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RESEARCH ARTICLE

Internal pigment cells respond to external UV radiation in frogs

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ABSTRACT

Fish and amphibians have pigment cells that generate colorful skins important for signaling, camouflage, thermoregulation and protection against ultraviolet radiation (UVR). However, many animals also have pigment cells inside their bodies, on their internal organs and membranes. In contrast to external pigmentation, internal pigmentation is remarkably little studied and its function is not well known. Here, we tested genotoxic effects of UVR and its effects on internal pigmentation in a neotropical frog, *Physalaemus nattereri*. We found increases in body darkness and internal melanin pigmentation in testes and heart surfaces and in the mesenterium and lumbar region after just a few hours of UVR exposure. The melanin dispersion in melanomacrophages in the liver and melanocytes in testes increased after UV exposure. In addition, the amount of melanin inside melanomacrophages cells also increased. Although mast cells were quickly activated by UVR, only longer UVR exposure resulted in genotoxic effects inside frogs, by increasing the frequency of micronuclei in red blood cells. This is the first study to describe systemic responses of external UVR on internal melanin pigmentation, melanomacrophages and melanocytes in frogs and thus provides a functional explanation to the presence of internal pigmentation.

KEY WORDS: Melanocytes, Melanomacrophages, Solar radiation, DNA damage, Anuran, *Physalaemus nattereri*

INTRODUCTION

Fish and amphibians have pigmented cells called visceral melanocytes and melanomacrophages in internal organs and membranes (Gallone et al., 2002; Franco-Belussi et al., 2011, 2012; Nilsson Sköld et al., 2013). Visceral melanocytes are in many ways similar to skin melanocytes (Zuasti et al., 1998). They originate from the ectodermal neural crest (Franco-Belussi et al., 2013; Colombo et al., 2011), have a dendritic shape, and contain large amounts of melanin (Oliveira and Franco-Belussi, 2012). Melanomacrophages are another type of pigmented cells that are present in hematopoietic organs. They derive from hematopoietic stem cells (Colombo et al., 2011) and have phagocytic activity similar to macrophages (Agius, 1980). Melanomacrophages are rounded (Sichel et al., 1997) and contain the catabolic substances hemosiderin and lipofuscin (Agius, 1981; Agius and Roberts, 2003). Both types of internal pigment cells have cytoprotective functions against free radicals (Mcgraw, 2005), produce melanin (Gallone et al., 2002, 2007) and detoxify pollutants (Fenoglio et al., 2005) in addition to functions related to the immune system (Franco-Belussi et al., 2013) and pathologies (Roberts, 1975). There is evidence that melanin participates in innate immunity and regulates cytokine activity (Mackintosh, 2001). Melanin itself also has a bactericide function (Wolke et al., 1985), which is a potentially important function in ectothermal animals since their enzyme activities are restricted at lower temperatures (Wolke et al., 1985).

In addition to immunological and antioxidative functions, studies also suggest other functions for internal melanin. For example, there are several types of pigment cells in the eyes of vertebrates and some of them respond to stimuli by changing color (Wucherer and Michiels, 2014; Sköld et al., 2015). Changes in internal color in many species of fish can be due to responsive peritoneal chromatophores and the degree of such a response correlates with levels of body transparency (Nilsson Sköld et al., 2010). Such extracutaneous pigment cells may have a role in animal camouflage and instances of social signaling (Nilsson Sköld et al., 2010; Wucherer and Michiels, 2014). However, the presence of internal melanocytes in non-transparent animals suggests that these cells have additional functions. Such functions might relate to the innate immune system or UV protection because melanin in melanocytes can absorb UVR (Ortonne, 2002) and melanin can act as an antioxidant and protect against DNA damage. In addition, UVR is immunomodulatory and can indirectly activate mast cells and promote immune responses in the skin (Hart et al., 2000).

In human skin, UVR causes cellular DNA damage and increases melanogenesis (Gilchrest and Eller, 1999). In addition, pigment cells in the peritoneum are responsive to UVR in transparent fishes (Nilsson Sköld et al., 2010). However, it is not known if and how UVR affects internal melanin pigmentation. Since melanin can protect tissues from DNA damage, we hypothesize that it responds to external UVR by increasing not only external pigmentation, but also internal pigmentation. Here, we tested the systemic effects of external UVR on internal pigmentation in addition to the genotoxic effects in internal cells, body darkness and external pigmentation. The testicular melanocytes and hepatic melanomacrophages were distinguished in comparisons of the changes in melanin. Analyses were conducted using a wild tropical anuran as an ecologically relevant model system and with natural levels of UVR.

