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Effects of experimental lead exposure on testis of the Chestnut Capped Blackbird *Chrysomus ruficapillus*

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Abstract

Lead (Pb) effects on testis histology, as well as sperm quality and oxidative status were evaluated in male Chestnut Capped Blackbird (*Chrysomus ruficapillus*). Wild blackbirds were captured, immediately sampled (field group) or kept in captivity and treated with a single intraperitoneal injection of saline solution (control) or saline solution with Pb acetate (50 or 100 mg/Kg Pb). Seven days after injection, whole blood, ductus deferens and testis samples were collected. Increased Pb concentrations were observed in whole blood and testis of Pb-exposed blackbirds with respect to those from field and control blackbirds. Sperm cells of Pb-exposed blackbirds showed loss of membrane integrity, mitochondrial functionality, and DNA integrity. Also, oxidative damage was observed in testis of blackbirds injected with 100 mg/Kg Pb. These findings indicate that Pb is accumulated in testis of *C. ruficapillius*, inducing severe morphological and biochemical injury that can compromise the reproductive performance of male blackbirds. Although the exposure scenario (Pb acetate, high dosage and intraperitoneal injection) tested in the present study would likely not occur in the wild, it was adequate to show potential and relevant toxic effects of Pb in wild birds.

Keywords: blackbird; histopathology; lead toxicity; reproduction; sperm.

Introduction

Uncontrolled discharge of lead (Pb) in atmospheric, aquatic and terrestrial ecosystems represents a global issue. In fact, Pb is the second chemical listed among the 275 most dangerous substances in the Priority List of Hazardous Substances (ATSDR 2017). Continuous environmental and occupational exposure to Pb can cause adverse effects, including toxicity to the nervous, excretory, cardiovascular, hepatic, hematological and reproductive systems in both humans and animals (Burger and Gochfed 2004). In mammals and birds, adverse effects of Pb exposure on male fertility can result from its direct interaction with the reproductive organs and/or endocrine system. They include changes in sperm motility and maturation, reduced spermatogenesis and testosterone level, as well as disturbances of other functions, depending on the integrity of the male reproductive system (Almansour 2009; Garu et al. 2011).

Despite the large number of studies describing the harmful effects of Pb on animal reproduction, there are only few studies reporting the toxic effects of this heavy metal on the reproductive system of wild birds (Dauwe et al. 2004). Furthermore, results from studies with free-living animals are more difficult to be interpreted than those arising from experimental

studies (Włostowski et al. 2010). In this context, blackbirds have been used as biomonitors of environmental contamination with organic pollutants and heavy metals (Hong et al. 2014). They are omnivorous birds, sensitive to environmental stressors, and have been exposed to several chemical pollutants, including Pb (Furness and Greenwood 1993). In fact, increased levels of blood Pb were reported in blackbirds, especially in individuals collected in heavily polluted areas (Tsipoura et al. 2008; Chapa-Vargas et al. 2010; Coeurdassier et al. 2012; Beyer et al. 2013). Therefore, there is a need for evaluating the toxic effects of Pb contamination in blackbirds for a better understanding of the potential risks to the health of these animals, especially those associated with the reproductive system.

The Chestnut Capped Blackbird *Chrysomus ruficapillus*, hereafter referred as blackbird, is widely distributed in South America. It is found in a variety of habitats, including natural marshes, reed beds, roadside ditches, sewage treatment sites, small eucalyptus plantations, and farmlands. In rice fields, populations of blackbirds are expanding. This expansion is associated with the protection, food availability, and optimal conditions for reproduction provided by this habitat (Cirne and López-Iborra 2005).

In light of the background described above, the aim of the present study was to evaluate the effects of experimental Pb exposure on reproductive parameters of male blackbirds. It is worth noting that the chemical form (Pb acetate; high Pb bioavailability), via of exposure (single intraperitoneal injection; high Pb absorption and tissue distribution) and doses (50 and 100 mg Pb/Kg) used in the present study allowed us to achieve experimentally levels of Pb in the blood of blackbirds similar to those found in several species of wild birds (Franson and Pain 2011; Carvalho et al. 2013; McDowell et al. 2015; Riecke et al. 2015), including blackbirds (Tsipoura et al. 2008). Therefore, results reported in the present study have great environmental relevance.

