LPS-induced alterations in reproductive organs and liver pigmentation in *Rhinella diptycha* (Bufonidae) toads"

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"LPS-induced alterations in reproductive organs and liver pigmentation in *Rhinella diptycha* (Bufonidae) toads"

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"LPS-induced alterations in reproductive organs and liver pigmentation in *Rhinella diptycha* (Bufonidae) toads"

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Abstract

Anurans can be naturally infected by *Escherichia coli* (Escherich 1885) bacteria, which contain lipopolysaccharides (LPS) in their external membrane. LPS affects reproductive functions in mammals and extracutaneous pigmentary system of anurans. Besides the functional gonad, Bufonidae amphibians have an undeveloped female organ (Bidder's organ), whose function is poorly known. We aimed to investigate the effects of LPS on testis and Bidder's organ germ cells and on liver pigmentation in *Rhinella diptycha* (Cope 1862) toads. Animals were inoculated with 0.1 mL of saline solution (control) or LPS solution (18 mg/kg) and then euthanized after 24 or 48 hours. The organs were processed for histology and then evaluated with morphometric analysis. The LPS decreased the area of bundle spermatozoa and increased lumen spermatozoa, while in Bidder's organ the number of degenerating oocytes decreased. Liver melanin increased after 24 hours and decreased after 48 hours. Our results suggest that LPS delays spermatozoa release, possibly due to alterations in serum levels of testosterone and/or gonadotropins, which may also explain fewer degrading cells in Bidder's organ. Increased melanin in the liver was expected due to the bactericidal role of this pigment. Thus, LPS affected reproductive organs and induced cytoprotective responses in the liver of amphibians.

Keywords: Amphibia; LPS infection; Spermatogenesis; Bidderian oogenesis; Melanomacrophages; *Rhinela diptycha*; Cururu toad.

Introduction

Lipopolysaccharide (LPS) is an endotoxin present in the external membrane of Gram-negative bacteria, such as *Escherichia coli*, and is pathogenic to other organisms during infection (Madigan and Martinko 2006). Anurans can be infected naturally by *E. coli* (Dipineto et al. 2010), and it is therefore important to understand the effects of this endotoxin on the health of these animals. In rodents, there are several studies evaluating the effects of LPS on systemic parameters, such as reproductive aspects (Kajihara et al. 2006; Reddy et al. 2006; Liew et al. 2007; Metukuri et al. 2010; Calejman et al. 2011; Collodel et al. 2012; Magata et al. 2014). However, only a few studies have investigated the effects of LPS on anurans to date (Franco-Belussi et al. 2013; Franco-Belussi et al. 2014; Cornuau et al. 2014).

In rabbits, treatment with LPS caused morphological lesions in testes and in ejaculated spermatozoa, indicating this endotoxin is a good model for studying effects of inflammation on spermatogenesis (Collodel et al. 2012). In rats, LPS can cause several testicular complications such as lower concentration and motility of spermatozoa (Kajihara et al. 2006), mitochondrial dysfunction and induction of apoptosis and/or other mechanisms of cell death (O'Bryan et al. 2000; Kajihara et al. 2006; Metukuri et al. 2010), hormonal alterations (Gow et al. 2001) and oxidative stress (Reddy et al. 2006). In amphibians, a long-term study in the urodele *Lissotriton helveticus* (Razoumovsky 1789) showed no effects of LPS on reproductive morphology or behavior (Cornuau et al. 2014). However, in anurans such as *Physalaemus nattereri* (Jan 1857), acute exposure to LPS altered testicular germ cell and locules volumes and also increased the number of testicular mast cells (Franco-Belussi et al. 2014) and superficial pigmentation of some organs (Franco-Belussi and De Oliveira 2011).

Anurans from the Bufonidae family are characterized by the presence of an undeveloped female organ called "Bidder's organ" in both sexes (Pough et al. 2001). Some authors have considered it to be a rudimentary ovary (Pough et al. 2001), but it is composed of cells with intense metabolic activity (Freitas et al. 2015) and responds to external stimuli (Brown et al. 2002; McCoy et al. 2008; Gregorio et al. 2016). The Bidder's organ also shows seasonal variation in many features such as estradiol production and its receptor expression (ERβ), aromatase activity and proliferation of follicular cells (Scaia et al. 2013; Scaia et al. 2016). Studies investigating Bidder's organ have increased in number recently, but its role is still not fully understood its role, and the effects of LPS infection on this organ have not been previously examined.

Amphibians and other cold-blooded animals have an extracutaneous pigmentary system composed of cells containing melanin, which occurs in several organs such as testis, kidney, lungs and liver (Gallone et al. 2002). In hematopoietic organs, pigmented cells called melanomacrophages have phagocytic activity (Agius 1980; Agius and Agbede 1984). These cells have cytoprotective roles (McGraw 2005) and are involved in defensive responses against bacterial compounds (Christiansen et al. 1996). Studies with *P. nattereri* exposed to LPS showed increased the melanin-pigmented area in histological sections of liver and testis, as well as on the surface of testis, heart and kidneys, probably due to the bactericidal properties of melanin (Franco-Belussi and De Oliveira, 2011; Franco-Belussi et al. 2013). These results suggest that the pigmented cells responses can be used as biomarkers of environmental contamination, including contamination by bacterial LPS (De Oliveira et al. 2017).

