Filipe Miguel Teixeira de Sousa Bernardo

COMPARAÇÃO DE DANO TESTICULAR ENTRE RATOS PROVENIENTES DE EXPLORAÇÕES AGRÍCOLAS ORGÂNICAS E CONVENCIONAIS

Testicular damage comparison between mice from organic and conventional farms

Mestrado em Ambiente, Saúde e Segurança
Universidade dos Açores
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Trabalho desenvolvido sob orientação de:
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To all those who came before me,
To all those who’ll come after me,
But especially to those who I’m fortunate to walk with,
“We are all connected.
To each other, biologically.
To the earth, chemically.
To the rest of the universe, atomically."

Neil deGrasse Tyson

Abstract

The main goal of this study was to assess the effects of chronic exposure to different agricultural practices (conventional and organic farming) on male reproductive disruption, particularly testicular damage. Three groups of 12 male specimens of *Mus musculus*, each belonging to natural occurring populations in the studied sites, were captured alive for later evaluation of testicular damage and determination of the internal hepatic doses of essential and trace metals, like Se, Pb, Hg, and Cd. The first group was captured in a conventional farm in which soils are frequently treated with agrochemicals. The second group was captured in a certified organic farm, where soil treatments are limited to organic fertilizers and the third group (control) was captured in a natural reserve forest that was never used for farming. After euthanization, each mice had its liver and testicles extracted. The testicles were fixed and processed for posterior histological analysis, while the liver was oven dried for further quantification of trace metals. Testicular damage was assessed by studying the relative volumetric density of different spermatogenic stages, of interstitial space and luminal space, the seminiferous tubules injury (based on qualitative scores), and by quantifying apoptotic spermatogenic cells in the seminiferous tubules (by TUNNEL assay). The proportion of interstitial space was significantly higher in the seminiferous tubules of mice from both farming sites in relation to control group, being greater in the conventional farming group. The proportion of late spermatids and sperm cells was significantly decreased in mice from the conventional farming group, which not only revealed a significantly increased amount of seminiferous tubules lacking sperm cells and with evidence of structural damage, but also a significantly increased amount of spermatogenic cells undergoing apoptosis, in comparison with the other two groups. These results suggest that both farming practices, especially conventional farming, enhance testicular damage in mice that are naturally and chronically exposed to trace metal enriched farming environments, confirming *Mus musculus* as an appropriate bioindicator of the potential effects and resulting risks for male fertility of farmers continually exposed to the same environments.

Keywords: Apoptosis; Farming; Spermatogenesis; Testicular damage;

Resumo

O principal objetivo deste estudo consistiu em avaliar os efeitos da exposição crónica a diferentes práticas agrícolas (agricultura convencional e orgânica) sobre o sistema reprodutor masculino, particularmente em termos de dano testicular. Três grupos de 12 machos da espécie *Mus musculus*, pertencentes a populações naturais dos locais de estudo, foram capturados vivos para posterior avaliação do dano testicular e determinação das doses hepáticas de metais essenciais e traço, entre os quais Se, Pb, Hg e Cd. O primeiro grupo foi capturado numa exploração agrícola convencional na qual os solos são frequentemente tratados com agroquímicos. O segundo grupo foi capturado numa exploração orgânica certificada, em que o tratamento dos solos está limitado a fertilizantes orgânicos. O terceiro grupo (controlo) foi capturado numa reserva natural cujos solos nunca foram utilizados para fins agrícolas. Após eutanização, os testículos de cada indivíduo foram extraídos, fixados e processados para análise histológica, enquanto o resíduo seco dos fígados foi sujeito à quantificação de metais traço. A avaliação do dano testicular foi levada a cabo através da análise da densidade volumétrica relativa de diferentes estágios celulares espermatogénicos, de espaço intersticial e espaço luminal, juntamente com uma avaliação qualitativa do dano nos túbulos seminíferos e com a quantificação de células espermatogénicas em apoptose (*TUNNEL assay*). A proporção de espaço intersticial foi significativamente superior nos ratos de ambas as explorações em relação ao grupo controlo, sendo superior no grupo da exploração convencional, enquanto a proporção de espermátides tardias e espermatozoides foi significativamente inferior neste mesmo grupo, o qual não só apresentou um número significativamente superior de túbulos seminíferos desprovidos de espermatozoides e com evidências de dano estrutural, mas também uma quantidade significativamente superior de células apoptóticas, em comparação com os outros dois grupos. Estes resultados sugerem que ambas as práticas agrícolas, especialmente a agricultura convencional, originam dano testicular em ratos que se encontram naturalmente e cronicamente expostos a ambientes agrícolas enriquecidos em metais traço, confirmando a adequação da espécie *Mus musculus* como bioindicadora dos potenciais efeitos e consequentes riscos para a fertilidade masculina de agricultores continuamente expostos a esses ambientes.

Palavras-chave: Apoptose; Agricultura; Dano testicular; Espermatogénese.