Materials and Methods

Experiments performed in the present study were approved by the Ethics Committee of the Federal University of Rio Grande (license #23116.006225/2011-39). Adult males *C. ruficapillus* (wet body mass: 36.1 ± 2.79 g; n = 50) were captured in the field during the reproductive period (October) using mist nets (ICMBio, Brazilian Ministry of Environment, license #30228-1). The use of mist nets is legal internationally, including in Brazil, since *C. ruficapillus* is a bird species not listed in the IUCN red list of threatened species. Five male blackbirds were randomly sampled immediately after being captured in the field (field group: n = 5). Blood samples were collected by puncture of the tarsal vein using disposable syringes and needles. Blackbirds were then euthanized by cervical dislocation, following the 2007 Report of the AVMA Panel on Euthanasia, and had their testes collected via laparotomy. Whole blood and testis samples were immediately stored a -80^{o}C until analysis. The other blackbirds collected (n = 45) were transferred to the laboratory, kept in captivity, experimentally treated with Pb and sampled, as described below.

In laboratory, blackbirds were kept in cages (2 birds/m^2) with a covered floor to avoid contamination from soil. Cages were provided with shrubs and trees, shade area, water mirror and feeder, following the criteria established by the Federal Normative Instruction 04/2002 issued by the Brazilian Institute for Environment (IBAMA). Every day, food and water were completely renewed over the whole experimental period (7 days). Blackbirds kept in captivity were randomly divided into three groups: (1) blackbirds treated with a single intraperitoneal (IP) injection (1 mL) of saline (0.9% NaCl) solution (control group; n = 12); (2) blackbirds treated with a single IP injection (1 mL) of saline (0.9% NaCl) solution containing Pb acetate (dose = 50 mg/Kg Pb; n = 15); and (3) blackbirds treated with a single IP injection (1 mL) of saline (0.9% NaCl) solution containing Pb acetate (dose = 100 mg/Kg Pb; n = 18), following procedures described by Burger and Gochfeld (2005).

Seven days after treatment, blackbirds were euthanized, as described above for the field

group. Ductus deferens and testes were collected via laparotomy. Isolated ductus deferens were sectioned longitudinally, individually immersed in saline solution, and kept at room temperature (~20ºC) for 10 min to allow sperm migration to the incubation medium. Seminal quality parameters were evaluated immediately after semen dilution, as described below. The right testis was dissected and fixed in 4% paraformaldehyde for histological analysis, as described below. The left testis was dissected and immediately divided into three subsamples. One subsample was kept on ice (2-4ºC) for reactive oxygen species (ROS) and antioxidant capacity against peroxyl radicals (ACAP) analysis, as described below. The other two subsamples were stored in ultrafreezer (-80°C) for further analyses of lipid peroxidation (LPO) and Pb content, as described below.

Quality parameters were assessed in 200 sperm cells per slide. Samples were analyzed under an epifluorescence microscope (400x magnification; Olympus BX 51, América, São Paulo, SP). Plasma membrane integrity was evaluated using fluorescent probes (carboxyfluorescein diacetate and propidium iodide; Sigma, St. Louis, MO, USA), as described by Harrison and Vickers (1990). Mitochondrial functionality was assessed after cell staining with 0.2 mM rhodamine 123 (Rh123; Sigma, St. Louis, MO, USA) and 7.3 mM propidium iodide, according to Mclean et al. (1993). Sperm cell DNA integrity was assessed after cell staining with acridine orange (Sigma, St. Louis, MO, USA) in dried slides. Sperm cells exhibiting green fluorescence were considered as being normal cells (bicatenary DNA) whereas red- or yellow-stained sperm cells were considered as being abnormal cells, with denatured DNA (monocatenary DNA). Data were expressed as percentage of sperm cells showing DNA integrity with respect to the total number of cells analyzed.

Testes were fixed in 4% paraformaldehyde for 2 h and processed using an automated vacuum processor (ASP 200, Leica, Germany), following standard histological techniques. Samples were impregnated and embedded in ParaplastXtra® (Sigma, St. Louis, MO, USA). Glass slides with tissue sections (3-µm thickness) were stained with hematoxylin and eosin (Carson and Hladik 2009). Histological analysis was performed under bright-field light microscope (model BX51) equipped with a digital camera (model DP73) (Olympus, Japan). For all samples, histological alterations were quantified based on blind analysis. Abundance or absence of the different cell types in the seminiferous epithelium was scored.