The aim of this study was to investigate the effect of *E. coli* LPS on testis and Bidder's organs germ cells and also on liver pigmentation in the toad *Rhinella diptycha*, using morphometric analyses. We expected that LPS would cause changes in the testis, compromising the germ cells, since it was shown to affect testicular morphology in several animals. This in turn could indirectly affect the development of Bidderian oocytes. We also hypothesized that the melanin pigmented area in the liver would increase after LPS treatment, due to the cytoprotective role of melanin and melanomacrophages.

Materials and Methodos

Animals

We collected 15 *R. diptycha* toads as they were vocalizing during their reproductive activity period in the summer of 2011 from different populations of southeast Brazil (20°47′07.05″S; 49°02′42.09″W). This species is distributed throughout South America (Frost, 2017), and is very common in the region used for this study. The animals were kept in pairs or trios in terraria (104 × 50 × 40 cm – length × width × height, respectively) containing approximately 5 cm of soil at the bottom and water containers, with food supplied twice a week. The animals were maintained in a covered external environment for seven days for acclimation, away from direct light and rain, with a photoperiod of approximately 14:10h light:dark and day-time temperature of 27±0.5ºC, similar to their natural environmental conditions.

Animals were collected under license Nº 18573-1, authorized by the Brazilian Institute of Environment and Renewable Natural Resources. The experimental design was approved by the Committee on Ethics and Animal Experimentation of the São Paulo State University (CEUA-IBILCE/UNESP #038/2011), following the recommendations of the

Guide for the Care and Use of Laboratory Animals (National Research Council Committee – US).

Experimental Design

Animals in the treatment groups (*N*=5 each) received an intraperitoneal injection of 18 mg per kg body weight of *E. coli* LPS (Serotype 0127:B8; Sigma, St. Louis, MO), diluted in 0.4 mL of sterile physiological solution with osmolarity adjusted for amphibians (60% of mammals), according to the procedure adapted from Flores-Quintana and Moraes (2001). The treated animals were euthanized 24 or 48 hours after exposure to LPS (hereafter "LPS24h" and "LPS48h" groups, respectively). The control group (*N*=5) received an injection of sterile physiological solution only, at the same 0.4 mL volume, and was euthanized after the LPS24h group. The concentration and experiment times were based on pilot tests conducted by Franco-Belussi and De Oliveira (2011), aiming to evaluate the acute systemic effects of LPS in *P. nattereri*, which showed fast responses. The euthanasia procedure was made by deep anesthesia using benzocaine (5 g/L) and then the animals were weighed and dissected to collect testis, Bidder's organ and liver samples. The organs were also weighed using a precision analytical balance (0.001 g readability) to determine the viscero-somatic index (organ weigh ÷ body weight).

Histological Procedures

The testis, Bidder's organ and liver were fixed in Karnovsky solution (5% paraformaldehyde and 2.5% glutaraldehyde in Sörensen's phosphate buffer 0.1M, pH 7.2) for 24 hours, embedded in historein (Leica Microsystems, Wetzlar, Germany) and then sectioned in the medial portion (3 µm thickness) using a microtome (RM 2265, Leica

Microsystems, Heerbrugg, Switzerland). The sections were dehydrated in a series of alcohol concentrations and then stained with Hematoxilin-Eosin (H/E) to quantify the germ cells of testis and Bidder's organ and melanin area in the liver.

Morphometric Analysis

Twenty-five histological fields were examined for each animal to quantify testicular germ cells area (20x objective lens), Bidderian oocytes count (10x objective lens) and liver melanin pigmented area (20x objective lens). In the testis, we quantified the area corresponding to primary and secondary spermatogonia, primary and secondary spermatocytes, and primary and secondary spermatids, which were all grouped together as "Cystic Germ Cells". Spermatozoa were classified as either bundles or in the lumen, according to their condition inside the seminiferous locules. The locular area was also measured and the total locular area per image was subtracted from the total histological field area to estimate the interstitial area (region between the locules). Bidderian oocytes were classified and counted according to Freitas et al. (2015). We quantified diplotene oocytes (including initial and final stages) and degenerating/atretic oocytes in the Bidder's organ, and calculated their proportions (%) in the tissue. Oogonia were not counted due to their small size, as they were barely visible at the magnification used (10x). In the liver, the melanin pigmented area was measured by color difference, since brown coloration of melanin contrasts with non-pigmented tissue (pink and purple due to H/E staining). All images were captured using light microscopy (Leica DM4000 B) and an image capture system (Leica DFC280). Quantifications were performed using Image Pro Plus v6.0 software (Media Cybernetics Inc., Silver Spring, MD, USA).

Statistical Analysis

The viscero-somatic indices fitted the assumptions of normality and homogeneity and were tested using One-Way ANOVA. To analyze the testicular germ cell results, data modeling was performed using the Generalized Linear Mixed Model (GLMM), molding the response variable (testicular germ cells) by the treatment of the animal as the intercept, and using the *beta* distribution. Data for Bidder's organ germ cells (response variable) were analyzed using the Generalized Linear Model (GLM), with the proportion of each cell affected by treatment and, in this case, with the *binomial* distribution. Results for the melanin pigmentation of the liver was also analyzed with GLM, with the response variable (melanin area) transformed by log, molded by the treatment, using *Gaussian* distribution.

We tested the assumptions of all models using diagnostic plots from *sjplot* package of R software (R Core Team, 2016). The residuals showed variance homogeneity and normal distribution in all cases.

