmentation after 3 hours of the experiment. At this period, the darkening of the skin was still high and the increase in testicular pigmentation was maximum (*P*=0.02). The testicular pigmentation was positively correlated to the liver pigmentation after one hour of the hormonal treatment (*P*=0.04) (Fig. 8).

DISCUSSION

The native hormone α-MSH causes the dispersion of melanin granules in the interior of pigmented cells. In our study, the α-MSH increased the pigmentation not only of the skin but of the internal organs of *P. nattereri*. Although the hormonal mechanisms that produce changes in visceral pigmentation are poorly studied, we corroborated our hypothesis that the three types of cells are responsive to the native hormone α-MSH. We observed that cutaneous melanophores, testicular melanocytes and hepatic melanomacrophages of *P. nattereri* responded to the hormone and had an increase in pigmentation after one hour of the hormonal administration, with a similar response time among them.

The skin of *P. nattereri* presents melanophores, xanthophores and iridophores. Melanophores contain melanin in the cytoplasm and xanthophores present carotenoids and iridophores with purines, which produce the animal integument coloration. By analyzing the skin of *Lithobates pipiens* (former *Rana pipiens*), Bagnara et al. (1968), named this group of pigmented cells as dermal chromatophore units. In our study, we observed these unities in samples of the dorsal region of *P. nattereri* skin. They have also been observed in the lumbar region of anurans of the genera *Pleurodema* and *Sumuncuria* (Leiuperidae) (Ferraro et al. 2013) and in a comparative study of the dorsal region integument of the genera *Ololygon* and *Scinax* (Hylidae) (Silva et al. 2017). Thus, we noted that

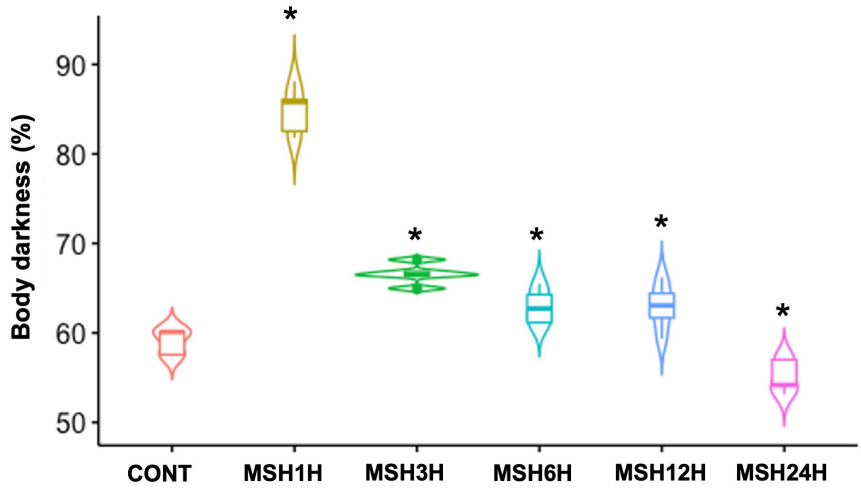

Figure 3. Body darkness analysis in *P. nattereri*, after exposure to α-melanocyte-stimulating hormone. Body darkening presented maximum values after 1h of exposure. The skin whitening occurred gradually in the subsequent times. Box represents the first and third quantiles and lines inside represent the median. Violin showing the data distribution and the points the outliers. ANOVA followed by Tukey test ($P<0.05$). (*) shows differences between control and treated groups.

