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Testicular damage and farming environments — An integrative ecotoxicological link

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HIGHLIGHTS

- Mice from conventional farming show severe alterations in histological and cellular parameters.
- Mice exposed to conventional farming environments bioaccumulate higher Pb hepatic loads.
- Pb hepatic loads are associated with testicular structural and functional disruption.
- Integrated Biomarker Response revealed that conventional practices entail higher risk to male fertility.

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ABSTRACT

The exposure to agrochemicals during farming activities affects the function of the reproductive system, as revealed by the increasing worldwide evidence of male infertility amongst farmers. The main objective of this study was to untangle the link between agricultural practices and male reproductive impairment due to chronic exposure to xenobiotics (such as agrochemicals) in conventional and organic farming environments. For this purpose, male wild mice (*Mus musculus*) populations from sites representing two distinct farming practices (conventional and organic farming systems) were used as bioindicators for observable effects of testicular damage, namely on a set of histological and cellular parameters: (i) relative volumetric density of different spermatogenic cells and interstitial space; (ii) damage in the seminiferous tubules and (iii) apoptotic cells in the germinal epithelium. Results showed that mice from the conventional farming site bioaccumulated higher Pb hepatic loads, while mice from the organic farming site tend to bioaccumulate higher Cd hepatic loads. In general, for the analyzed testicular damage related parameters, mice from the organic farming site showed a similar performance than mice from the reference site. Mice from the conventional farming site stood out not only by underperforming in most studied parameters, while displaying an association between Pb hepatic loads and the observed testicular structural and functional disruption, but also by the increased stress index (Integrated Biomarker Response value). This study highlights the potential damaging effects of conventional farming practices on testicular structure and function, under natural conditions, raising concern about ensuing fertility risks for farmers.

1. Introduction

Agricultural practices are one of the most significant anthropogenic activities that greatly affect both environment and human health (Horrigan et al., 2002). Human population is expected to grow by one third until 2050, according to FAO estimates (FAO, 2009). How to meet the resulting food demand by sustainable

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means, within acceptable environmental impacts, remains a great challenge, since so far prevailing production methods have relied in the intensive use of agrochemicals to enhance soil fertility and crop productivity (Gomiero et al., 2011). On the downside, long-term and extensive land use for agricultural purposes frequently results in the incorporation of several types of pollutants into soil ecosystem, such as pesticides residues and metals, even if they are applied in small amounts (Krami et al., 2013). Present-day agricultural volcanic soils have a unique metallic footprint mostly derived from the volcanic parent rock, modulated by the metal inputs of long-term agricultural practices (Parelho et al., 2014). As a consequence, some of these pollutants slowly accumulate in the ecosystem, contributing to a higher ecotoxicological risk under these environments.

The effects of agrochemical exposures on male reproduction are a topic of considerable concern in environmental, occupational and reproductive toxicology (Mehrpour et al., 2014), as the testicle is considered one of the most vulnerable organs to agrochemicals that often act as endocrine disruptors (Mohapatra et al., 2013). Occupational exposure to agrochemicals in farms can occur by directly dealing through mixing, loading and spraying activities (Shadnia et al., 2005) or simply by frequent attendance of such environments, whose soils can act as a sink for several pollutants (Saaltink et al., 2013). Since many potential pollutants exist in these environments, occupational exposure is not easy to be proved. However, the decreased fertility rate observed in men occupationally exposed to agrochemicals is significantly above the expected amongst the general population (Ashiru and Odusanya, 2009).

Most of the well-known adverse effects of agrochemicals upon the testicle are reported in animals under laboratory conditions, following the administration of a single element in high-dose or short-term exposure (e.g. Rajawat et al., 2015). These effects include genotoxicity (e.g. Urióstegui-Acosta et al., 2014), tissue necrosis (e.g. Cavalli et al., 2013), inflammation and structural seminiferous tubular damage (e.g. Li et al., 2013), ultimately entailing a decrease of male reproductive health. However, the cumulative effects of multiple compounds in low-levels, akin to an occupational exposure in a farm, are still not well established (Mantovani et al., 2008) and available data is not enough to determine whether long-term exposure to agrochemicals entails a significant risk to male fertility under field conditions.