Results

The testis, Bidder's organ and liver were located in the abdominal cavity of the animals. Bidder's organ was found in all animals, positioned at the anterior part of the testis, which is located over the kidney (Figure 1A). The liver was divided into three lobes, with the gallbladder positioned between them, and was located above the stomach and below the heart and the lungs (Figure 1B). No macroscopic alterations were observed after LPS treatment in any organ analyzed. There were also no alterations in their viscerosomatic indices in any time of exposure (Table 1, Figure 2). However, the morphometric parameters of testis, Bidder's organ and liver were altered 24 and/or 48 hours after LPS treatment.

In the testis, treatment with LPS affected significantly the area of bundle and lumen spermatozoa (Table 2, Figure 3). At both 24 and 48 hours after exposure, there was an increase in bundle spermatozoa area and a decrease in lumen spermatozoa area, while the cystic germ cells were unaffected at either time-point. At 48 hours, the locular area was also decreased, consequently increasing the interstitial region (Table 2, Figure 4).

Bidderian oocytes were also affected by LPS, showing alterations at both time-points (Table 3, Figure 5). The counts of each oocyte stage are complementary and inversely correlated, since the data are relative and always amount to 100%. At 24 hours after treatment, there was an increase of 4.56% in the proportion of diplotene oocytes compared to the control group, with a corresponding decrease in degenerating/atretic cells. After 48 hours, an 11.03% increase was observed in diplotene oocytes, and a decrease in degenerating/atretic oocytes compared to control group.

In the liver, the melanin-pigmented area was also altered after both 24 and 48 hours post-LPS exposure (Table 4, Figure 6). In the control group, the mean pigmented area corresponded to 0.44% of the tissue. After 24 hours, this mean area increased to 0.69% (an increase of 0.25%). In contrast, a slight decrease of 0.06% was observed in liver pigmentation after 48 hours, so the mean melanin area corresponded to 0.39% of the liver tissue at this time-point.

Discussion

Our results showed that both reproductive organs (testis and Bidder's organ) were affected by *E. coli* LPS treatment, with the more pronounced responses occurring at 48 hours after injection. The mechanisms triggered by LPS infection were not evaluated in this study, but this compound is possibly affecting pathways that act inversely in testis and

Bidder's organ, since the treatment caused testis impairment and Bidder's organ stimulation.

In the testis, the increase in bundle spermatozoa and the decrease in lumen spermatozoa indicate a delay in the process of spermatozoa release. Some studies with rats showed that acute exposure to LPS can cause a decrease in serum testosterone and interstitial fluid (O'Bryan et al. 2000; Gow et al. 2001; Reddy et al. 2006; Zhan et al. 2014), . Luteinizing hormone (LH) secretion has been reported to be affected by LPS in rats (O'Bryan et al. 2000; Gow et al. 2001; Herman et al. 2010; Zhan et al. 2014) as well as follicle-stimulating hormone (FSH) (Zhan et al. 2014). Gonadotropins and steroid hormones play an important role in spermatogenesis in anurans: androgens are important in several reproductive aspects, such as spermatid differentiation, and gonadotropins are important in spermiation induction, with FSH being the most effective hormone involved in this process (Norris and Lopes 2011). Thus, LPS may affect these hormonal levels in anurans in a way similar to rodents, affecting the spermatogenetic process and possibly causing the delay in spermatozoa release in *R. diptycha* testis.

At 48 hours after LPS injection, the locular area decreased with a corresponding increase in the interstitial area. The reduction of spermatozoa area in the lumen is probably one of the causes of the locular decrease, since these cells occupied a large area of the tissue in the control group. In *P. nattereri*, the same LPS dosage used in this study caused a decrease in the locular and germ cell areas at 12 hours after exposure (Franco-Belussi et al. 2014). In rats, LPS exposure can also cause the death of germ cells by apoptosis or other mechanisms (O'Bryan et al. 2000; Kajihara et al. 2006; Metukuri et al. 2010), which could contribute to the observed decrease in locular area. The size of the locules is directly related to germ cell production, since this is where the germ epithelium is located, surrounded by

connective tissue (De Oliveira et al. 2005). Thus, the decrease in locular area may be a result of the observed decrease in the release of spermatozoa.

In the Bidder's organ, LPS caused an increase in the proportion of diplotene oocytes, and a decrease in degenerating oocytes. Previous studies have shown that testis removal and suppression of testicular function both induce the development of Bidderian oocytes, even though the organ did not become a functional gonad (Pancak-Roessler and Norris 1991). It was also observed in seasonal studies that Bidder's organ volume increased during non-reproductive periods when testosterone levels were lower in males, with an inverse decrease in volume observed during the reproductive period (Calisi 2005), showing the negative influence of testosterone on Bidder's organ development. We hypothesize that LPS is possibly decreasing androgens and gonadotropins levels, which could explain the delay in spermatozoa release and the further development of oocytes in the Bidder's organ, reducing the proportion of degenerating cells.

In addition, studies in several species have shown that steroidogenic enzymes are present in this organ, converting androgens to estrogens (Colombo and Colombo-Belvedere 1980; Gosh et al. 1982; Pancak-Roessler and Norris 1991; Scaia et al. 2011), suggesting that Bidder's organ may not be just a rudimentary organ. In agreement with the previous studies, the decreased amount of degenerated oocytes after LPS exposure observed in our study is another indicator that Bidder's organ can be stimulated by external factors. Rudimentary organs or structures are often nonfunctional and/or underdeveloped, as observed, for example, in human outer ears muscles, which are not functional for us as in other mammals, and reduction of our tail bones (coccyx). Therefore, we believe it is inappropriate to regard Bidder's organ as a rudimentary structure because, in addition to

being present in all the animals used in this study with well-developed cells, it was also responsive to LPS inoculation, which delayed the oocyte degradation.