Regarding oxidative status parameters, ROS measurements were performed in supernatants of sample homogenates using the fluorescent 2',7'-dichlorofluorescein diacetate (H_2DCF-DA; Molecular Probes, USA). Fluorescence reading was performed every 5 min up to 60 min using a fluorescence microplate reader (Victor2, Perkin-Elmer, USA). Fluorescence area was calculated integrating fluorescence units (FU) over time after adjusting data to a second order polynomial function. ROS content was expressed as FU per milligram protein in the supernatant of the sample homogenized (Myhre and Fonnum 2001). ACAP was also determined in supernatants of sample homogenates. Measurements were performed following procedures described by Amado et al. (2009). Readings were done every 5 min up to 60 min using the fluorescence microplate reader (Victor2, Perkin-Elmer, USA). FU were integrated over time after adjusting data to a second order polynomial function. Results were calculated as the difference in FU area per minute in aliquots of the same sample tested in the presence or the absence of 2',2'- azobis (2-methylpropionamidine) dihydrochloride (ABAP), and then normalized considering the ROS area obtained in the absence of ABAP (background area). The relative difference between the ROS area in the presence and the absence of ABAP is considered as a measurement of the sample antioxidant capacity. Therefore, sample antioxidant capacity decreases as the relative area increases. Finally, LPO in testis samples was determined using the FOX method (Hermes-Lima et al. 1995). Results were expressed as nmoles CHP per gram of wet tissue.

Whole blood and testis samples of five blackbirds were randomly selected from each experimental group and had their Pb content analyzed. Tissue Pb content was determined by

atomic absorption spectrophotometry (AAS-932 Plus, GBC, Australia), following procedures and quality assurance controls described by Carvalho et al. (2013). Measurement accuracy and standard curves were obtained using a standard Pb solution (Standard Reference Material 3114; National Institute of Standards & Technology, Gaithersburg, MD, USA). For whole blood and testis samples, the detection limit was 12.5 µg/L and 0.0064 µg/g ww, respectively. Pb recovery was calculated based on standard reference material (European Reference Material ERM-CE278, Geel, Belgium), prepared as described for tissue samples analysis. It corresponded to 98.3%. For whole blood and testis, results were expressed on a volumetric (µg/L) and a wet weight basis (µg/g ww), respectively.

Data were expressed as mean ± standard error. Mean values among treatments were compared using analysis of variance (ANOVA) followed by the LSD test. ANOVA assumptions were checked using the normal probability plot of raw residuals (data normality) and the Cochran C test (homogeneity of variances). Data on whole blood Pb content in blackbirds from field group were not included in the ANOVA, since Pb levels were below the detection limit (<DL) of the technique employed. For sperm quality parameters, percentage data were mathematically transformed (arcsine) before analysis. Data of histological examination were compared using the chi-square test. In all cases, the level of confidence adopted was 95% ($\alpha = 0.05$; $P<0.05$). All analyses were performed using the software Statistix 9.0 (Analitical Software, Tallahassee, FL, USA).

Results and Discussion

Pb levels in the whole blood of blackbirds from the field group were below the detection limit of the technique employed (12.5 µg/L). In addition, very low levels of Pb were found in whole blood of blackbirds from the control group (20.9 µg/L). Also, it is important to note that similar values of Pb concentration were found in testis of control (0.383 µg/g ww) and field (0.325 µg/g ww) blackbirds. These findings suggest that no significant Pb contamination occurred during maintenance of blackbirds in captivity over the experimental period. Furthermore, no mortality was observed in control blackbirds during this period. However, three blackbirds were found to be dead two days after treatment with 100 mg/Kg Pb. This could be associated with the fact that the dosages employed were high. Furthermore, Pb was tested as Pb acetate, thus being 100% bioavailable. Additionally, Pb was intraperitoneally injected. These facts would characterize an exposure scenario that could likely not occur in the wild.

Despite the experimental approach employed in the present study to test the Pb effects in *C. ruficapillus*, this metal was found to be significantly accumulated in the whole blood and testis of blackbirds injected with Pb acetate. Indeed, Pb concentration was 6.5- and 11-fold higher in the whole blood of blackbirds injected with 50 and 100 mg/Kg Pb than in the whole blood of control blackbirds, respectively. In testis, Pb concentration was ~1.9- and 2.4-fold higher in testis of blackbirds injected with 50 and 100 mg/Kg Pb than in the testis of control blackbirds, respectively (Fig. 1). In both tissues, Pb content was significantly higher in blackbirds injected with 100 mg/Kg Pb than in control blackbirds. These findings show that Pb reached the blood stream of *C. ruficapillus* after being intraperitoneally injected and accumulated in the testis of blackbirds. After entering the blood stream, Pb acetate can cross the blood-testis barrier and accumulate in testis of animals and humans (Doumouchtsis et al. 2009).