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1) Introduction

1.1. Framework

Agricultural and livestock provisions remain the basis of the human nutrition pyramid, just as they have been in the past millennia, and will predictably continue so in the foreseeable future, despite existing efforts in establishing alternative nourishing supplies, since global human population is expected to grow by over a third until 2050, according to FAO's 2009 estimates. In fact, the fast-paced human technological advent, subsequent to the western Industrial revolution, has introduced an incessant loop: increasing food availability allowed an overall enhanced nutritional state of human populations, leading to their growth. The additional food demand then triggered the intensification and sophistication of agricultural practices, along with further land occupation for farming purposes, with increasingly worrying environmental impacts, left unattended for centuries.

While addressing one of the most basic challenges to its survival and in order to sustain its exponential growth, humanity's efforts, at the expense of environmental disruption, raised many other subsequent challenges that required an integrated harmonious approach. Fortunately, in the middle of the XX century, influential work and irreducible stances from some notable researchers, in face of fierce opposition, helped raising awareness on anthropogenic impacts upon the environment, namely Rachel Carson, with her famous book *Silent Spring*, which ultimately led to DDT banning from all agricultural usage in the USA, or Clair Patterson's campaign against lead contamination, with his 1965 publication *Contaminated and Natural Lead Environments of Man*, whose efforts ultimately led to the widespread removal of lead as additive from all standard automotive gasoline in the USA, by the end of 1986. The public health implications of these and other studies served as remarkable examples for future researchers and encouraged the establishment of modern ecotoxicology's foundations. Nowadays, special concern with the extensive use of agrochemicals remains, such as pesticides and fertilizers, for raising plant and animal crop production efficiency, commonly associated with harmful ecosystemic effects.

Despite recent regulatory attempts, many hazardous agrochemicals are still used globally, due to the higher cost of production of safer alternatives with the same or improved efficiency. Therefore, one of the utmost challenges is to attain a level of sustainable agriculture and rationalization of natural resources usage that is enough to attend the continuously growing demand.

The context of the Azores islands is particularly pertinent within this ecotoxicological interest. Counting with an entrancing natural patrimony of volcanic origin, the Azores have depended on farming and livestock productions on its naturally fertile soils for centuries and even today, they constitute the root of the archipelago's economy, with a significant amount of local inhabitants working in the primary sector. Until recent decades, traditional agricultural practices were carried out mostly in smallholdings, where agrochemicals were unrestrictedly applied. Even with the contemporary implementation of EU directives for the application of agrochemicals, research is only beginning to be done in order to assess the real extent of the environmental impacts of farming practices in the islands, today and in the past, especially considering that volcanic soils are known to be particularly vulnerable to anthropogenic inputs (Parelho *et al.*, 2014). Therefore, studies should focus on potential negative impacts on local microbial, plant, animal communities and ultimately on health risks for the farmers under the somewhat overlooked prolonged occupational exposure to farming environments.

Given the traditional predominance of the male gender in farming activities, the vast amount of studies associating reduced male fertility with particular constituents of common synthetic agrochemicals, and the preponderance of the agriculture sector in Azores, the current study is pertinent and was designed in order to assess if, in fact, prolonged exposure to farming environments entails greater risks to male fertility, by studying the testicular damage in wild mice specimens (*Mus musculus*). Testicular damage was assessed by studying the relative volumetric density of different spermatogenic stages and of interstitial space, the diameter of seminiferous tubules, the seminiferous tubules injury (percentage of luminal area occupied by spermatozoa and germinal epithelium structural organization), and by quantifying apoptotic spermatogenic cells.

1.2. Background and pertinence

The widespread environmental contamination with human-made chemicals and its repercussions has been subject of increasingly concern amongst public health authorities and has motivated the establishment of several research lines (Monsees *et al.,* 2000). Amongst other biological functions, their effects upon reproduction, in wildlife and humans, have sparkled intense debate and from the broad array of compounds that could interfere with reproductive function, specially the highly sensitive male fertility, agrochemicals have deserved particular scrutiny (Mehrpour *et al.*, 2014). The testicle is considered one of the most vulnerable organs to environmental agents, particularly agrochemicals (Oliva *et al.,* 2001), that often act as testicular toxicants. Besides, agrochemicals are still heavily applied worldwide, persisting in the environment and have long lasting effects in biosphere (Bustos-Obregón, 2001; Bustos-Obregón and Hartley, 2008; Cheng *et al.*, 2011).

Beside the thousands of yearly deaths due to accidental poisoning, according to WHO, millions of people are potentially suffering from effects of agrochemicals, such as pesticides, some of them known to disrupt spermatogenesis primarily through low level acute or chronic environmental or occupational exposures (Perry, 2008). Many animal experimental studies have linked decreased reproductive capacity with even moderate to low exposure to endocrine-altering chemicals (Telisman *et al.*, 2000). However, research for cumulative effects of multiple compounds in low-levels, akin to an occupational exposure in a farm, is still not as well established (Mantovani *et al.*, 2008) in the literature as the vastness of studies associating particular agrochemical constituents to testicular damage. Most adverse consequences are described in animal studies performed on laboratory, following the administration of a single element in high-dose or short-term exposure, not applicable to practical situations (Damek-Poprawa and Sawicka-Kapusta, 2003; Pizent *et al.*, 2012).