In the liver, LPS caused an increase in melanin pigmentation of melanomacrophages 24 hours after treatment, but pigmentation decreased after 48 hours. Melanomacrophages are associated with the phagocytosis of cellular material originating from catabolism (Ellis et al. 1976) and they also play a role in detoxification processes (Herráez and Zapata 1986). It is known that LPS can stimulate the formation of lipid peroxidation products, inducing oxidative stress (Nowak et al. 1993; Portolés et al. 1993) and leading to the production of free radicals (Shimamoto et al. 2011), which are neutralized by melanin (Zuasti et al. 1989; Agius and Roberts 2003). These processes can justify, at least in part, this initial increase in liver pigmentation. Moreover, melanin has been reported to have a bactericidal role in ectothermic vertebrates (Christiansen et al. 1996). Consistent with our results, Franco-Belussi et al. (2013) showed a similar increase in the melanin area of the liver in the anuran *P. nattereri* 24 hours after exposure to LPS. Thus, hepatic melanin was expected to increase due to the defensive role of this substance, in particular against bacterial infections.

In contrast, a decline in the pigmented area was observed 48 hours after inoculation. Recently, melanomacrophages have been shown to be involved in engulfment of basophils, which in turn would affect the production of melanin since the histamine secreted by basophils stimulates melanogenesis (Gutierre et al. 2017). In addition, the liver is considered to be the main organ responsible for metabolism (Saleh 1982), so it may respond rapidly to external stimuli. Thus, the liver melanomacrophages showed a rapid response against the damage caused by LPS exposure, which is the probable cause of the slight decrease in melanin pigmentation observed after 48 hours.

In conclusion, this study showed that LPS compromises testicular germ cells, in particular by delaying the liberation of spermatozoa, as well as stimulating Bidder's organ and liver melanin pigmentation. The testicular impairment likely occurred due to hormonal alterations by LPS, while the lower proportion of degrading cells in Bidder's organ, is probably an indirect effect. The integrity of reproductive organs is fundamental for the maintenance of a species, so it is important to conduct further investigation with environmentally- relevant doses of LPS to investigate if it could compromise the reproductive performance of *R. diptycha* males in a real situation. In addition, the rapid increase in liver pigmentation suggests that this parameter is a good bioindicator of acute contamination by LPS, and this also corroborates the importance of melanomacrophages in defending against bacterial infections. In general, our results showed that *R. diptycha* anurans are sensitive to contamination by *E. coli* LPS, interfering with their reproduction and health, but more studies are needed to investigate the mechanisms involved and other systemic effects of this endotoxin in this species.

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Tables

Table 1. Statistical results for viscero-somatic indices of testis, Bidder's organ (log_{10}) and liver. No statistical difference ($p< 0.05$) from the control group was observed.

	Pearson x^2 Normality Test		Bartlett Test of Homogeneity of Variances		One-Way ANOVA Variance Test	
	P	***p***	**K^2**	***p***	**F**	***p***
Testis	1.7	0.34	5.79	0.35	0.78	0.52
Bidder's organ	5.2	0.27	0.10	0.99	0.35	0.79
Liver	1.7	0.66	5.79	0.20	1.08	0.38

Table 2. Statistical results for testis data, analyzed with GLMm and *beta* distribution. Statistical difference ($p< 0.05$) is indicated by an asterisk (*).

Testicular Area	Comparison	Contrast	Estimate	z ratio	*p* value
Cystic Cells	Control - LPS24h	0.13	0.10	1.31	0.56
	Control - LPS48h	-0.13	0.10	-1.29	0.57
Bundle Spermatozoa	Control - LPS24h	-0.02	0.07	-0.30	0.02 *
	Control - LPS48h	-0.21	0.07	-3.25	0.01 *
Lumen Spermatozoa	Control - LPS24h	-0.02	0.07	-0.30	0.02 *
	Control - LPS48h	-0.21	0.07	-3.25	0.01 *
Locular Area	Control - LPS24h	-0.003	0.06	-0.06	1.00
	Control - LPS48h	0.26	0.06	4.34	<0.01 *
Interstitial Area	Control - LPS24h	-0.003	0.06	0.06	1.00
	Control - LPS48h	0.26	0.06	-4.34	<0.01 *

Table 3. Statistical results for Bidder's organ germ cells, analyzed with GLM and *binomial* distribution. Statistical difference ($p < 0.05$) is indicated by an asterisk (*).

Germ Cell Proportion	Comparison	Contrast	Estimate	z ratio	*p* value
Diplotene Oocytes	Control - LPS24h	-0.10	0.04	-2.65	0.04 *
	Control - LPS48h	-0.14	0.04	-3.67	<0.01 *
Degenerating Oocytes	Control - LPS24h	0.39	0.06	6.24	<0.01 *
	Control - LPS48h	0.62	0.07	9.17	<0.01 *

Table 4. Statistical results for melanin area in liver, analyzed with GLM and *gaussian* distribution. Statistical difference ($p < 0.05$) is indicated by an asterisk (*).