MATERIALS AND METHODS

Animal model

Adult males of *Physalaemus nattereri* Steindachner 1863 (Anura) were collected in wetlands and temporary lakes in southeastern Brazil (20 47′07.05″S, 49°02′42.09″W). Animals were kept in a laboratory at room temperature and daylight regime (27±0.5°C and 12 h light:12 h dark) for 7 days before experiments. All animals were treated humanely according to the Guide for Care and Use of Laboratory Animals and procedures approved by Committee on Ethics and Animal Experimentation of the São Paulo State University (CEUA-IBILCE/UNESP 096/2014).

Exposure to UVR

Frogs were exposed to UVR at wavelengths from 280 to 400 nm, peaking at 313 nm. Animals were grouped into eight different radiation treatments (N=6 per group) with different exposure times

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of 3, 6, 9, 12, 15, 18, 21 and 24 h. For the experimental groups, animals were randomly separated. We used Philips TL20 lamps W/12 RS, which emit 60% UVB and 40% UVA, less than 3% of which is gamma radiation (UVC), with irradiance of 2.4 W m^{-2} of UVB (adapted from Shirley et al., 2012; Schuch et al., 2015). The doses used were approximately 10% of natural UVB and UVA levels, as measured in an average summer day in southeastern Brazil (Schuch et al., 2015). *Physalaemus nattereri* remains buried during the day and are exposed to UVR only in the evening when they go out to eat or reproduce. The low dose in this study was chosen to reflect the natural range of exposure they face. During the experiments, animals were placed in an incubator (EletroLab, Model 121 FC) with controlled temperature and photoperiods (27 ±0.5°C and 12 h light:12 h dark, with a Philips TLT20W/75RS fluorescent lamp). Control animals (N=6 per group) were kept under the same laboratory conditions but without UVR exposure. After exposure, animals were anesthetized and killed with a lethal dose of benzocaine diluted in water (5 g l^{-1}).

Analysis of tissue melanin content

To conduct the histological analysis, liver and testes were removed and fixed in Karnovsky fixative solution (0.1 mol l^{-1} phosphate buffer, pH 7.2, containing 5% paraformaldehyde and 2.5% glutaraldehyde) at 4°C for 24 h. Then, samples were washed in water, dehydrated in alcohol series and embedded in historesin (Leica-Historesin embedding kit, Leica Microsystems). Sections of 2 µm were obtained using a RM 2265 microtome (Leica, Switzerland). For examination, sections were stained with Hematoxylin and Eosin. Quantification of hepatic and testicular dispersion followed De Souza Santos et al. (2014) using a microscope (Leica DM4000 B) equipped with an image capture system (Leica DFC 280). To analyze pigmentation on organ surfaces, we classified the intensity of melanin coloration, from the absence of color (category 0) to intense dark coloration (category 3), following Franco-Belussi et al. (2009).

For quantification of melanin inside the liver, the organ was homogenized in 1 mol l^{-1} NaOH with 10% dimethyl sulfoxide (DMSO) and heated at 80°C for 2 h. The mixture was centrifuged at 2500 rpm for 15 min, the supernatant was collected (1 ml) for analysis of melanin content. Levels of melanin in samples were measured at 475 nm in a flat bottom plate 200 µl sample in an ELISA plate reader (adapted from Abdel-Naser et al., 2003). Synthetic melanin (Sigma-Aldrich) was used as a reference and made up as a stock solution (10 mg in 1 ml of 1 mol l^{-1} sodium hydroxide with 10% DMSO, heated at 80°C for 2 h). Absorbance values for synthetic melanin were determined in duplicate and plotted as a function of concentration in a standard curve. From the determined linear regression, the mathematical relationship between absorbance and concentration was established and used to quantify melanin in the liver.

Mast cell quantification

Liver sections were stained with Toluidine Blue and borax (Bancroft and Gamble, 2001). Using 10 histological liver sections per animal, the number of mast cells was counted in average areas of 8 mm^2 per section. Data show mean cell frequency per histological section area for each animal.