Data collected from vertebrates living in/exposed to contaminated areas are of utmost importance since they can bring pertinent information to be used in human risk assessment (Pereira et al., 2006). Furthermore, field studies using animal models provide crucial ecotoxicological data given that, under laboratory conditions, it is difficult to mimic the complexity of field conditions (Pereira et al., 2006; Sánchez-Chardi et al., 2009). Among these vertebrates, small mammals, such as mice, are preferentially selected, particularly for studies regarding terrestrial pollution (Shore and Douben, 1994).

The assessment of physiological effects of chronic exposure to agrochemicals using integrative multi-biomarker approaches is essential to evaluate testicular damage in natural populations under farming environments. The Integrated Biomarker Response index (Beliaeff and Burgeot, 2002) has been developed as effect-based monitoring approach and may be used to integrate a set of early warning responses into a more simple and realistic stress model, a valuable tool for ecological risk assessment.

According to Mehrpour et al. (2014) meta-analysis, the majority of pesticides affect the male reproductive system by mechanisms such as reduction of sperm density and motility, inhibition of spermatogenesis, reduction of testes weights, reduction of sperm counts, motility, viability and density, inducing sperm DNA damage and increasing abnormal sperm morphology. These authors also refer other possible effects, such as: epididymis, seminiferous tubule, seminal vesicle and ventral prostate degeneration; changes in plasma levels of testosterone, follicle-stimulating hormone, and luteinizing hormone; decreases in the level and activity of the antioxidant enzymes in testes, and inhibition of testicular steroidogenesis.

Hence, given the worldwide increasing evidence of male infertility amongst farmers, the main objective of this study is to assess if chronic exposure to agrochemicals in distinct farming environments, whose volcanic soils are naturally enriched with metals and anthropogenically molded by agricultural practices, entails elevated risks to male reproductive health. For this purpose, three groups of male wild mice *Mus musculus* from sites representing two distinct farming systems (conventional and organic) and from a site without records of farming activity (reference group), were used as bioindicators for the observable histological and cellular alterations associated with testicular damage. All the information is intended to provide new insights, under field conditions, into the link between agricultural practices, occupational exposure to agrochemicals and male testicular damage.

2. Materials and methods

2.1. Study sites

The fieldwork was conducted in S. Miguel Island (Azores, Portugal), where agricultural practices have gone through significant changes during the last decades, with the transformation of the majority of traditional smallholdings into farms for commercial purposes and where intensive agriculture is practiced following the EU directives, either with the use of synthetic agrochemicals (conventional farming) or not (organic farming).

The selected study sites (Supplementary material – Fig. 1) correspond to two representative farms with different agricultural practices [conventional (CF) and organic (OF)]. In the local context, CF refers to agricultural practices in which the use of synthetic agrochemicals (both pesticides and fertilizers) is legally framed by European and national guidelines. OF systems are certified by the European Commission, therefore the use of synthetic agrochemicals is prohibited and soil amendments are confined to organic fertilizers (compost and manure). The selected farms have been explored under the same farming system for at least 10 years. A reference site (RF) was also included in the study. RF corresponds to a forest reserve of centennial Japanese cedar (*Cryptomeria japonica*), with no historical records or evidence of farming activity. All study sites are located in the same geological complex (Picos Fissural Volcanic System), ensuring the same bedrock and pedological conditions, being differentiated only by the type or absence of agricultural soil management. Supplementary material – Table 1 shows the characterization of each studied farm and reference site, regarding the years of exploitation under the corresponding farming system and use of agrochemicals.

2.2. Mice sampling and preparation of samples for histology

Mus musculus fulfill the criteria of selection as bioindicator species, namely: high abundance, wide life expectancy, enough to estimate possible long-term effects (Marcheselli et al., 2010) and relatively small home range [in average 145 m^2 (Lidicker, 1966)], which is significantly smaller than the surface areas of the chosen study sites (the farming areas have, in average, 5000 m^2).