Pigment Area	Comparison	Contrast	Estimate	z ratio	*p* value
Melanin	Control - LPS24h	-0.34	0.09	-3.79	<0.01 *
	Control - LPS48h	0.27	0.09	3.00	0.02 *

Figures Captions

Figure 1. Abdominal cavity of male *R. diptycha* showing **A.** the testis (T) over the kidney (K), with the Bidder's organ (Bi) in its anterior portion and **B.** the lobed liver (Li) with the gallbladder (Gb) between the lobes, bellow the heart (H) and the lungs (Lu). Scale bar: 0,5 cm (A) and 1 cm (B).

Figure 2. Testis, Bidder's organ and liver viscerosomatic indices in control and treated groups. No significant difference was observed. Mean ± SE.

Figure 3. A. Testicular germ cells areas from control and treated groups. The asterisk (*) indicates significant difference from the control group. Mean ± SE. **B-I.** Germ epithelium of *R. diptycha* testis: primary (**B**) and secondary spermatogonia (**C**), primary (**D**) and secondary spermatocytes (**E**), primary (**F**) and secondary espermatids (**G**), bundle spermatozoa (**H**) and lumen spermatozoa (**I**). Staining: Hematoxylin-Eosin. Scale bar: 5 µm.

Figure 4. A. Testis locular and interstitial areas from control and treated groups. The asterisk (*) indicates significant difference from the control group. Mean ± SE. **B.** Testis histological section showing the locular (surrounded) and interstitial (arrow) areas. Staining: Hematoxylin-Eosin. Scale bar: 25 µm.

Figure 5. A. Bidderian germ cells from control and treated groups. The asterisk (*) indicates significant difference from the control group. Mean ± SE. **B.** Histological section of Bidder organ showing the cortical region (C) with oocytes and the medular region (M) with somatic cells. **C-F.** Counted germ cells: Initial diplotene oocytes (**C**), final diplotene

oocytes (**D**), degenerating oocytes (**E**) and atretic oocytes (**F**). Staining: Hematoxylin-Eosin. Scale bar: 50 µm (B), 5 µm (C, D and F) and 10 µm (E).

Figure 6. A. Melanin pigmented area in the liver from control and treated groups. The asterisk (*) indicates significant difference from the control group. Mean ± SE. **B-D.** Histological sections of *R. diptycha* liver representing the mean pigmented area from control (**B**), LPS24h (**C**) and LPS48h (**D**) groups, showing the melanomacrophages containing melanin (arrow). Staining: Hematoxylin-Eosin. Scale bar: 25 µm.

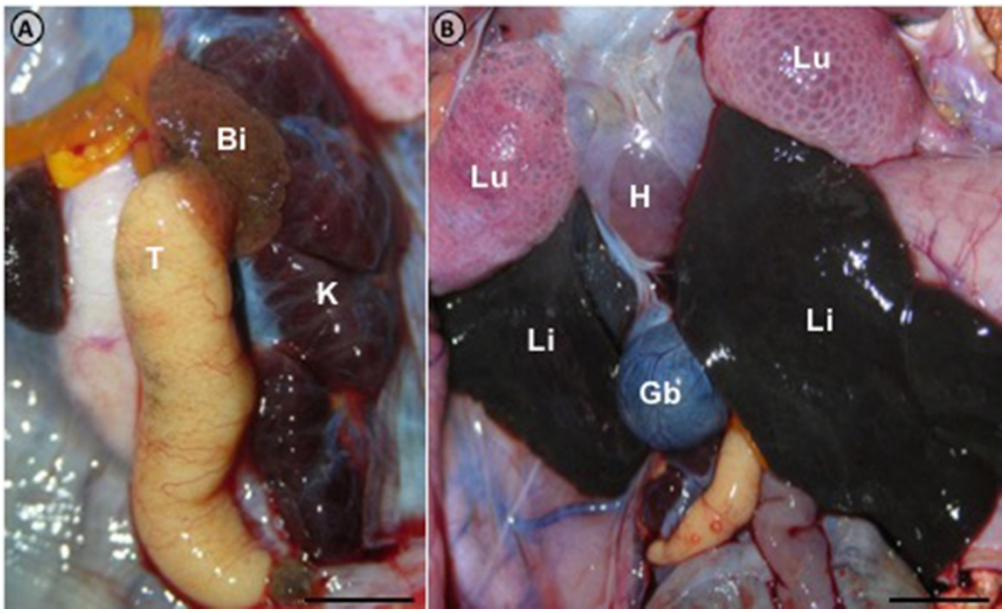

Figure 1. Abdominal cavity of male R. diptycha showing A. the testis (T) over the kidney (K), with the Bidder's organ (Bi) in its anterior portion and B. the lobed liver (Li) with the gallbladder (Gb) between the lobes, bellow the heart (H) and the lungs (Lu). Scale bar: 0,5 cm (A) and 1 cm (B).