Analysis of micronuclei and other nuclear abnormalities in blood cells

Circulating blood cells were collected on slides and a blood cell assay to address genotoxicity was performed by fixing with cold methanol (4°C) for 20 min and then staining with Giemsa solution (7.5%) for 15 min. Erythrocytes were analyzed and data were expressed in total number of micronuclei or other nuclear abnormalities per 1000 cells. Analysis was carried out as described by Pérez-Iglesias et al. (2014).

Body darkness analysis

Images of the whole animal taken under standardized light conditions were analyzed using Adobe Photoshop 6.0, following Svensson et al. (2005). Digital images were first converted to CIE $L^*a^*b^*$, where L^* is the lightness parameter. The L^* channel was normalized by setting the total black image (L^*=0) and the total white image (L^*=255). Landmarks were defined to ensure that the same area was analyzed in all animals. We analyzed 2 cm^2 of dorsal skin below the insertion of forelimbs using the rectangular marquee tool. The mean values of lightness of the selected area 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 photographs were analyzed and the average was used.

Statistical analysis

The area occupied by melanin, melanin concentration, body darkness, mast cell frequency and micronucleus (response variables) were tested against the time of exposure to UVR (predictor). The data were square-root transformed to meet the assumptions of homogeneity of variance and normality. Then, we ran separated one-way ANOVA to test the effect of UVR on each response variable. ANOVA analysis was followed by a Tukey *post hoc* test.

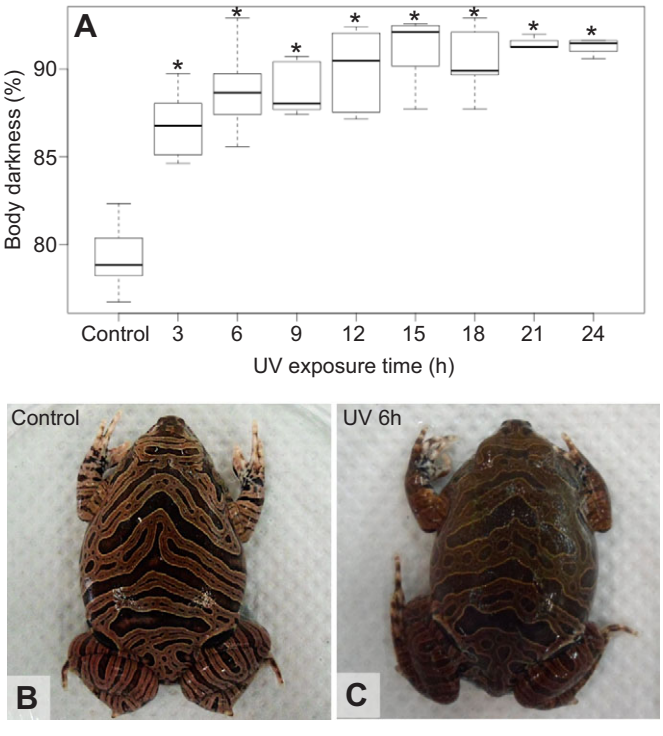

Fig. 1. UVR exposure increases body darkness of *Physalaemus nattereri* over all experimental times. UVR exposure increases body darkness over all experimental times. (A) Box plot showing percentage body darkness for each experimental time. The line represents the median value, box is the first and third quartile. Bars represent highest and lowest values. (B) Frog from control group. (C) Frog after exposure to UVR for 6 h. Values are means+s.e.m. of N=6 samples for each group. *P<0.05 compared with control group.

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To compare differences among categories of melanin coloration in each organ or region after treatment, we used a *G*-test for goodness of fit, with Yates' correction. This test was implemented using the code provided by Peter Hurd (available at www.psych.ualberta.ca/~phurd/cruft/g.test.r). Pearson's correlation was used to analyze the potential link between internal pigmentation (e.g. the area of pigmentation in testes and liver and the quantity of melanin in the liver) and levels of blood cell micronuclei. In addition, we correlated body darkness and internal pigmentation (e.g. the pigmented area in testes and liver and the quantity of hepatic melanin), micronuclei and mast cells. Analyses were performed using R software version 3.2.0 (R Development Core Team) and a significance value of $P<0.05$.