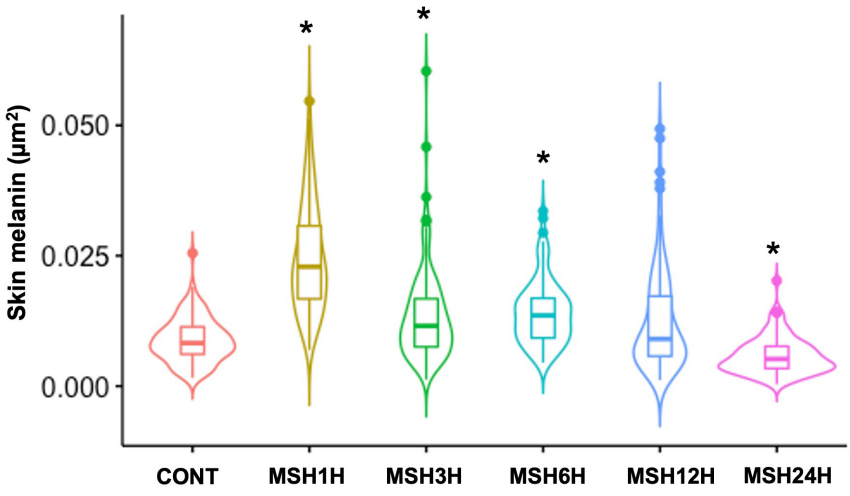

Figure 4. Histological aspects of dispersion and aggregation of cutaneous melanin in *P. nattereri*, after exposure to α-melanocyte-stimulating hormone. Melanin-containing area presented maximum values after 1h of exposure. The skin whitening occurred gradually in the subsequent times. Box represents the first and third quantiles and lines inside represent the median. Violin showing the data distribution and the points the outliers. ANOVA followed by Tukey test ($P<0.05$). (*) shows differences between control and treated groups.

the species *P. nattereri* has the same pattern of chromatophores distribution as other anurans.

There was an increase in the dispersion of melanin granules in the melanophores of *P. nattereri*, which led to the darkening of the animals' body (body darkness) in a short period of exposure to the hormone (i.e., 1h). In the unities formed by pigmented cells located in the skin, melanin disperses and iridophores contract at the same time (Bagnara & Matsumoto 2006). This phenomenon is common to vertebrates and is related to the background adaptation (camouflage) and to communication signals (social status and nuptial coloration) (Aspergren et al. 2009b, Kindermann et al. 2013). The darkening of animals' body can also be related to thermoregulation (Crusella-Trullas et al. 2007, Santos et al. 2014) and to protection against ultraviolet radiation (Franco-Belussi et al. 2016, 2018).

Besides α-MSH, studies show that its superpotent analog and other substances, such as pituitary extract, serotonin, melatonin and ACTH act on pigmented cells. These substances act in an agonist or antagonist way to the melanotropin producing, in a short period of time, the dispersion or retraction of the melanin-containing organelles (melanosomes) in the

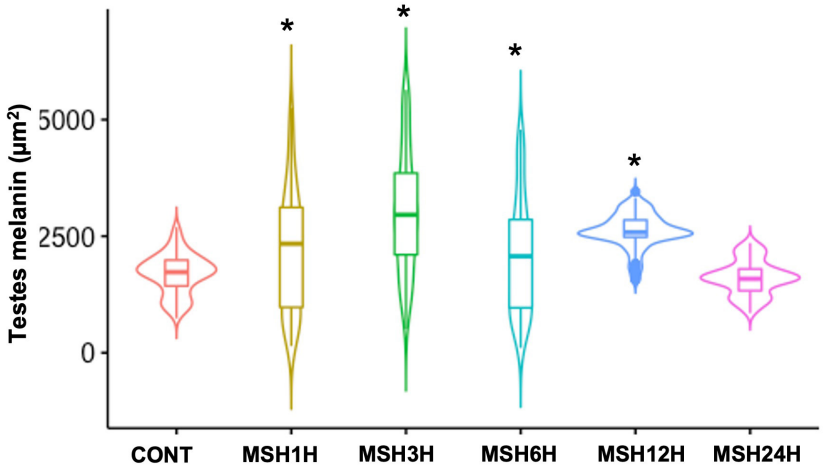

Figure 5. Effect of α-MSH in the pigment-containing area of testes of *P. nattereri*, showing the melanin dispersion variation within cells, analyzed after 1, 3, 6, 12 and 24 hours after hormonal treatment, compared with the control group. Box represents the first and third quantiles and lines inside represent the median. Violin showing the data distribution and the points the outliers. ANOVA followed by Tukey test ($P<0.05$). (*) shows differences between control and treated groups.