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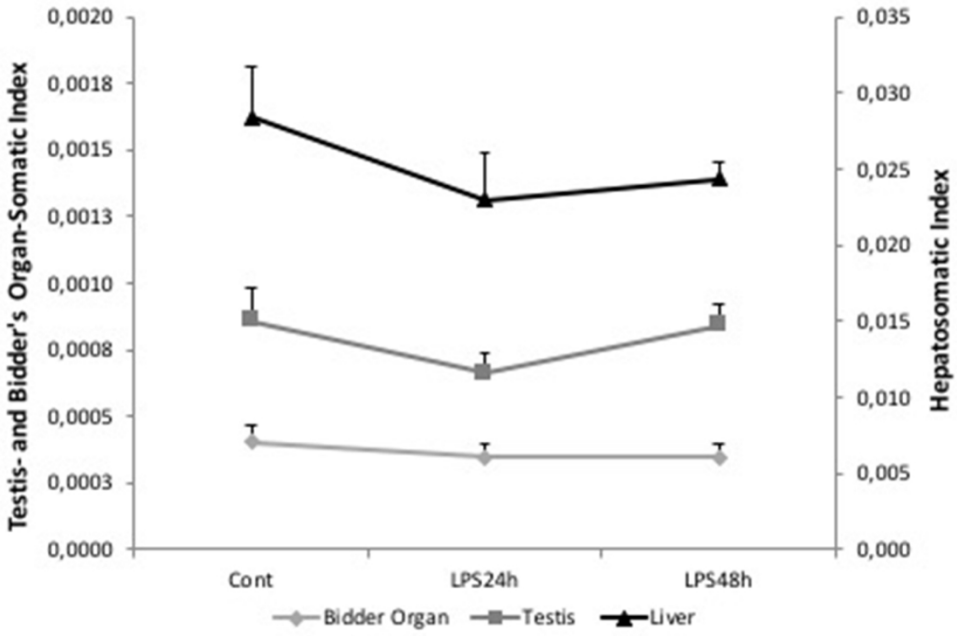

Figure 2. Testis, Bidder's organ and liver viscerosomatic indices in control and treated groups. No significant difference was observed. Mean ± SE.

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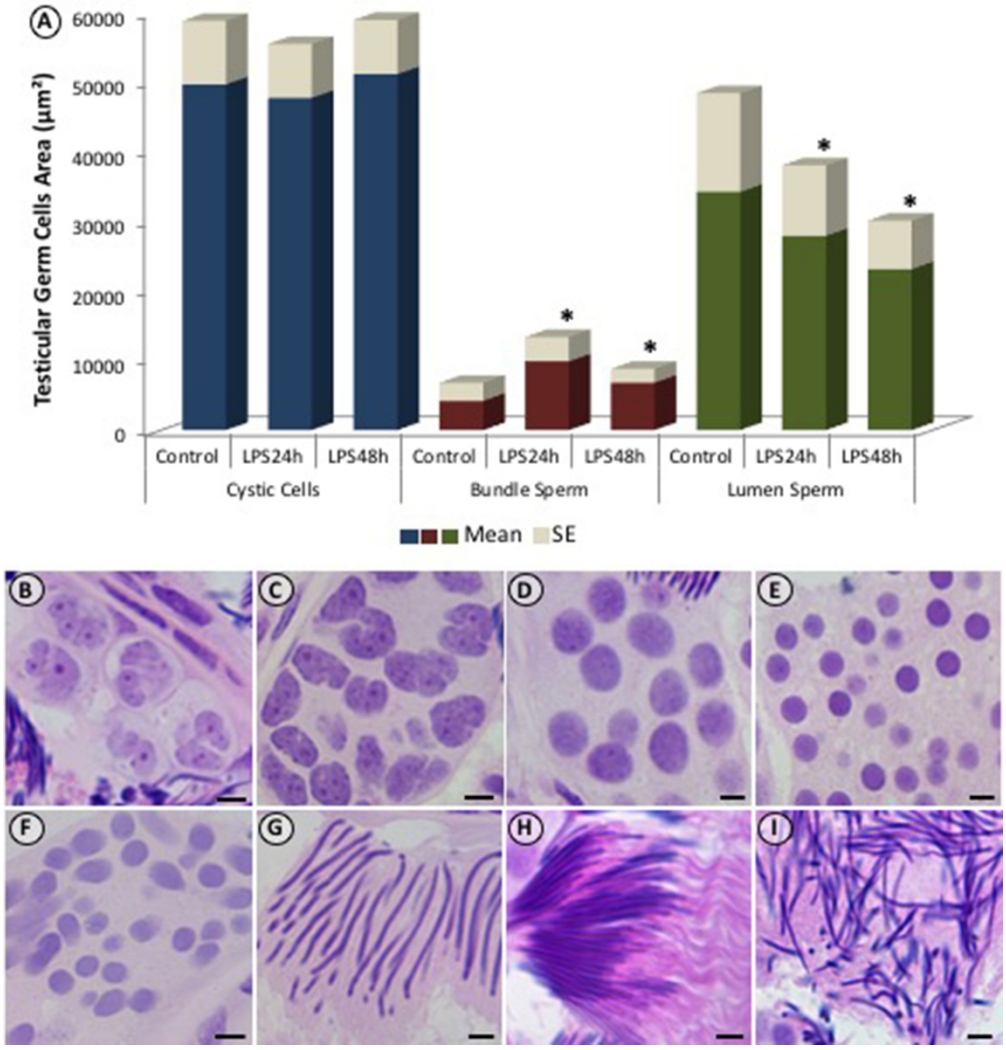