Three separate sets of 12 adult male *M. musculus* (one set per site) were captured using live-catch traps, housing the mice for no longer than 24 hours before euthanization. The relative age of each mouse was determined via dry crystalline lens mass, following the methodology proposed by Quéré and Vincent, 1989. Only mice with a minimum weight of 10 g and sexually mature (evaluated by microscopic observation of the epididymal content) were considered for the study. The group of mice from RF was significantly (Tukey HSD, $P < 0.05$) older and heavier than mice from CF and OF groups [(age (days): 191.75 ± 69.26 (RF); 109.13 ± 34.85 (CF); 115.96 ± 46.26 (OF); and, weight (g): 17.04 ± 2.56 (RF); 13.61 ± 1.57 (CF); 13.44 ± 1.46 (OF)]. Once euthanized with isoflurane, mice were necropsied, having their testicles and liver extracted. Both testicles were fixed in 4% buffered formaldehyde for standard histology processing and afterwards dehydrated and embedded in paraffin wax. For each testicle, a set of histological slides was prepared for morphometric measurements and for the evaluation of the damage in the seminiferous tubules. These slides consisted of several sections of 4 μm in thickness, which were stained with hematoxylin and eosin, according to Martoja et al. (1970). Another set of histological slides was prepared for TUNEL assay in order to assess the number of apoptotic cells in the seminiferous tubules.

This study was carried out in accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123), 86/609/EEC Directive and Portuguese legislation (DL 129/92).

2.3. Hepatic trace metal content in mice

The study by Parelho et al. (2014) revealed that agriculture is a diffuse source of trace metal (TM) soil pollution in agricultural Andosols, particularly of Cu, Zn, As, Mo, Cd, and Pb. Therefore, these TM were selected to determine their concentration in mice liver. The concentration of Se was also determined, since this element is known to have a protective role in the male reproductive system (Wlodarczyk et al., 1995; Kalender et al., 2013) and the studied volcanic soils are naturally enriched with this element. Trace metal contents were determined by mass spectrometry with inductively coupled plasma (ICP/MS, Activation Laboratories Ltd., Canada). Quality control was assured by analysis of duplicate samples, blanks and reference materials (DOLT-3 and DORM-2). For statistical purposes, the values below the detection limit were assigned as equal of its lower detection limit.

2.4. Testicular damage biomarkers

2.4.1. Histological morphometric parameters

For each specimen, 10 testicular histological sections (5 per testicle) were randomly chosen for relative volumetric density determination of different spermatogenic stages [spermatogonia (Sg), spermatocyte (Sc), early spermatids (ESt), late spermatids (LSt), spermatozoa (Sz)] and interstitial space (IntS), using the M168 Weibel Multipurpose Test System (Weibel, 1979). The selected histological sections were observed at 250× magnification. Additionally, the spermatogenic stages were further grouped as: germinal epithelium (GE), that includes Sg, Sc and ESt, and sperm cells (SC), that includes LSt and Sz.

2.4.2. Evaluation of seminiferous tubules injury

Seminiferous tubules injury (STI) was assessed on 30 micrographs per testicle (60 per individual), at 200× magnification, centered on a single, randomly picked and transversally sectioned seminiferous tubule. All micrographs were evaluated and scored from 1 to 4 by three independent observers, using double-blinded rank evaluations, according with criteria based on the percentage of luminal area occupied by spermatozoa and germinal epithelium structural organization (adapted from Ferreira et al., 2015). A more detailed description of the methodology employed in STI evaluation is provided on Supplementary Material. The higher the STI value, the worse is the state of integrity of the seminiferous tubule.

2.4.3. Evaluation of apoptosis in spermatogenic cells

Histological slides were analyzed for apoptotic expression by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) staining, using the DeadEnd™ Fluorometric TUNEL System (Promega, USA). Ten seminiferous tubules (5 per testis) were randomly selected, observed by fluorescence microscopy and photographed at 200× magnification. The evaluation of apoptotic expression was performed by an observer, who counted the total number of TUNEL-positive cells [apoptotic cells (ApC)] in each seminiferous tubule.