Meanwhile, in the field, many toxicants with reported or suspected reproductive effects are still in regular commercial use, presenting exposure risks primarily to farmers (Bian *et al.*, 2004) and ending up mixed together in soils and water. Likewise, residential proximity to agricultural activity has been described to explain reproductive and developmental abnormalities in epidemiological studies (Mnif *et al.,* 2011). There is no doubt that agricultural activity is a major source of environmental contamination. But because agrochemical compounds are so diverse in their chemical structure and the bioactive ones address a multitude of biological pathways, it not easy to predict which exact mixtures or concentrations present the greatest risk (Mantovani *et al.,* 2008).

Thus, an interesting alternative approach in order to counteract such limitation is to use animal models that are naturally subjected to the same ecotoxicological context as bioindicators, in order to assess their internal doses of trace metals, known to be in the constitution of agrochemicals. Such doses should be effectively the result the total amount of what has been bioavailable out of the whole load that has been introduced into the surrounding environment, through all possible uptake routes and exposure sources, becoming the most relevant measure. Then, by quantifying biological parameters of effect, a much closer estimate of the occupational risk presented to human reproductive system under similar circumstances is attainable.

Mus musculus, the species chosen for this study, is frequently proposed in several studies as an adequate bioindicator for biomonitoring environmental pollutants, not only because it shares the same environmental exposure with humans, but also due to their phylogenetic proximity. Prominently important within the context of this study is the fact that they are in direct contact with soil's surface during most of their lifetime, being easily exposed to any deposited compounds and incorporating them through ingestion or dermal absorption. Being closer to the ground level implies mice they can be considered as animal sentinels, "organisms that react to an environmental contaminant before it affects humans" (van der Schalie, 1999), in the sense that any damaging effects might be detected at much lower levels than it would be required to prove an effect in humans.

1.3. Objectives

The main goal of this study is to assess the potential for male reproductive disruption of two different agricultural practices (conventional and organic farming) in contrast with a background scenario of a control site not associated with farming. Conventional farming resorts to intensive application of synthetic agrochemicals on soils (pesticides, synthetic and organic fertilizers), while in the certified organic farming, synthetic agrochemicals are not allowed and any soil amendment is limited to organic fertilizers. For that end, the common house mice (*Mus musculus*) is used in this study as a bioindicator species for observable effects of testicular damage on a set of histological and cellular parameters.

2) Material and methods

2.1. Fieldwork

2.1.1. Sampling sites location

Given the objectives of this study, two distinct farm properties, where different agricultural systems take place (therefore implying a dissimilar management of their respective soils that determines their impact upon the local environment) were selected for mice capture - a conventional farming (CF) site in São Roque civil parish, and an organic farming site (OF) in Capelas civil parish. In fact, both sites share key similarities that stand behind their selection. Besides being amongst the main producers of vegetables in São Miguel island, they belong to the same geological complex, denominated Picos Fissural Volcanic System (Figure 1) and thus share the same bedrock and pedological conditions. Also, both sites are located at a similar altitude (50-100m), minimizing rainfall variability between sites and rain shadow effects, and have been explored under the same farming system for at least a decade, being differentiated strictly by the abovementioned type of agricultural management, which involves the legally framed application of synthetic agrochemicals (pesticides and fertilizers) in CF, while in OF, such application is forbidden and any other soil amendments are restricted to organic fertilizers (Parelho *et al.*, 2014).

In order to ensure a control group of mice, guaranteed to live in the wild and isolated from any major known source of anthropogenic interference, in present or past, particularly from either agricultural system in question, a site was established inside a forest reserve of centennial cryptomeria, known as Pinhal da Paz and henceforward abbreviated as reserve control (RC) site. Pinhal da Paz is mostly a primeval area in Fajã de Cima civil parish, with no historical records of farming activity.

All three civil parishes (São Roque, Capelas and Fajã de Cima), where the designated sites are located, belong to the Ponta Delgada municipality, the largest of the Autonomous Region of the Azores, although remaining outside the radius of its central urban area. The CF site is the closest to the city center, being adjacent to a highway directly leading to the city, while OF site is the furthest one, on the opposite shore, consisting of a property from the residential area of Capelas civil parish. Pinhal da Paz is roughly linearly equidistant between both farming sites and, as natural reserve, is the most isolated and pristine area out of the three sites.