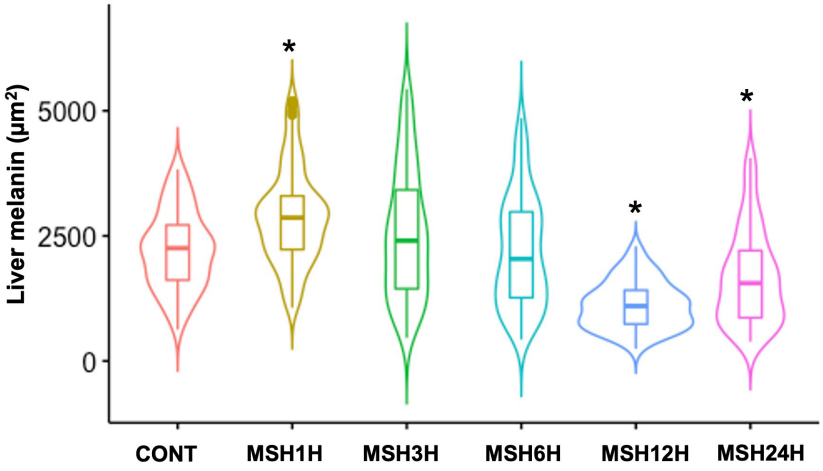

Figure 6. Effect of α-MSH in the pigment-containing area of liver of *P. nattereri*, showing the melanin dispersion variation within cells, analyzed after 1, 3, 6, 12 and 24 hours after hormonal treatment, compared with the control group. Box represents the first and third quantiles and lines inside represent the median. Violin showing the data distribution and the points the outliers. ANOVA followed by Tukey test ($P<0.05$). (*) shows differences between control and treated groups.

interior of melanophores. Such movements of granules result in physiological color changes in the skin cells of amphibians, fish and reptiles (Wilkes et al. 1984, Castrucci et al. 1997). In this context, amphibian melanophores are under the direct influence of the neuropeptide α-MSH (Aspengren et al. 2009a), which can be considered the main melanophore dispersing agent (Sköld et al. 2012). Our study supports these findings and we provide evidences that the α-MSH has an important role in the dynamic of animal skin color, as we observed the rapid action of the hormone through the darkening of *P. nattereri* body.

Regarding the viscera, we selected the liver and the testes, as these organs are related to metabolism and reproduction, respectively, and present pigmented cells. In *P. nattereri*, both liver and testes are highly pigmented and their melanin-containing cells are subjected to the hormone effect, as we observed in this study. Environmental factors (i.e., UV radiation, photoperiod, temperature, xenobiotics and inflammatory agents) can modulate the pigment occurrence and intensity in several anuran. This demonstrates the adaptive and protective functions of the internal melanin under stress conditions (Franco-Belussi et al. 2017). Such conditions can increase the plasmatic level of