2.5. Linking testicular damage to trace metals and farming environments

Biomarkers and TM hepatic contents were further explored under Principal Component Analysis (PCA). PCA was used to reduce the initial set of TM (7), highlighting only those that are essential to explain the observed variability of testicular damage between studied mice groups. Components with eigenvalues >1 and explaining >10% of the variance were retained. Oblimin with Kaiser Normalization was applied as the rotation method in the analysis.

Testicular damage biomarkers (GE, SC, IntS, STI and ApC) were combined into the Integrated Biomarker Response index (IBR), as described by Beliaeff and Burgeot (2002), to evaluate testicular stress. A more detailed description of the IBR methodology is provided on Supplementary Material.

2.6. Statistical analysis

Analysis of variance (ANOVA) was carried out in order to evaluate the significant differences in testicular damage biomarkers and TM hepatic contents between mice groups. When ANOVA showed significant differences ($P < 0.05$) between data sets, paired comparisons of each mean using Tukey HSD test was done. Before ANOVA, the data were tested for normal distribution and for homogeneity of variance and, wherever appropriate data were transformed with a decimal log operator to meet normality criteria. All statistical analyses were performed with IBM SPSS Statistics® v. 21.0.

3. Results

3.1. Hepatic trace metal content in mice

Mice from the RF group had significantly higher concentrations of Cu and Se in the liver than mice from the farming sites, while no significant differences were found between sites for Zn, As and Mo

Table 1
Mean values (±SD) for the biomarkers of testicular damage [relative volumetric density of sperm cells (SC); germinal epithelium (GE); interstitial space (IntS); seminiferous tubule injury index (STI); apoptotic cells (ApC)]; reference site (RF), organic (OF) and conventional (CF) farming sites. Means within each line followed by different letters are significantly different at $P < 0.05$ (Tukey test).

	Mice from RF (n = 12)	Mice from OF (n = 12)	Mice from CF (n = 12)
SC	0.15 ± 0.01 a	0.19 ± 0.03 b	0.11 ± 0.04 c
GE	0.52 ± 0.04	0.51 ± 0.04	0.54 ± 0.07
STI	144.63 ± 11.84 a	132.18 ± 26.09 a	175.33 ± 13.29 b
IntS	0.09 ± 0.02 a	0.13 ± 0.04 ab	0.14 ± 0.03 b
ApC	0.38 ± 0.34 a	0.65 ± 0.73 a	3.68 ± 2.75 b

hepatic loads; mice from OF displayed the highest mean hepatic concentrations of Cd, differing from the other studied sites (Table 2). Pb hepatic concentration increased from RF < OF < CF, although not being significantly different.

3.2. Testicular damage biomarkers

Mice from the OF and RF groups showed, in general, well-structured seminiferous tubules, full spermatogenesis and well defined luminal boundaries (Fig. 1A and B), while mice from the CF group showed severe alterations in their seminiferous tubules: disorganization of the germinal epithelium, frequent detachment of Sg cells from the basement membrane, macrovacuolization, pronounced alteration of spermatogenic process, with noticeable reductions of Sz in the lumen, lack of luminal definition, dilatation of blood vessels and enlargement of the interstitial space (Fig. 1C1 and C2).

The stereological analysis of testis (Table 1) showed that mice from the CF site had a significantly lower relative volumetric density of SC (0.11 ± 0.04) and a higher relative volume of IntS (0.14 ± 0.03). In contrast, the individuals from OF had a significantly higher relative volume of SC (0.19 ± 0.03) and overall showed a pattern of biomarker response similar to the RF mice.

Seminiferous tubule injury (STI) was significantly higher in mice from CF (Table 1).

In all the studied mice groups, the apoptotic DNA-fragmented cells marked by TUNEL assay were mainly detected in Sg and Sc stage (Fig. 1D, E and F). The average amount of these cells per tubule was significantly higher (7.1 folds) in mice from the CF group than in mice from OF and RF (Table 1).