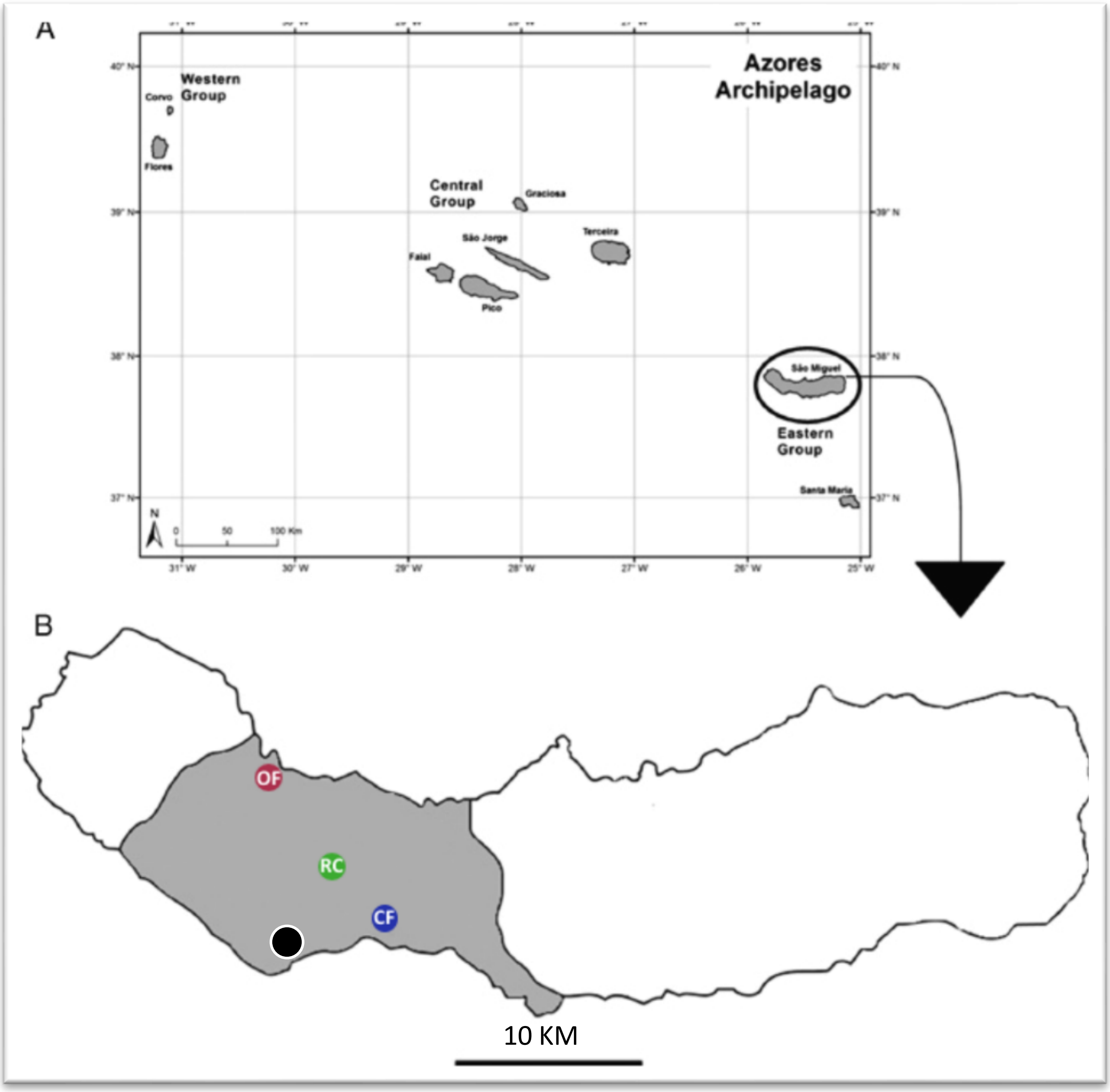


Figure 1 – Location of São Miguel island in the Azores archipelago (A) and of the three locations (B) where the studied groups of mice were captured [conventional farming (CF) site (São Roque), organic farming (OF) site (Capelas) and control (RC) site (Pinhal da Paz)]. Picos Fissural Volcanic System boundary (200km^2), derived from Moore (1990), is highlighted in grey. The black spot represents the location of Ponta Delgada city center. Adapted from Parelho *et al.*, 2014.

2.1.2. Sampling sites environmental description

The work and findings of a previous study by Parelho *et al.,* (2014), provide the best possible up to date description of the local environments of each farming site and the control site, where the studied mice groups were captured. The work by Parelho et al., (2014) explicitly reveals the impact on the soil composition of the different agricultural systems, not only the current agricultural pratices, but also over the course of the last couple of centuries in trace metal soil pollution, more so than any other previous study, considering the particular scope of the current study. This study manages to successfully provide a clear distinction between geogenic and anthropogenic contributions to soil trace metal contents and even detail the input of each farming system, which is the main discriminator factor between the mice groups in analysis. Also, this is one of the few studies of its kind, carried out for agricultural soils derived from volcanic parent bedrock materials, despite their intensive use for agricultural purposes.

It is imperative to note that volcanic soils are particularly susceptible to trace metals' accumulation. Due to their unique physicochemical properties and composition, volcanic soils possess a higher binding and retention capacity for certain trace metals. Concomitantly, volcanic soils, being naturally fertile, are very attractive for farming activities (Adamo *et al*., 2003). Therefore, volcanic soils are amongst the most vulnerable to anthropogenic inputs and are indeed preferentially under exhaustive exploration for such trace metal enriching activities, which means any organisms along the food chain, facing exposure with those soils, are at greater risk of potential toxic effects derived from trace metal contamination.