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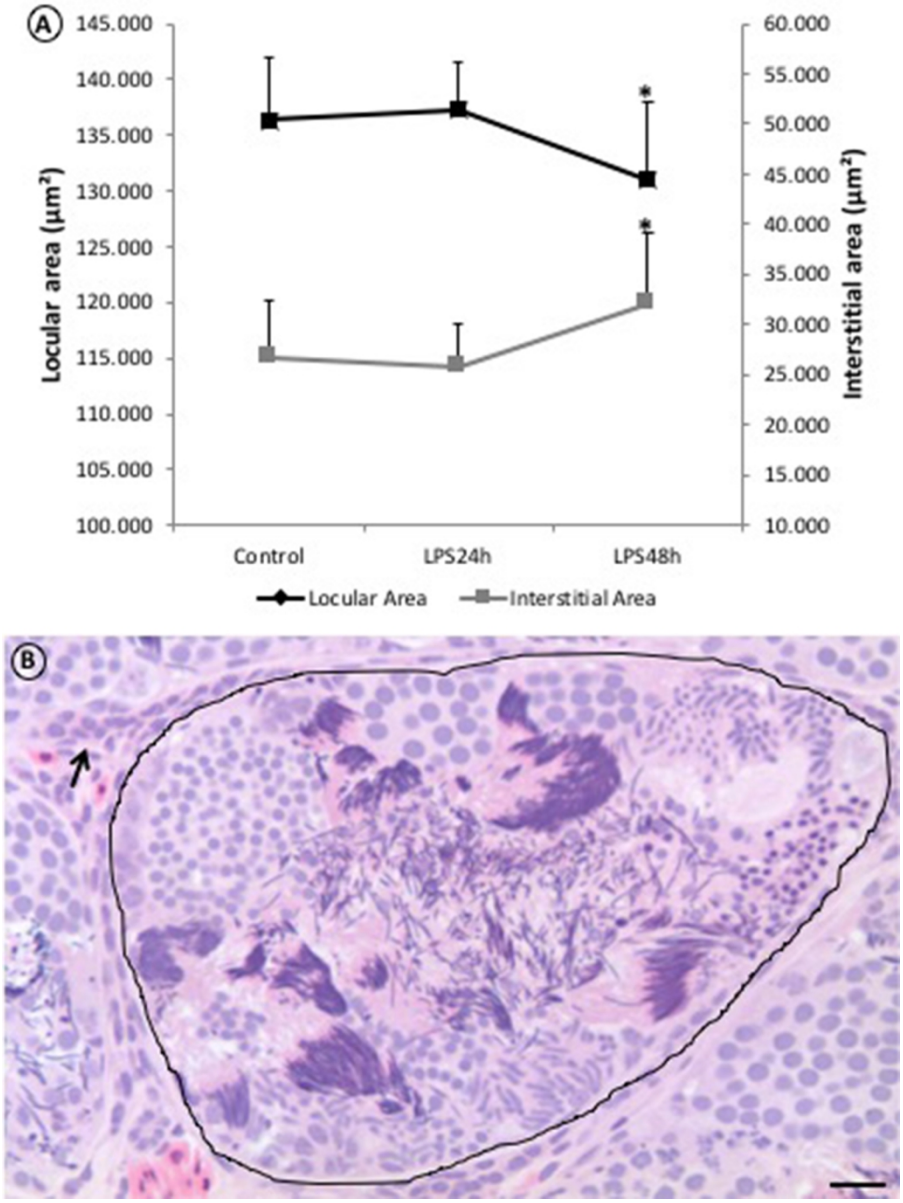

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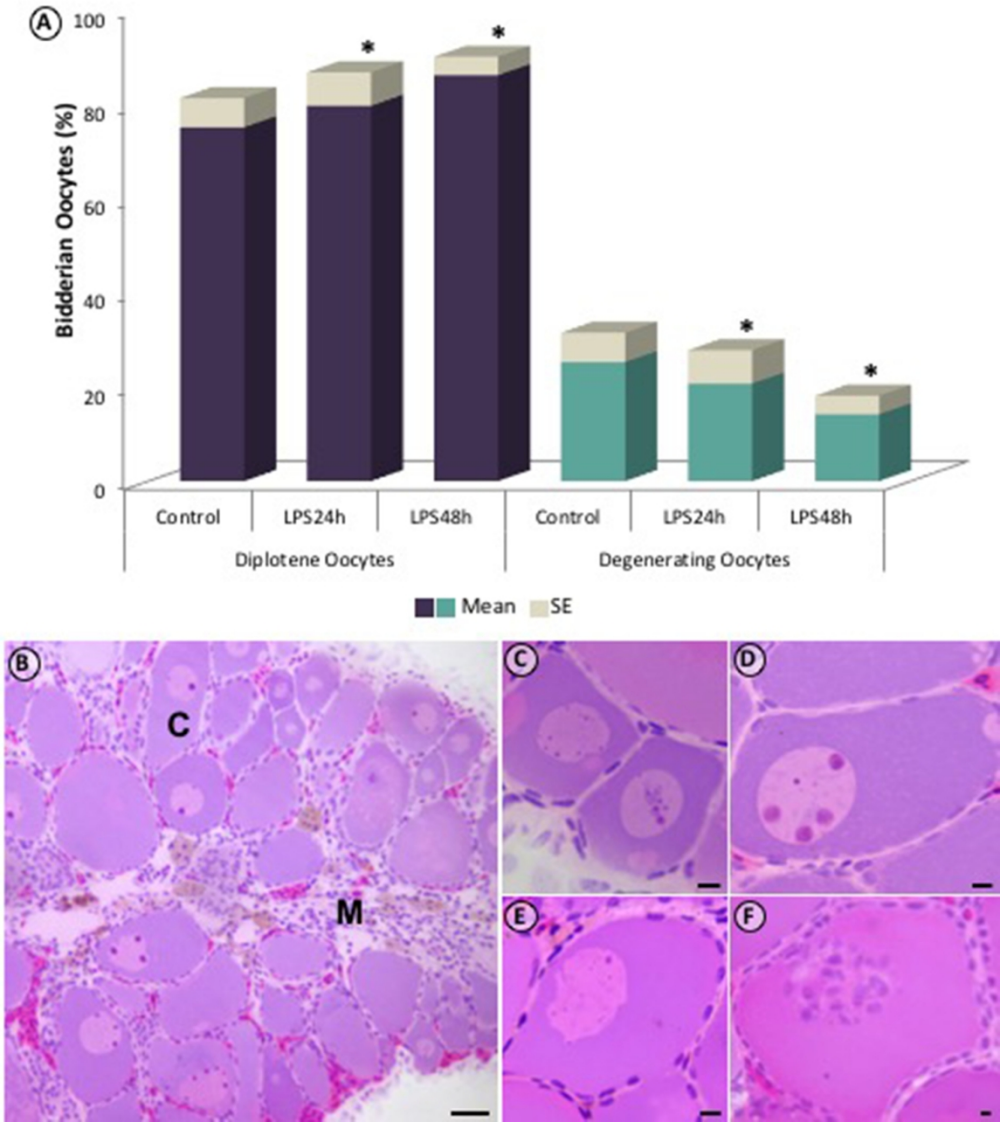