It is important to notice that despite forming the older group, mice from RF displayed no signs of testicular damage (Table 1 and Fig. 1) and, therefore a higher difference in testicular damage would be expected between the groups of mice from the farming sites (OF and CF) and RF, if the former were similar in age to mice from RF. The age-normalized bioaccumulation factor index (BAF_{age}) calculated for each TM and mice (see Supplementary Material Table 2) corroborate the hypothesis that the higher bioavailability of TM in the reference site is due to particular local conditions of its soil, regardless of the age of the animal.

3.3. Linking hepatic trace metal content and testicular damage

Using PCA, the correlated testicular damage biomarkers and TM were replaced by 3 independent factors (PC1, PC2 and PC3) that accounted for 76.8% of the cumulative variance, indicating that the results are statistically consistent (see Supplementary material – Table 3). PC1 explained 43.2% of the variance and had significant positive loadings for STI, ApC, GE, IntS and Pb, and significant negative loading for SC. PC2 explained 20.1% of the variance, with significant positive loadings for Cu and Se and significant negative loadings for IntS and ApC. PC3 explained an additional 13.5% of the variance, with significant positive loadings for Zn, Pb and ApC and, significant negative loading for SC (Supplementary material – Table 3).

Table 2
Mean (±SD) concentration of trace metals (mg kg^{-1}, d.w.) in mice liver; reference site (RF), organic (OF) and conventional (CF) farming sites. For trace metal concentrations, means within each line followed by different letters are significantly different at $P < 0.05$ (Tukey test).

	Mice from RF (n = 12)	Mice from OF (n = 12)	Mice from CF (n = 12)
Cu	17.95 ± 2.46 a	14.25 ± 1.62 b	15.52 ± 2.83 b
Zn	125.49 ± 26.17	115.53 ± 35.90	125.58 ± 35.13
As	3.76 ± 7.73	1.45 ± 1,12	1.72 ± 3.10
Se	4.06 ± 0.82 a	2.03 ± 0.76 b	1.74 ± 0.93 b
Mo	3.57 ± 0.31	3.41 ± 0.78	4.03 ± 0.81
Cd	0,17 ± 0.14 a	0.56 ± 0.37 b	0.18 ± 0.11 a
Pb	0.47 ± 0.15	0.70 ± 0.51	1.02 ± 0.92

3.4. Testicular stress evaluation

Five biomarkers for testicular damage (GE, SC, IntS, STI and ApC) are represented in the star plot (Fig. 1G). According to the IBR value, mice from CF are the ones under the most stressful testicular conditions, with the highest IBR score for all biomarkers: CF > OF = RF (better and worse scores are respectively represented by lower or higher values). Mice from RF and OF groups always presented a zero score (A = 0) for all the biomarkers, except for the IntS (OF) and STI and SC (RF), thus resulting in a total IBR value of zero (Fig. 1H).

4. Discussion

Mice from the conventional farming site (and thus chronically exposed to xenobiotics like agrochemicals) revealed a higher bioaccumulation of Pb (the hepatic loads increased from RF < OF < CF). Hepatic tissues are known for being a target of Pb bioaccumulation, due to chronic exposure to metallic agrochemical residues, especially when this persistent metal is incorporated through ingestion, the most important exposure route in small mammals (Shore and Rattner, 2001). As a general mechanism, when metal concentrations exceed the binding capacity of metallothioneins in liver, free metallic ions may accumulate in other tissues, such as the male reproductive system, acting as endocrine disruptors (Georgescu et al., 2011).