Regarding biological effects, testis of blackbirds from the control group showed well-organized seminiferous tubules with normal size lumen, containing Sertoli cells and germ cells. Also, Leydig cells in all stages of the spermatogenesis were seen (Fig. 1a). On the other hand, testis of blackbirds treated with a single IP injection of 50 mg/Kg Pb showed seminiferous tubules displaying from mild to moderate testicular degeneration, which was characterized by the presence of Sertoli cells finely vacuolated and reduced spermatogenesis,

thus showing a decreased sperm production (Fig. 1b). In turn, testis of blackbirds treated with 100 mg/Kg Pb showed seminiferous tubules with moderate thickening of the basement membrane. Also, moderate to severe testicular degeneration was observed. This was characterized by the presence of Sertoli cells finely vacuolated, as well as a reduced number of spermatogonia, spermatocytes, spermatids and sperm cells (Fig. 1c). These findings indicate that Pb accumulated in the testis following a single IP injection of Pb acetate (50 and 100 mg/Kg Pb) induced damage to the male reproductive tract of *C. ruficapillus*.

Biological injuries were not restricted to the histological changes discussed above. Also, a significant decrease in membrane integrity and mitochondrial functionality were observed in sperm cells of Pb-injected blackbirds when compared to those from the control group. A similar result was observed in blackbirds injected with 50 and 100 mg/Kg Pb. However, sperm cells of blackbirds injected with 100 mg/Kg Pb showed a significantly reduced DNA integrity with respect to those from control blackbirds or those injected with 50 mg/Kg Pb. No significant difference was observed between control blackbirds and those injected with 50 mg/Kg Pb (Fig. 3a). These findings are in complete agreement with those reported by Acharya et al. (2003). These authors reported increased percentage of spermatic pathologies and reduced percentage of motile sperm in rats intraperitoneally injected with 100 mg/Kg Pb, one of the doses tested in the present study. The similar damage observed in membrane integrity and mitochondrial functionality in sperm cells of blackbirds injected with 50 and 100 mg/Kg Pb is in agreement with the similar level of Pb accumulated in the testis of these birds.

The negative biological effects induced by the IP injection of Pb acetate can be associated with an oxidative stress condition in testis of *C. ruficapillus*. Although no significant change in ROS content was seen, an increased LPO level was observed in the testis of Pb-injected blackbirds. It is important to stress that this increased LPO was paralleled by a reduced ACAP. Indeed, these effects were dose-dependent, being significant in blackbirds injected with 100 mg/Kg Pb with respect to those from the control group (Fig. 3b). In birds, sperm cell membrane is rich in poly-unsaturated fatty acids (PUFAs), thus being subject to ROS-induced LPO (Cerolini et al. 2010). Oxidative degradation of PUFAs has been considered as the main cause of membrane fluidity and permeability losses, culminating with damage to germ and sperm cells (Mishra and Acharya 2004). Therefore, the higher LPO level found in testis of blackbirds exposed to 100 mg/Kg Pb can be a reasonable explanation for the observed lower integrity of cell and nuclear membranes in sperm cells of *C. ruficapillus*. It can also explain the consequent reduction in sperm quality observed in blackbirds subjected to this experimental condition. In addition, it can be the basis of the structural disorder and atrophy observed in testis of blackbirds exposed to Pb, as earlier discussed.

Sperm cells parameters analyzed in the present study were important to evaluate the Pb impact on the whole reproductive process in blackbirds. In some domestic animals, such as pig and sheep, it is reported that a decrease in membrane integrity reduces sperm viability (Harrison and Vickers 1990). Furthermore, membrane lesions induced by Pb exposure would facilitate the metal access into the cells and its interaction with organelles and nucleus, bringing them more susceptible to Pb-induced damages. In fact, a significant reduction in mitochondrial functionality and DNA integrity was observed in Pb-injected blackbirds. Regarding DNA, Hernandez-Ochoa et al. (2006) also reported a high frequency (60%) of sperm cells showing DNA lesion after treating mice with a high dose of Pb acetate. In fact, these authors demonstrated that Pb can be incorporated into sperm cell nucleus during development in the testis or during maturation in the epididymis. Therefore, Pb effects on sperm quality (membrane integrity, mitochondrial functionality and DNA integrity) observed in *C. ruficapillus* seem to result from a direct interaction of the metal with the testis and sperm cell nucleus (Doumouchtsis et al. 2009).