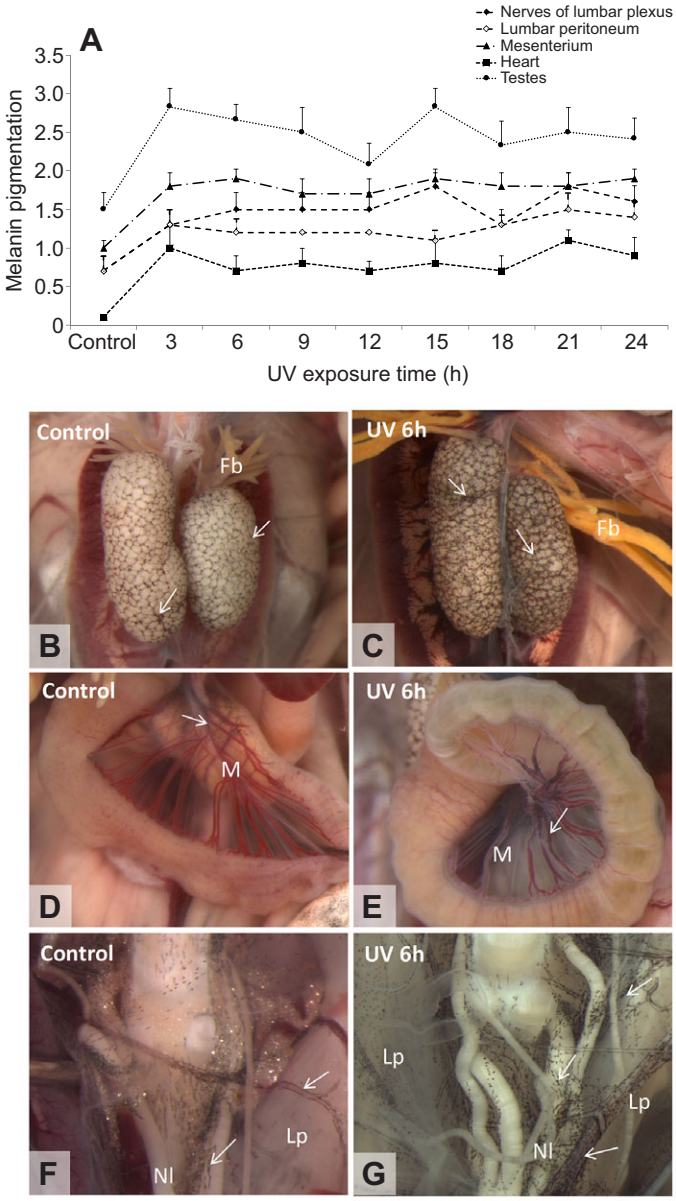

Fig. 2. UVR exposure increases visceral melanin pigmentation in *P. nattereri*. (A) Temporal response to UVR exposure on internal pigmentation. 0=absence of color; 3=intense dark coloration. (B–G) UVR exposure increases pigmentation in testes surfaces (arrows in B,C), mesenterium (arrows in D,E), nervous lumbar plexus and lumbar parietal peritoneum (arrows in F,G). Fb, fatty body; Lp, lumbar parietal peritoneum; M, mesenterium; Nl, lumbar plexus.

RESULTS

Effects of UVR on body coloration and external melanin pigmentation

After 3 h or more of UVR exposure, body darkness increased and the outer skin became darker ($F_{8,36}=16.76$, $P<0.01$; Fig. 1).

Effects of UVR on internal melanin pigmentation

External UVR exposure induced a rapid increase in visceral melanin pigmentation on the surface of the testes, heart, mesenterium, lumbar peritoneum and nerves of lumbar plexus ($G_{24}=41.79$, $P<0.01$; Fig. 2). There was also an increase in melanin content in the liver, with a peak at 3 h of UVR exposure ($F_{8,81}=5.05$, $P<0.01$; Fig. 3A).

UVR also increased the pigmented area by dispersing melanin in hepatic melanomacrophages ($F_{8,1115}=62.24$, $P<0.01$, Fig. 3A,D,E) and in melanocytes on the testis ($F_{8,1115}=21.24$, $P<0.01$, Fig. 3A,B,C). The maximum increase of melanin dispersion in the liver was after 12 h of exposure, whereas the maximum increase of melanin dispersion in the testes was after 3 h.