Our results showed that mice chronically exposed to conventional farming environments underperformed in most tested parameters for testicular damage, expressing atrophy and degeneration of the germinal epithelium, together with interstitial fibrosis. STI index, stereological analysis and TUNEL assay revealed an elevated disruption in seminiferous tubules, increased interstitial tissue, lack of sperm cells in luminal space and higher amount of germinal cells undergoing apoptosis in mice from CF, in comparison with mice from OF and RF groups. The increased number of apoptotic cells in earlier stages of spermatogenesis, coupled with the decrease of late spermatids and depletion of sperm cells in luminal space of mice from CF, in relation to OF and RF, indicates that germ cell survival and meiotic proliferation are negatively affected, particularly in environments where synthetic agrochemicals are commonly used. Many agrochemicals have been associated with similar spermatogenesis impairment, though their mechanisms of toxicity upon the proliferation of the germinal epithelium might be very diverse, ranging from straightforward mechanisms, like inducing irreparable DNA damage by promoting oxidative stress, to subtler mechanisms, acting as endocrine disruptors interfering with estrogen or androgen-mediated processes (Mehrpour et al., 2014). Moreover, the increased volume occupied by interstitial tissue is commonly associated with chronic inflammation of the testes (Apa et al., 2002; Damek-Poprawa and Sawicka-Kapusta, 2003) and implies a decreased volume occupied by the parenchyma (Barth et al., 2008), the portion effectively involved in sperm cell formation, since fibrosis is the aftermath of testicular parenchyma replacement by fibroblasts and collagen, following necrosis and inflammation (Creasy et al., 2012). These signs fit within a pattern of biological response that has been described in several studies associating exposure to agrochemicals with decreased reproductive capacity (Joshi and Sharma, 2011; Martenies and Perry, 2013). In face of these results, a worse testicular functionality is expected when considering conventional