Considering the Pb effects described above on sperm quality, a lower reproductive potential would be expected in male *C. ruficapillus* acutely exposed to Pb. In fact, male

blackbirds exposed to Pb acetate (50 and 100 mg/Kg Pb) showed a reduced number of spermatogonia, spermatocytes, spermatids and spermatozoa. Therefore, it is clear that the acute Pb exposure tested in the present study negatively affected spermatogenesis in blackbirds, leading to a reduced availability of all spermatogenic cells types. Furthermore, Leydig cells, responsible for testosterone synthesis, were also reduced in blackbirds injected with Pb acetate (50 and 100 mg/Kg Pb). A similar result was observed in mice treated with Pb acetate (Fahim et al. 2013). This finding suggests that a lower level of circulating testosterone would be occurring in Pb-exposed blackbirds, thus negatively affecting germ cells.

In a broad view, biochemical damages observed in sperm cells were reflected at a higher level of biological organization, i.e., cellular structural disorder and testis atrophy. Actually, vacuole formation was observed in testis of more than 50% of the Pb-exposed blackbirds analyzed. Similar findings were reported in adult male Common Quail *Coturnix coturnix* exposed to Pb acetate in drinking water (0.1, 0.25, 0.5 and 1%) for 1 to 6 months (Almansour 2009). In this case, chronic exposure induced histopathological alterations including sperm cell hyperplasia, Leydig cell degeneration, decreased spermatocyte number and tubular atrophy. These findings suggest that damages observed in testis of *C. ruficapillus* are likely related to the observed losses of sperm function and viability, as discussed above.

In light of the Pb-induced effects observed in *C. ruficapillus* and discussed above, it is important to note that blood concentrations tested in the present study (<200 µg/L Pb) are lower than the range of concentrations (200-500 µg/L Pb) considered as inducing subclinical signs of Pb poisoning in birds (Pain 1996). In fact, the blood concentration of 710 µg/L Pb is proposed as a critical concentration of protecting birds from Pb toxicity (Buekers et al. 2009). On the other hand, values ranging from 50 to 80 µg/L Pb are proposed as reference Pb blood concentrations for the European blackbird *Turdus merula* (Scheifler et al. 2006).

In summary, data reported in the present study show that Pb administered via IP injection was accumulated in testis of *C. ruficapillus* and induced toxicity. Effects on the reproductive tract of male blackbirds included loss of membrane integrity, mitochondrial functionality and DNA integrity in sperm cells, as wells as cellular structural disorder and testis atrophy. These effects were quite severe and likely resulted from an oxidative stress condition induced by Pb exposure. Therefore, our results show that Pb can negatively affect testis function and sperm quality in male *C. ruficapillus*, being a potential threat for the reproduction of blackbirds.

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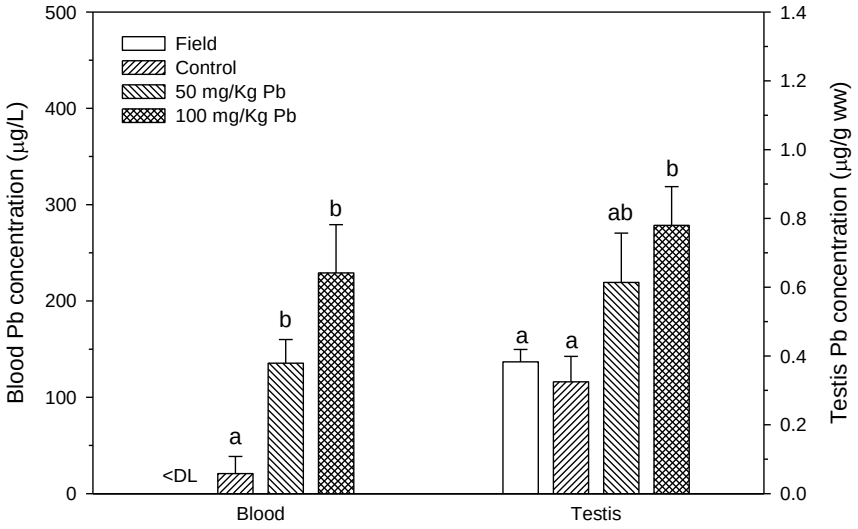