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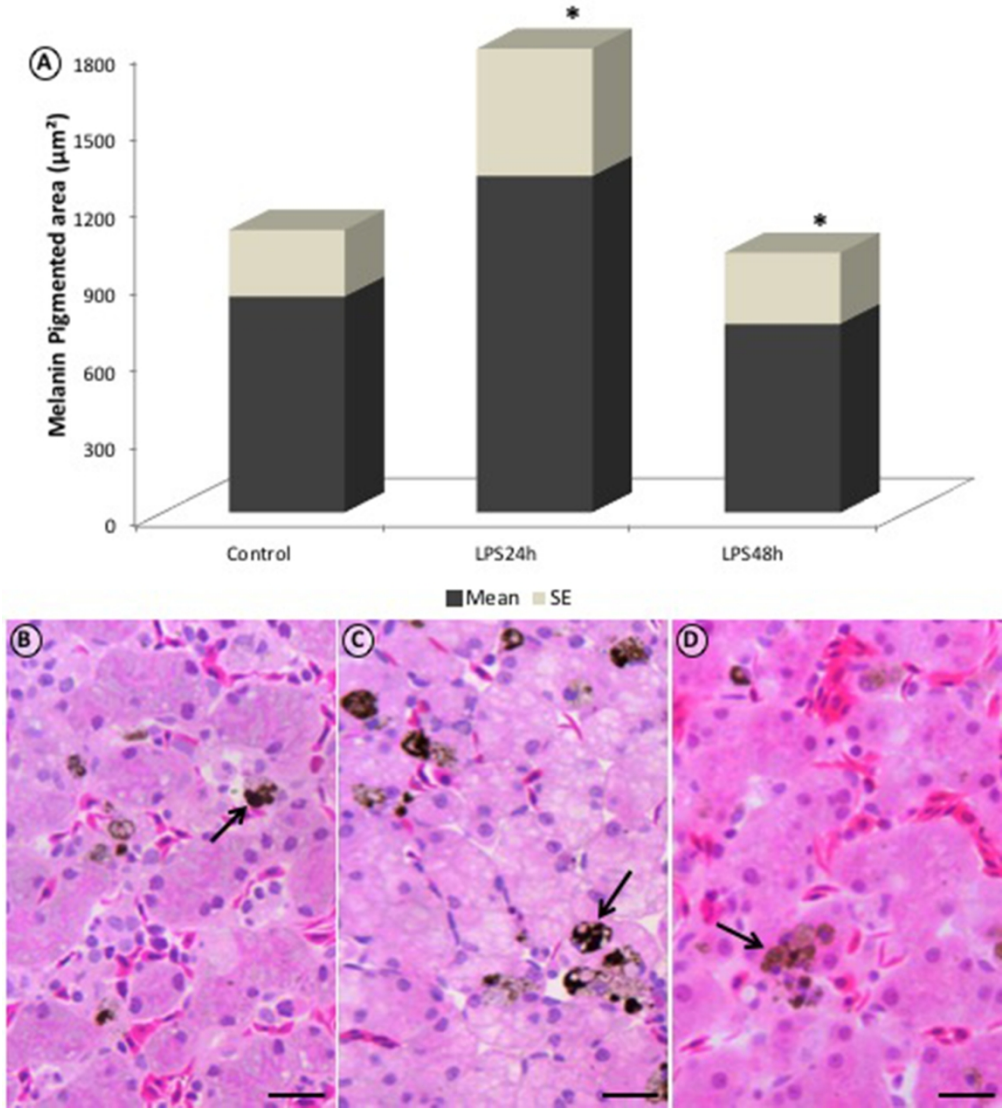

Figure 6. A. Melanin pigmented area in the liver from control and treated groups. The asterisk (*) indicates significant difference from the control group. Mean ± SE. B-D. Histological sections of R. diptycha liver representing the mean pigmented area from control (B), LPS24h (C) and LPS48h (D) groups, showing the melanomacrophages containing melanin (arrow). Staining: Hematoxylin-Eosin. Scale bar: 25 µm.

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