Effects of UVR on mast cells and the cell nucleus

The number of micronuclei in blood erythrocytes increased 10-fold following 18, 21 and 24 h of UVR exposure ($F_{8,36}=6.08$, $P<0.01$; Table 1). Other nuclear abnormalities, such as nuclei buds, erythroplastids, and binucleated nuclei were also observed

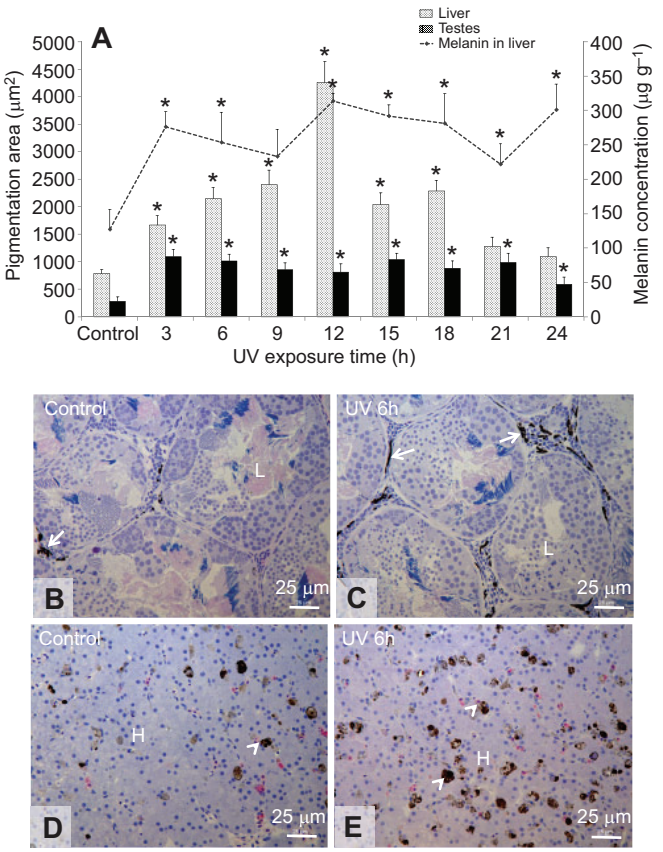

Fig. 3. UVR exposure increases melanocyte and melanomacrophage dispersion in testes and liver tissue of *P. nattereri*. (A) Temporal response to UVR in liver and testes. (B–E) Histological effects of UVR in testes (B,C) and liver (D,E). Arrows indicate testes melanocytes around the locular seminiferous. Arrowheads indicate hepatic melanomacrophages. Sections are stained with Hematoxylin and Eosin. L, locular seminiferous; H, hepatocytes. Values are means+s.e.m. of $N=6$ samples for each group. *$P<0.05$ compared with control group.

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Table 1. Frequency (‰) of nuclear abnormalities in blood erythrocytes of *P. nattereri* exposed to UVR

Treatment	Micronuclei	Nuclei buds	Erythroplastids	Binucleated
Control	0.2±0.13	0.2±0.13	0.2±0.13	0.2±0.13
UV 3 h	0.6±0.23	0.2±0.11	0.2±0.12	0.6±0.20
UV 6 h	0.8±0.20	0.2±0.12	0.2±0.11	0.4±0.18
UV 9 h	1.0±0.31	0.2±0.14	0.2±0.11	0.8±0.27
UV 12 h	0.6±0.24	0.2±0.10	0.2±0.10	0.2±0.19
UV 15 h	0.6±0.22	0.4±0.24	0.4±0.24	0.4±0.21
UV 18 h	2.4±0.23***	0.8±0.28	0.6±0.20	0.6±0.21
UV 21 h	2.2±0.29**	0.4±0.21	1±0.29	1.2±0.27
UV 24 h	1.8±0.27*	0.4±0.23	0.6±0.22	0.8±0.12

N=6 animals and *n*=6000 cells for all animals of one experimental group; all values are means±s.e.m. Control, animals not exposed to UVR. Significant differences with respect to control values are shown as **P*<0.05, ***P*<0.01 and ****P*<0.001.

(Fig. 4). However, these other genotoxic effects did not correlate with increased dispersion in pigmented cells nor with an increase of melanin content (r=0.27, P=0.67).

Mast cell frequency in the liver increased about 10 times after 3 h of UV exposure ($F_{8,441}$=21.65, P<0.01; Fig. 5) and was 5 times greater than in the control group after 6 h of exposure ($F_{8,441}$=21.65, P<0.01). There was no increase in the number of mast cells after longer exposure times.