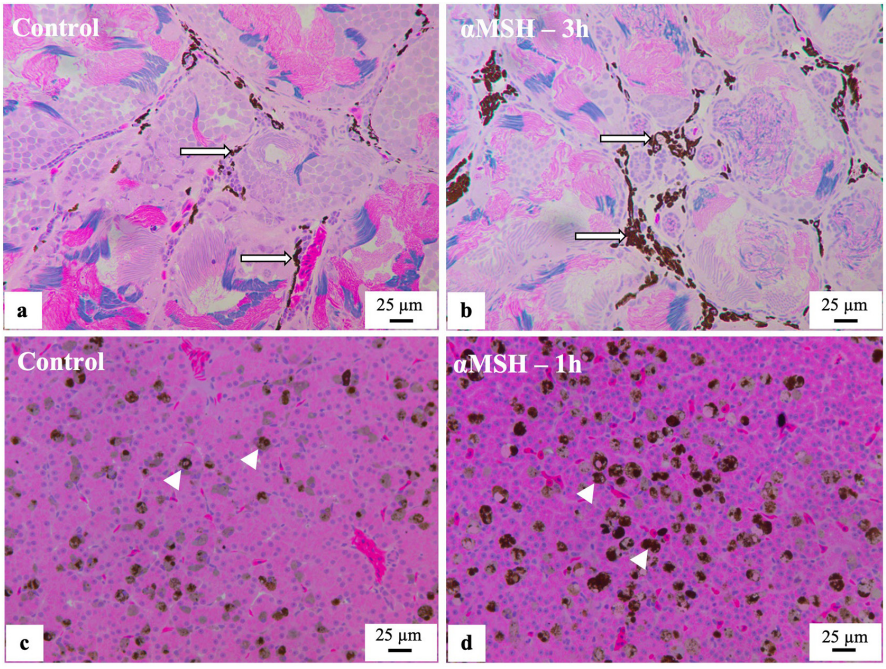

Figure 7. Morphofunctional representative illustration of the action of α-MSH in the testicular (a and b) and hepatic pigmented cells (c and d) of *P. nattereri*. Testicular melanocytes (arrows) presented maximum darkening after 3h, while hepatic melanomacrophages (arrow head) reached the peak of coloration after 1h of exposure.

α-MSH and the gene expression of melanotropin receptors. This has been observed in hepatic cells of mice, including liver-resident macrophages, during the acute phase response produced by muscle injury (Malik et al. 2012). In addition, this peptide has a neuroimmunomodulatory function and participates in the control of inflammatory processes, fever and migration of defense cells, and its structure is highly conserved between groups of vertebrates and higher organisms, including humans (Lipton & Catania 1997).

The MMs from the liver of *P. nattereri* are isolated, globular cells immersed in the hepatic parenchyma. This organization seems common to anurans, but in fish these cells are organized in clusters (MMCs), which are similar in structure and function to the germinal centers (GCs) present in mammals' lymph nodes (Agius & Roberts 2003). Such characteristics seem to indicate an evolutionary proximity among them, although this hypothesis needs further studies (Steinel & Bolnick 2017, Stosik et al. 2019).

The hepatic melanomacrophages of *P. nattereri* were also responsive to the native hormone α-MSH and reached maximum darkening in a short period of time (i.e., 1h). We know little about the factors that regulate the melanogenic pathway in these cells. However, *in vitro* studies with MMs from *P. esculentus* showed typical tyrosinase and dopa oxidase activities in melanosomal proteins when treated with Nle4, D-Phe7-α-MSH, which is the non-biodegradable synthetic analog of α-MSH (Guida et al. 2004, Gallone et al. 2007). In melanocytes, these enzymes are induced by an increase in the cellular level of cAMP, but in MMs this mechanism is different and still needs to be better understood. Franco-Belussi et al. (2013), demonstrated the effects of the superpotent analog on the testicular pigmentation of *P. nattereri*, but they did not observe changes in the hepatic pigmentation. This suggests that the synthetic analog of α-MSH acts only on cells of melanocytes' lineage, not on that of melanomacrophages. In our study, we used the native hormone (α-MSH) in the same animal model but in a concentration about 1,000 times higher. We observed its short-term effect of increasing pigmentation in the liver as well as in the skin and testes. As such, we demonstrated