Within this particular ecotoxicological context established in volcanic soils under farming exploration, which is the case for both CF and OF sites, it makes sense to formulate the hypothesis that mice captured in farming sites are in fact greatly exposed to enriched soils and may express some of the observable biological effects broadly described in literature, regarding the male reproductive system, for several toxic metals.

Results from Parelho *et al.* (2014) for physicochemical properties showed that, in average, CF site's soils had lower pH value, higher electric conductivity and that agricultural soils in general had very low organic matter content in comparison with reference soils. The abundance of amorphous aluminosilicate minerals in these agricultural soils increases their metal binding properties, preferentially for Cu, Zn, Ni and Cd, while the acidic nature of CF soils might promote the bioavailability of these metals (Parelho *et al.*, 2014).

As for trace metals contents and respective source, most of their total loads were increased in CF soils, confirming its agricultural input, namely for Li, attributable to lithium perfluorooctane sulfonate in pesticide polymers, K and Mo, likely associated with intensive use of phosphorus inorganic fertilizers, and Cu and Zn, attributable to animal manure, while Cu is also frequently present in the chemical composition of pesticides and fungicides. Both Cu and Zn exceeded the maximum limit for agricultural soils. In addition to similar excessive loads of Cu and Zn, in comparison with CF soils, the OF soils are also enriched with greater loads of As, Cd and Pb, though only As concentrations differ significantly and none of the three the exceeds stipulated maximum limits for agricultural soils. These trace metal loads are attributable to continuing soil amendments, particularly of chicken and pig manure applications. Nonetheless, apart from these few, OF soils were depleted of all other considered trace metals. Finally, total trace metal loads for the RC site are within the average values for European volcanic soils, being lower than in both farming sites except for V and Ba (Parelho *et al.*, 2014).

In resume, V, Ba and Hg are mainly of geogenic origin, derived from volcanic parent materials, while Li, P, K, Cr, Mn, Ni, Cu, Zn, As, Mo, Cd and Pb loads can be recognised as products of agricultural inputs over the course of long-term intensive land exploration, with Li being tightly associated with CF practices, whereas in OF soils there is a depletion for the majority of trace metals, except of Cu and Zn, that are present in high loads (Parelho *et al.*, 2014). This confirmation of the impact of agricultural inputs in modulating the existing local environmental soil conditions is key to ensure the adequacy of each farming site for the objectives of this study.

2.1.3. Mice capture

A campaign to capture wild mice (*Mus musculus*) took place sequentially for each designated site, starting with the CF property in São Roque, between November and December of 2013. Next in line, already into 2014, was the OF property in Capelas, starting at late January until mid-March and. Mice capture in the control site, Pinhal da Paz, started in the last week of March and ended in the last day of May. The campaign lasted over half a year, a total of 7 months and crossing three seasons (from autumn 2013 to the following spring). In each site, the captures were not declared finished until the reunion of a whole group composed by 12 adult sexually mature male mice, confirmed so via laboratorial optical observation of live sperm cells present in epididymis. All captured individuals, seemingly immature by size, were released on site, while those with less than 10 grams of body weight were not considered in the study later on. Ultimately, a total of 36 male mice was obtained, 12 from each farming site and 12 from the control site.

Mice capture was carried out using live-catch mousetraps in which the trapped mouse would be held alive until the following morning. Special care was taken in order to insure that mousetraps were positioned in sheltered places, raising mice's chances of survival through the night, in face of the characteristically unstable and often harsh Azorean weather. Also, cheese baits were covered in peanut butter to increase their efficiency in capturing the mice.

Previously to their capture, it was not possible to control or measure the precise intensity and duration of contact of each group of mice with agricultural soils, since they were living unrestrictedly. However, the influence of the particular conditions in which mice were captured, in each designated site, should not be underestimated and should in fact be regarded as clues for estimating the relative degree of exposure. For instance, while at first all mousetraps were evenly deployed, covering a considerable area of the CF site, including open fields, a warehouse and greenhouses, the great majority of mice ended up being captured and were presumably living most of the time inside the farming greenhouses, likely due to warmth and food availability. Most mice captured in the OF site were inside a warehouse where harvested vegetables were stored before commercialization, presumably spending the majority of their lifetime isolated from the rest of the farm, with only a few mice being caught inside farming greenhouses or open fields, where manure is actually applied in the soils. In the control site, mice were also captured in specific areas, albeit no apparent plentiful source of food or artificial shelter was identified around, which implies these mice were probably living in harsher and wilder conditions than their CF and OF counterparts.

2.2. Laboratorial procedure

2.2.1. Organ extraction and processing

Once brought to the laboratory, all mice were euthanized with chloroform, weighed and then necropsied, for the extraction of the liver and both testicles along with their epididymides. The testicles were readily fixed in 4% buffered formaldehyde. The subsequent histological processing of the testicles followed a standard protocol, using ethanol as a dehydrator agent, xylene as clearing agent and paraffin wax as an embedding material. Later on, the slides were stained with haematoxylin and eosin. In the end, each slide consisted of ten or more 5-µm thick sections from one of the testicles. For each specimen, both testicles were analysed.