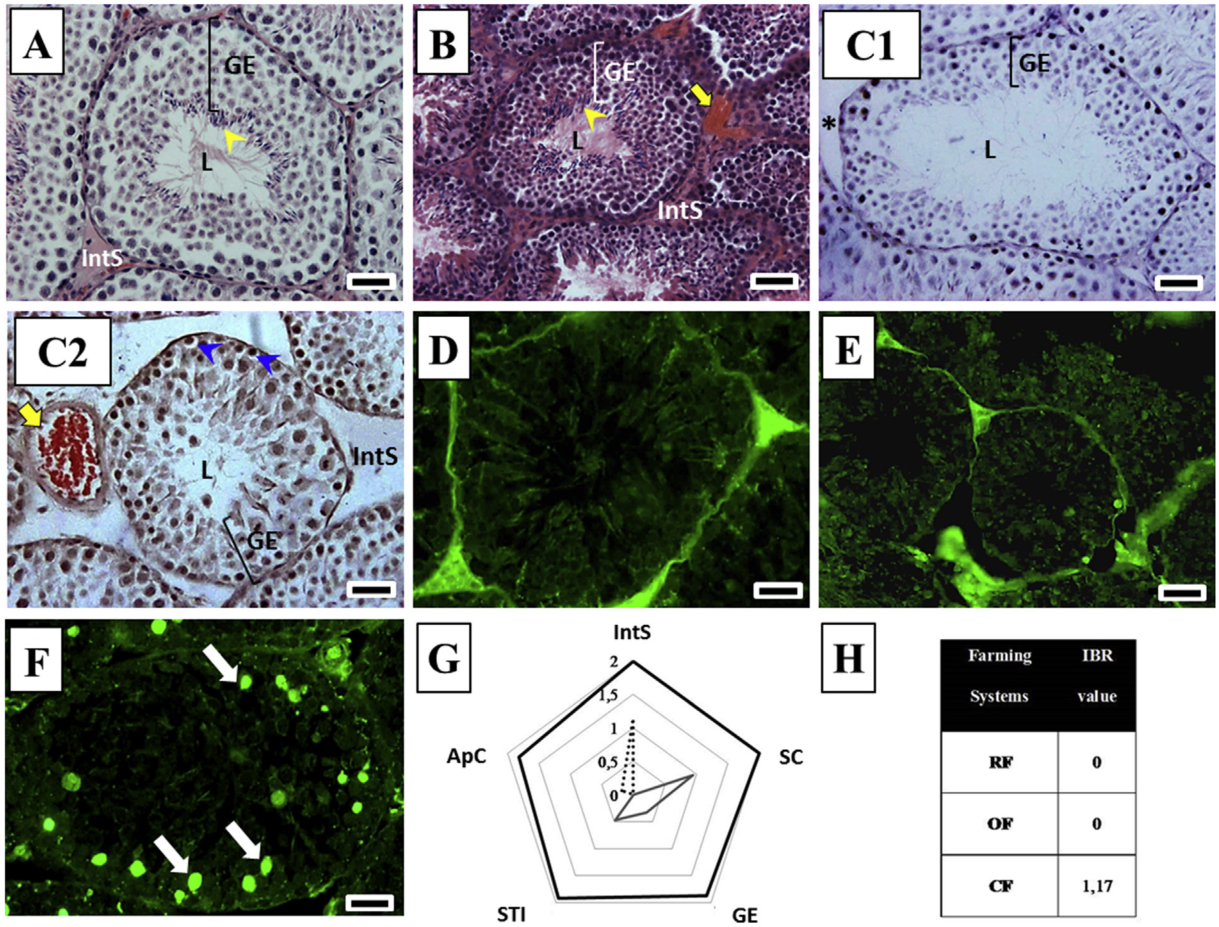

Fig. 1. Histology of the seminiferous tubules, TUNEL assay for apoptotic DNA fragmentation and Integrative biomarker response (IBR) for testicular damage biomarkers. Hematoxylin and eosin staining (A, B and C) and TUNEL assay (D, E and F) of the seminiferous tubules of mice from the reference site (A and D), organic (B and E) and conventional farming sites (C and F); germinal epithelium (GE), interstitial space (IntS), lumen (L). IBR score star plot (G) and IBR value (H) for testicular damage biomarkers [apoptotic cells (ApC); germinal epithelium (GE); interstitial space (IntS); sperm cells (SC); seminiferous tubule injury index (STI)] of mice from conventional (black line, CF), and organic (dotted line, OF) farming sites and reference site (grey line, RF). White arrows point out apoptotic nuclei; yellow arrows point out blood vessels; yellow arrow heads point out spermatozoa; blue arrow heads point out macrovacuolization. Scale bar = 25 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

farming environments under intensive agrochemical application.

Mice from the organic farming site revealed simultaneously a significantly higher mean Cd hepatic concentration and a higher proportion of interstitial fibrous tissue, often showing disrupted blood vessels, hemorrhage and edema, when compared to RF mice. The higher hepatic load of Cd in this mice group is supported by Parelho et al. (2014) previous findings that observed an elevated Cd accumulation in organic farming soils derived from the frequent application of organic fertilizers (compost and manure). Therefore, the higher Cd loads in mice from the OF may reflect the respective higher Cd loads present in these soils. Blood-testis barrier and spermatogenic cells have been shown to be extremely sensitive to Cd toxicity (Thompson and Bannigan, 2008), even at considerably low doses (Siu et al., 2009). Recent studies elucidate Cd involvement in vascular injury by mechanisms of endothelial oxidative stress (Angeli et al., 2013). While our study does not provide data on renal or testicular TM loads, with the former being the preferential target for Cd bioaccumulation (Damek-Poprawa and Sawicka-Kapusta, 2003) and the latter exceedingly sensitive to Cd toxicity (Siu et al., 2009), by noting the similarity between the signs of damage observed in OF mice and those described by Siu et al. (2009), namely concerning the interstitial tissue, it is plausible that these mice suffered of chronic Cd induced testicular toxicity. Nevertheless, the general performance of mice from the OF in most parameters is alike to that of the RF individuals.