Fig. 1 Pb content in whole blood and testis of *Chrysomus ruficapillus.* Data are expressed as mean ± standard error (n = 5 for each group). Pb content in the blood of blackbirds from the field group was below the detection limit (<DL) of the technique employed. Different letters indicate significant difference (P<0.05) among experimental groups for each tissue

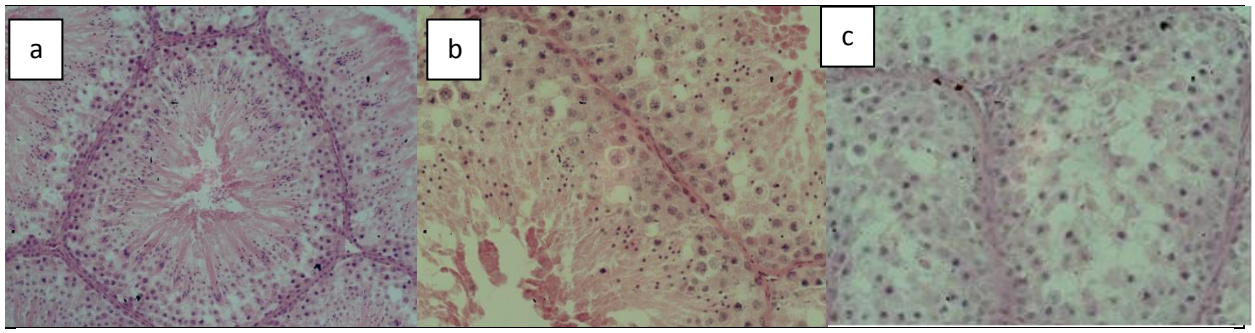

Fig. 2 Testis histology of *Chrysomus ruficapillus*. (a) Testis of control blackbirds showing normal organization of seminiferous tubules and all stages of spermatogenic and Leydig cells (HE; 40x magnification). (b) Testis of blackbirds injected with 50 mg/Kg Pb (HE; 100x magnification). (c) Testis of blackbirds injected with 100 mg/Kg Pb (HE; 100x magnification)

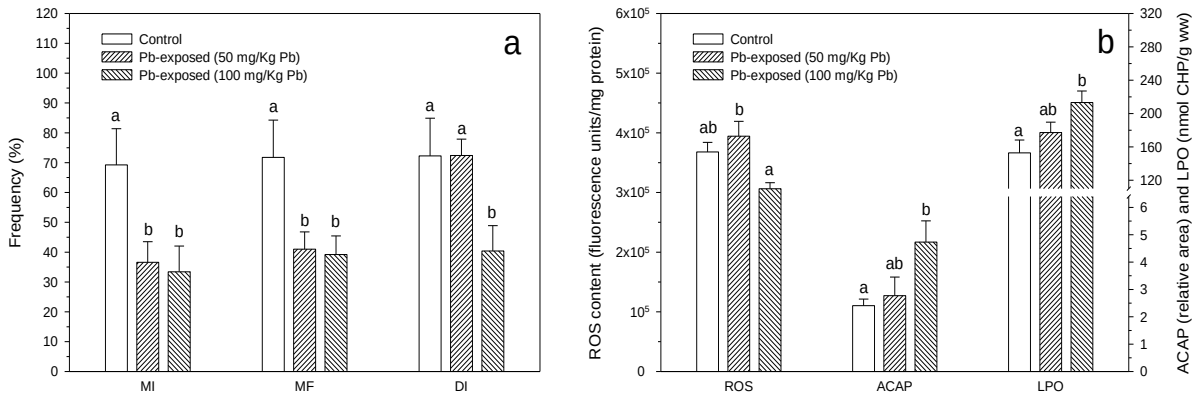

Fig. 3 (a) Sperm quality parameters in *Chrysomus ruficapillus* seven days after injection of 1 mL of saline solution (control group) or 1 mL of saline solution with Pb acetate (50 or 100

mg/Kg Pb). Data are expressed as mean ± standard error (control group: n = 12; 50 mg/Kg Pb group: n = 15; and 100 mg/Kg Pb group: n = 15). MI: membrane integrity; MF: mitochondrial functionality; DI: DNA integrity. (b) Reactive oxygen species (ROS), total antioxidant capacity (ACAP), and lipid peroxidation (LPO) in testis. Data are expressed as mean ± standard error. Different letters indicate significant different mean values among experimental groups ($P<0.05$) for each parameter