Joint effects of UVR exposure

Body darkness as an effect of UVR exposure correlated linearly with the pigmentation in testes and heart surfaces, i.e. when body darkness increased animals also became darker internally (r=0.87, P=0.003 for testes; r=0.67, P=0.03 for heart, Fig. S1A,B, respectively). Body darkness also correlated with the pigmented area inside the testis (r=0.93, P=0.02, Fig. S1C), where there was an increase in dispersed melanin inside the melanocytes. No correlated melanin effects were observed for the liver.

Darkening of the body further correlated with an increased number of micronuclei (r=0.93, P=0.03, Fig. S1D). The increase of mast cells correlated positively with elevated dispersion of melanin in liver melanomacrophages after 6 h of UVR exposure (r=0.87, P=0.04, Fig. S1E). However, there was no correlation between genotoxic effects and an increase in pigmentation of the liver or testes.

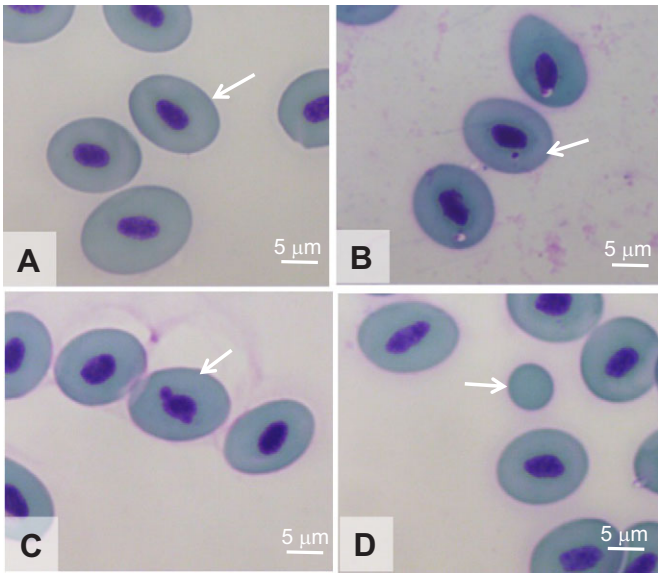

Fig. 4. Blood cells of *P. nattereri* exposed to UVR for 21 h show nuclear abnormalities. (A) Normal erythrocytes of control frog. (B,C) Erythrocytes of frogs exposed to UVR contained micronuclei (arrow in B) and nucleus buds (arrow in C). (D) Erythroplastids (arrow) are also observed in blood of UVR-exposed frogs.

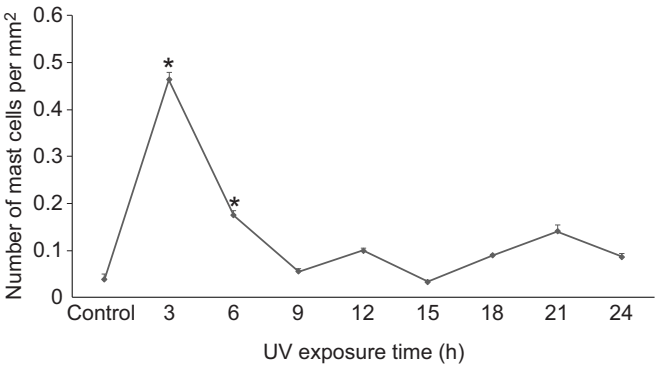

Fig. 5. UVR exposure in *P. nattereri* causes an early increase in hepatic mast cells. Values are means+s.e.m. of *N*=6 samples for each group. **P*<0.05 compared with control group.

DISCUSSION

Ectothermic animals have melanin and pigment cells in and around internal organs and their potential relationship with environmental variables is poorly explored. However, liver pigmentation in frogs varies according to environmental temperature (De Souza Santos et al., 2014). In fishes, internal chromatophores are responsive to hormones, where the degree of body transparency correlates positively with an increasing responsiveness of pigment cells (Nilsson Sköld et al., 2010).

Here, we show that external ultraviolet radiation (UVR) results in a rapid (e.g. 3 h of UVR exposure) increase in body darkness through elevated skin pigmentation as well as an increase in internal melanin pigmentation. The increase in internal melanin was explained by dispersion of melanin granules inside internal melanocytes and melanomacrophages, in addition to an increase of melanin production in different tissues. Although the exact amount of UVR reaching the internal organs is unknown, the rapid increase in both skin and internal pigmentation indicate a direct effect of UVR.