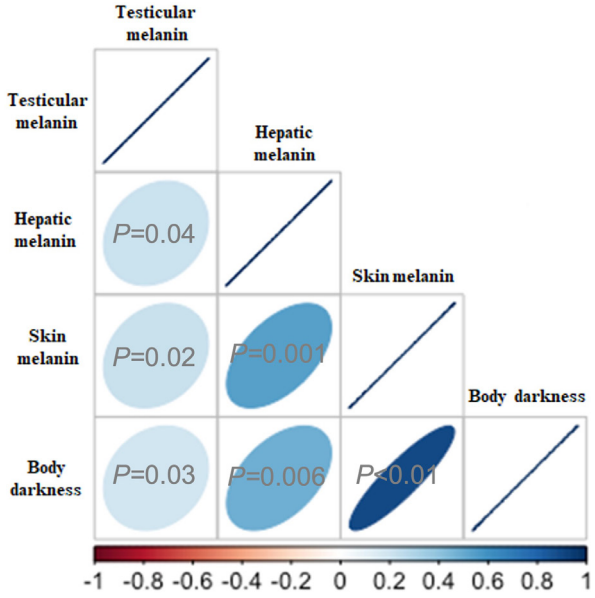

Figure 8. Correlogram showing the relationship among the variable-responses (melanophore, melanocytes and melanomacrophages) under effect of α-MSH at different exposure times (1, 3, 6, 12 and 24h). Color gradient indicates the strength of the correlation (r value) where blue circles show positive correlations. While shape shows significative correlation. All *P*-value are represented in figure.

for the first time the effect of the native hormone α-MSH on the internal melanin present in the viscera.

Although there was an increase in pigmentation in the testes of *P. nattereri* after the first hour of exposure to the hormone, this organ required more time to reach the darkening peak (i.e., 3h) and more than 12 hours to return to its original pigmentation. Studies with *Xenopus laevis* using a higher dose ($5x10^{-7}$ mmol/10g) of α-MSH showed that the melanophores remained darker for a longer time and the lightening process started only after 3 hours of a single dose (Hadley et al. 1985). Based on these observations, we verified that high concentrations, duration of the stimulus and potency of the hormone act both on the speed of the dispersion and on the maintenance of the dispersed pigments. In addition, the presence and quantity of serum containing specific enzymes also influence the duration of the stimulus on these cells, as observed in *in vitro* bioassays with serum of *L. pipiens* (Castrucci et al. 1984a, b). Thus, we suggest that the metabolic activities of liver and skin may influence the response speed of these organs in comparison to the testes. The same probably occurs with the plasmatic enzyme that act on the degradation of α-MSH in these organs.

CONCLUSIONS

In this study, we observed that the three types of pigmented cells responded very similarly to the treatment with the native hormone α-MSH administered to individuals of *P. nattereri*, a native species considered as a model to morphological bioassays about pigmentation. However, melanophores and melanomacrophages reached the maximum darkening in a shorter time (i.e., 1h) in comparison to testicular melanocytes. Both processes of dispersion (darkening) and aggregation of pigments (lightening) happened more rapidly in melanophores and melanomacrophages, probably due to intrinsic metabolic characteristics of each organ. This is the first time that changes in pigmentation are demonstrated *in vivo* in these three types of cells on vertebrate organisms. Also, it corroborates other bioassays made with cultures of pigmented cells of non-tropical species. Such evidences allow us to say that these cells are indeed part of a pigmentary system. Therefore, we believe that our results contribute to studies on the visceral pigmentation functions.

Acknowledgments

This work is part of postdoctoral research presented by Rodrigo Zieri to Department of Biology from Ibilce/Unesp. The authors thank Gabriela Baroni Leite and Juliane Silberschmidt Freitas for animal care and technical assistance. This study was supported by Conselho Nacional de Desenvolvimento Científico e

Tecnológico – CNPq/Edital Universal (grant# 014/2010; 473499/2010-0) and, Classius de Oliveira has been continuously supported by CNPq, grant #304552/2019-4. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. There was no additional external funding received for this study.

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How to cite

ZIERI R, FRANCO-BELUSSI L & DE OLIVEIRA C. 2023. Short-term effects of α-melanocyte-stimulating hormone in three distinct melanin-pigmented cell types of Anura. An Acad Bras Cienc 95: e20211581. DOI 10.1590/0001-3765202320211581.

Manuscript received on December 10, 2021;
accepted for publication on April 17, 2022

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Rodrigo Zieri, Lilian Franco-Belussi and Classius de Oliveira designed the project, conducted the work and analyzed the data. Rodrigo Zieri interpreted the data and wrote the manuscript. LFB performed data analysis. All authors read and agree with the final version of the manuscript.