For each individual, the major portion of fresh liver (except one of the lobules, that was separated and fixed for further histological studies) was promptly weighed upon extraction and then oven dried for at least 7 days in a constant temperature of 80ºC for dehydration. Due to the fact that many specimens, regardless of their origin, had their livers parasitized, a large spectrum of noticeable liver integrity was observed, from jellylike consistency to apparently fully healthy. Nevertheless, the aforementioned procedure was uniform to all livers and none of them was excluded, unless the dry residue did not attain the necessary weight requirements for analytical study. After dehydration, the dry residue was retrieved, weighed and then enclosed for further quantification of trace and essential elements, some of which have their average concentrations present in **Table 2**. This quantification was made in Canada (Activation Laboratories Ltd., Canada) by mass spectrometry with inductively coupled plasma (ICP/MS). Quality control was assured by analysis of duplicate samples, blanks and reference materials (DOLT-3 and DORM-2).

Meanwhile, the epididymides were immersed in 5-ml of phosphate buffered saline (PBS) solution right after extraction and then macerated, promoting sperm cells' release into the solution. A drop from the extract was observed through optical microscopy, in order to ensure the presence and abundance of sperm cells. If no sperm cells were observed, the procedure was repeated at least three more times. The epididymal extract content served the purpose of confirming the male's sexual maturity but was not used further in this study.

Finally, with the intention of more accurately estimate the age of each male (as an estimative of their time of exposure to the local environment, eye lenses were extracted and then processed, according to the methods followed by Rowe *et al.*, (1985). The mathematical equation used for age estimation in this study is the following:

$$\log10\ (\text{age} + 20\ \text{days}) = 1{,}019 + 0{,}175\ (\text{paired lens weight})$$

It is important to underline that the results of this equation are estimates and not fully accurate ages, since the effect of nutrition on mammalian lens weight is uncertain (Rowe *et al.,* 1985), was not controlled under this study and was likely dissimilar between groups, while genetic diversity could increase lens weight variability in wild populations. Therefore the lens growth equation presented here should be regarded with caution (Rowe *et al.,* 1985).

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) and 86/609/EEC Directive and Portuguese rules (DL 129/92).

2.2.2. Apoptotic cell quantification

For each specimen, a sampling selection of 10 seminiferous tubules (5 per testis) was analysed for apoptotic expression by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) staining, using the DeadEnd™ Fluorometric TUNEL System (Promega, USA). During the fluorescence window, the tubules were photographed at a 200x magnification and the amount of apoptotic cells in each tubule was counted. A mean value of the number of apoptotic cells per individual was then calculated and used in statistical analysis.

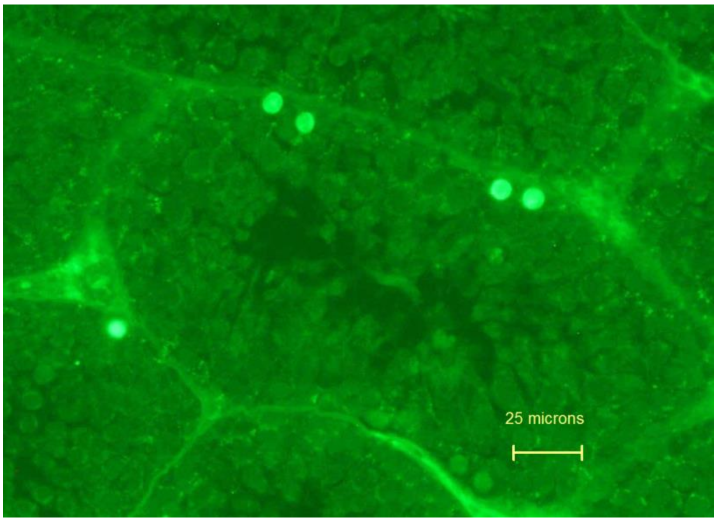


Figure 2 – Centred photomicrograph of a single seminiferous section from a specimen captured in the CF site (São Roque), containing 4 cells undergoing apoptosis, glowing in green (200x magnification; scale bar = 25 μm).

2.2.3. Stereological analysis

For each specimen, a total of 10 testicular histological sections (5 per testis) of the seminiferous tubules, stained with standard hematoxylin and eosin, were randomly chosen (except when severely disintegrated) for relative volumetric density determination. A custom stereological grid, consisting of 84 equidistant line segments, was then superimposed over the optical field of a light microscope at a 250x magnification and any time one of the designated elements crossed an end of a line segment, it would be marked. A maximum of 168 elements could be marked in each grid. The considered elements were: interstitial space;

spermatogonias; spermatocytes; round spermatids (early spermatids); elongated spermatids (late spermatids); sperm cells and luminal space. No distinction was made between spermatogonia A and B. The total number of times each element had been previously signalled in the 5 samples was determined and divided by the total of identified elements, thus generating a proportion for each testicle. For each individual, mean proportions for both testicles of the 7 designated elements were determined and then used in the statistical analysis. Due to the difficulty in accurately distinguishing between late spermatids and sperm cells, the proportions of both stages were combined into a single proportion.