Until now, an increase in internal pigmentation following UVR exposure has not been reported, and we believe this increase seen here in frogs is adaptive and due to the primary photo-protective role of melanin. Melanin absorbs UVR in both skin and internal cells and transforms it into heat to be dissipated (Ortonne, 2002). Our results show an increase in internal melanin production, which is similar to the response in human skin melanocytes, where an increase in melanin production has also been described after UVR exposure (Friedmann and Gilchrest, 1987). In humans, this increase was further related to higher tyrosinase activity (Friedmann and Gilchrest, 1987). However, in anuran melanomacrophages, there is a distinct pathway of melanin production, involving tyrosine hydroxylating enzyme (Gallone et al., 2002). This difference can potentially show how physiologically distinct melanocytes are from melanomacrophages (Gallone et al., 2007). Together, these responses demonstrate systemic and physiological effects of external UVR and suggest that internal pigmentation can respond to UVR in many vertebrate species.

We found that genotoxicity correlated with body darkness, with animals showing a darkened skin and increased number of micronuclei, but only after 18 h or more of UVR exposure. In

humans, short-term exposure to UVR increases skin pigmentation and has genotoxic effects, whereas prolonged exposure increases deleterious effects such as skin cancer (Agar and Young, 2005). In a recent study, tree-frog tadpoles exposed to UV-B irradiation showed morphological malformation (Schuch et al., 2015).

Interestingly, body darkness correlated with pigmentation in testes and heart, as well as dispersion of testicular melanocytes, but did not correlate with hepatic pigmentation. These differences may be explained by the different kinds of pigment cells present in the heart and testes versus those in the liver (Franco-Belussi et al., 2013). Visceral melanocytes on testes and heart originate from the ectodermal neural crest, similar to skin melanocytes (Zuasti et al., 1998, Colombo et al., 2011). However, hepatic melanomacrophages originate from hematopoietic stem cells (Zuasti et al., 1998, Colombo et al., 2011). Since melanocytes and melanomacrophages are distinct pigment cells, it is not surprising that they also show distinct responses to UVR.

The number of hepatic mast cells increased rapidly after UVR exposure (e.g. after 3 and 6 h). Similarly, the number of mast cells in the skin of mammals increases after UV-B exposure (Hart et al., 2000). Previous studies on mammals showed a peak of degranulation of mast cells within about 3–4 h of treatment and the authors suggested an indirect activation of cells from UV-B by isomerization of *trans*- to *cis*-urocanic acid (Hart et al., 2000). However, effects of UVR on mast cells in other tissues were not known. In this study, we describe for the first time the effects of UVR in hepatic mast cells in frogs. In fact, mast cells are found in many different tissues of frogs but their density varies (Baccari et al., 1998). In the heart of *Rana esculenta*, mast cells are located close to melanocytes and this association might indicate paracrine interactions between mast and melanocyte cells (Baccari et al., 1998). Here, we observed an increase in both mast and hepatic pigmented cells after UV exposure. Cutaneous melanocytes in mammals can potentially function as antigen-presenting cells based on their large and dendritic morphology and location in the skin (Plonka et al., 2009). If this is the case, then the increase in mast cells and increased dispersal of hepatic pigment cells may be explained by potential functional interactions between these cell types in the liver.

In conclusion, UVR exposure in anurans at doses that activate mast cells resulted in skin darkening and increased internal pigmentation in several organs, with an increase in melanin dispersion in hepatic melanomacrophages and testicular melanocytes as wells as increased melanin production in the liver. Only longer times of exposure caused genotoxic effects. This systemic response of internal melanin and pigment cells to external UVR is described here for the first time in vertebrates. Our results show effects of an external and environmentally relevant factor on internal coloration in frogs. The data in our study indicate that melanin in internal melanocytes as well as in internal melanomacrophages protects internal cells and organs against short-term external UVR exposure.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
L.F.-B. carried out the experiment, analyzed the data and wrote the manuscript. H.N.S. participated in the analyses and interpretation of data, and preparation of the manuscript. C.d.O. collected animals, participated in the analyses and interpretations of data, as well as commented on the manuscript.

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Supplementary information
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