Regarding mice from the reference site, the mean hepatic concentrations of Cu, As and Se were generally higher than in mice from the farming sites. This apparently controversial phenomenon can be partially explained by the higher bioavailability of all TM in this site due to particular local conditions of its soil (except for Se, as revealed by BAF_{age} results, Supplementary Material Table 2), such as the elevated organic soil matter content (Parelho et al., 2014). Even so, TM hepatic loads in these mice were always lower than values reported in the literature for small mammals from natural forest reserves (Tête et al., 2014). Moreover, a decreased proportion of late spermatids and spermatozoa was spotted in mice from RF group, though in the absence of major signs of testicular damage. This can be explained as a consequence of reduced food availability and generally harsher wild conditions of the reference site, contrasting with the great abundance of food in both farms. In addition, energy requirements are higher in the wild, because of the expense of acquiring food, defending territory, thermoregulation and provide for the offspring (Damek-Poprawa and Sawicka-Kapusta, 2003). It is well established that a condition of undernutrition is unfavorable to spermatogenesis (Cheah and Yang, 2011), which would explain spermatogenesis arrest and resulting decrease of spermatozoa in luminal space of RF mice, without relatable signs of testicular damage such as observed in the other studied mice groups.

While the global differences in biological responses observed between mice from farming sites are the net result of the joint-action of complex agrochemicals mixtures in variable amounts, rather than the effect of a single TM, the PCA allowed the establishment of a link between mice TM hepatic profile and particular testicular damage biomarkers, highlighting 4 metals (Cu, Se, Zn and Pb) as essential to explain the variability in terms of testicular damage. This multivariate approach associated the increasing Pb hepatic loads in mice from the studied groups (RF < OF < CF) with an increased seminiferous testicular injury, germinal epithelium, apoptosis rate and a decrease of sperm cells. These results are supported by PC3, which identified Pb and Zn as the main contributors to the increased number of apoptotic cells in seminiferous tubules and the decrease of sperm cells. Zinc is an acknowledged antioxidant factor, a core constituent of free radical scavenging enzymes, a recognized protector of sulfhydryl groups and is also thought to impair lipid peroxidation by displacing transition metals such as Fe and Cu from catalytic sites, causing free radical formation (Gaetke and Chow, 2003). Batra et al. (1998) reported the counteract effect of Zn supplementation in the oxidative stress created in the testes by exposure to Pb. Also, a metal-effect pattern between Se and Cu (PC2) was spotted, as simultaneous higher loads of Se and Cu are associated with lower interstitial space and apoptotic cells. Although the toxic effects of Cu over reproductive male function are well reported (e.g. Kheirandish et al., 2014), it has been previously demonstrated that Se has a protective role in the male reproductive system against metal induced damage (Wlodarczyk et al., 1995; Kalender et al., 2013). Therefore, higher Se internal loads are expected to positively correlate with Cu loads, preventing testicular damage. In fact, we can hypothesize that mice from the CF and even OF might have lacked similar protection, as shown by significantly lower mean Se hepatic loads in comparison with mice from RF, which would further corroborate the involvement of Pb and Cd, respectively, in the observed responses, even at considerably low concentrations.

Overall, results revealed significant differences in key aspects of mice testicular damage, which were overall consistent within their respective ecotoxicological context. Chronic exposure to conventional agricultural practices entailed higher risks to mice male fertility, in which testicular function was severely compromised and an increased stress index (IBR value) was observed. The IBR summarized the pool of biomarkers of testicular damage into a single stress index, providing an integrative view of the testicular damage that ranked as CF > OF = RF, supporting integrally the evidence discussed above.

5. Conclusion

This study showed that mice chronically exposed to conventional farming environments stood out by underperforming in most studied parameters of testicular damage, in comparison with the other groups. Mice from the CF group displayed higher Pb hepatic accumulation, further associated with testicular structure and function disruption. The considered testicular damage biomarkers indicated a suppression of testicular function that ultimately may lead to male fertility impairment. In fact, the Integrated Biomarker Response index results clearly showed that conventional agricultural practices entail a higher risk to male fertility, as revealed by the increased stress index (IBR value). These results also demonstrate that *M. musculus* is a suitable bioindicator for male fertility biomonitoring in farming environments, where collected information can be useful for a weight-of-evidence approach in risk assessment decisions.

This study further highlights the potential damaging effects of conventional farming practices on male reproductive health and ensuing fertility risks, raising concern about farmers that are occupationally exposed to these farming environments, especially those that deal directly with the application of agrochemicals.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.04.043.

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