2.2.4. Seminiferous injury evaluation

Each testicle of every specimen was analysed with 30 microphotographs, at a 200x magnification, centred on a single, randomly picked and transversally sectioned seminiferous tubule, present in the histological slides used for stereological analysis. All microphotographs were collectively analysed by the author, together with two other investigators, aware of the assignment, accordingly to two main criteria consensually established beforehand:

A- Presence of fully formed sperm cells in luminal space;
B- Evidence of major structural damage, not attributable to histological processing or any other conceivable source of artificial interference.

Evidence of structural damage, included in the second criteria, was considered in face of one or more of the following features:

- ✓ Macro vacuolization;
- ✓ Omission of one or earlier spermatogenic cellular stages;
- ✓ Exfoliation of the seminiferous epithelium;
- ✓ Low cohesion of the seminiferous epithelium;
- ✓ Detachment of the seminiferous epithelium from basal lamina;
- ✓ Generalized disintegration or disorganization of the seminiferous epithelium, with unclear delimitation between sequential stages.

It should be noted that, in cases where at least 10 sperm cells could be glimpsed in the luminal space, the first criterion was set to always prevail over the second, valuing the germinal efficacy of the observed tubule rather than its overall integrity, favouring a greater degree of delimitation of tubules resembling the worst case scenarios - compromised both structurally and functionally. Therefore, the conjugation of criteria A and B unfolds in 4 classifications, to which correspond the scores from 1 to 4, in descending order of seminiferous tubules integrity.

1- Relative abundance of sperm cells, comparatively with the total luminal area observed, in a relation of at least one fifth of the total area (including tails);
2- Observation of at least one sperm cell in the luminal area and simultaneous absence of any major evidence of structural damage;
3- Absence of sperm cells in luminal space and simultaneous absence of any major evidence of structural damage;
4- Absence of sperm cells in luminal space and simultaneous major evidence of structural damage

The collection of photomicrographs was uninterruptedly observed and assessed, resorting to the aforementioned scores, coherently attributed in accordance with the scenario each tubule best fit. All scores were consensually decided after deliberation and ensuing reasoning behind diverging assessments, until an agreement could be reached between the three assessors as to the ultimate score given. In the end, a combined total of 2160 seminiferous tubules were assessed.

In order to obtain a continuous data distribution for statistical analysis, a single value expressing the level of seminiferous injury of each individual was determined, according with the following expression, where letters a, b, c and d represent, respectively, the total number of tubules scored 1, 2, 3 and 4 in one of the testicles. Because 60 tubules were assessed for each individual, this index varies between 60 (all tubules scored 1) and 240 (all tubules scored 4). Therefore, a greater score implies a greater degree of overall seminiferous injury.

Seminiferous tubule injury index = $(a\times1+b\times2+c\times3+d\times4) + (a\times1+b\times2+c\times3+d\times4)$.

2.3. Statistical analysis

The main factors in analysis on this study were the two separate and unrelated farming sites where distinct agricultural practices are carried out, and the control site, where no agricultural activity was recorded in past or present day.

Testicular stereological proportions were compared between groups by one-way ANOVA, using farming sites (CF, OF and RC) as main factors, followed by Tukey HSD tests for paired comparisons, whenever ANOVA revealed significant differences ($P<0,05$) between data sets. Interstitial space data was successfully transformed with the expression ($\arcsin\sqrt{x}$) in order to comply with the assumption of normality and allow a parametric approach. The scores for seminiferous injury evaluation and the average number of apoptotic cells were compared, between each pair of groups, through non-parametric Kruskal-Wallis 1-way ANOVA pairwise comparison test.

One-way ANOVA followed by Tukey HSD tests or Kruskal-Wallis 1-way ANOVA pairwise comparison tests were also used to compare age and liver concentrations distributions. Some trace and essential elements data distributions were normalized by either Log10(x) or Log10(x+1). To verify the existence of any associations between pairs of studied variables, Pearson's or Spearman's rank correlations were done, depending on the compliance of subjected bivariate data pairs with the assumption of normality. Once again, transformed data was used whenever applicable. Because soil concentrations in trace and essential elements consisted of single total mean for each site, in relation with twelve individual liver concentrations of the same elements, only Spearman correlations were tested for the association between these two variables.

All statistical procedures were performed with IBM® SPSS® Statistics v22 for Windows (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.)

3) Results

3.1. Group characterization

Tables 1 and 2 summarize data regarding the characterization each group of mice. On average, mice from the control group are significantly older (F=9,286, df=2, *P*=0,001, 1-way ANOVA) and heavier (F=13,299, df=2, *P*<0,001, 1-way ANOVA) than the individuals from either CF site (age: *P*<0,001, Tukey-HSD test; weight: *P*<0,001, Tukey-HSD test) or OF site (age: *P*<0,001, Tukey-HSD test; weight: *P*<0,001, Tukey-HSD test), while both groups from farming sites have very similar and not significantly different ages and weights. The youngest specimen belongs to the OF site (31 days of age), while the oldest belongs to the control site (329 days of age). Bearing in mind the known lifecycle of *Mus musculus,* we can consider that generally all mice are relatively young yet old enough to achieve and sustain sexual maturity, an assumption that was then confirmed or denied, individually, by means of epididymal extract observation (section 2.2.1 – organ extraction and processing).

Each group was formed during a different season of the year, as explained above (section 2.1.3 – mice capture). Control group revealed the highest number of parasitized livers, with eight affected specimens out of twelve, while CF and OF groups had 3 and 5 parasitized specimens, respectively. Having in account that, much likely, the three groups didn't live with the same conditions of food availability, in order to get a rough indication of their nutritional state, a ratio between weight in grams and age in days was determined, for all individuals below 200 days old, given that most mice stop growing at between 4 and 6 months of age. Under the unverified assumption that their growing rate is linear, the control group has the lowest average ratio, while the OF group has the highest average ratio, though the ratios do not differ significantly between groups (F=0,322, df=2, *P*=0,727, 1-way ANOVA).

Table 1 – General characterization of each group of 12 mice from a conventional farming (CF) site (São Roque), organic farming (OF) site (Capelas) and the control (RC) site (Pinhal da Paz).

Origin	Period of capture	Mean (±SD) age (days)	Mean (±SD) weight (g)	Average Weight/Age
São Roque (CF)	11-12/2014	109,13±34,85	13,61±0,45	0,133
Capelas (OF)	01-03/2014	115,96±46,26	13,44±0,42	0,141
Pinhal da Paz (RC)	03-05/2014	191,75±69,26	17,04±0,74	0,121

For each mouse, the concentrations of 58 chemical elements were analytically determined in its liver. From those, twenty, all under the category of metals, except for B and As metalloids and non-metal Se, showed variable concentrations above the detection limit. From these 20, a few were selected and are shown in **Table 2** for being potentially involved in the observed differences in terms of testicular damage. Amongst other analysed elements which transversally fell below the detection limit, for all three groups, were Al, Be, Ho, P, V, Ni, Ba, Ag, Bi, Sn, Sb, Th and U. As a side note, only a few mice from CF site had measurable liver concentrations of Li (established as a tracer of agricultural contamination in conventional farming by Parelho *et al.,* 2014). Other elements, such as B, Na, Mg, K, Ca, Mn, Fe, Cs, Co, Zn, Rb, Cu, As, Cr and Sr, had variable concentrations between groups but their values were either not significantly different or were not particularly concerning or meaningful.

Table 2 – Mean (±SE) liver concentrations (mg.kg^{-1}, d.w.) for 4 analysed trace and essential elements in mice from a conventional farming (CF) site (São Roque), organic farming (OF) site (Capelas) and the control (RC) site (Pinhal da Paz). Significantly different means at *P*≤0,05 (Tukey-HSD tests/Kruskal-Wallis 1-way ANOVA pairwise comparison tests) are indicated with different letters.

Element (mg.kg^{-1})	CF	OF	RC
Cd	0,18±0,033 A	0,56±0,107 B	0,17±0,041 A
Hg	0,01±0,00 A	0,03±0,02 B	0,71±0,36 B
Se	1,74±0,267 A	2,03±0,268 A	4,06±0,237 B
Pb	1,017±0,26	0,700±0,14	0,467±0,04

From the elements in table 2, Cd (H=10,353, df=2, *P*=0,006, Kruskal-Wallis test), Hg (H=23,354, df=2, *P*<0,001, Kruskal-Wallis test) and Se (F=27,278, df=2, *P*<0,001, 1-way ANOVA) presented significant differences at *P*<0,05 between groups, with the control group detaining significantly higher mean concentrations of Se in relation to both CF (*P*<0,001, Tukey-HSD test) and OF (*P*<0,001, Tukey-HSD test) groups, while having significantly greater concentrations of Hg in relation with the CF group (H=-20,208, *P*<0,001, Kruskal-Wallis 1-way ANOVA pairwise comparison test). The OF group has significantly greater mean concentrations of Cd in relation to both CF (H=-11,718, *P*=0,017, Kruskal-Wallis 1-way ANOVA pairwise comparison test) and control (H=11,917, *P*=0,015, Kruskal-Wallis 1-way ANOVA pairwise comparison test) groups. Furthermore, mean hepatic concentrations of Pb are higher in CF group, though no significant differences were found.

3.2. Stereological parameters

Amongst the studied testicular elements, the proportion of interstitial space was significantly affected by the site (F=9,977, df=2, *P*<0,001, 1-way ANOVA). The proportion of interstitial space was significantly higher in both groups from farming sites than in the control group, being the highest in the CF group (0,144±0,009), although not significantly higher (*P*=0,167, Tukey-HSD test) than the OF site group (0,122±0,011). The control group revealed a significantly lower proportion of interstitial space (0,092±0,006) in relation to both CF (*P*<0,001, Tukey-HSD test) and OF groups (*P*=0,014, Tukey-HSD test) (Figure 3).

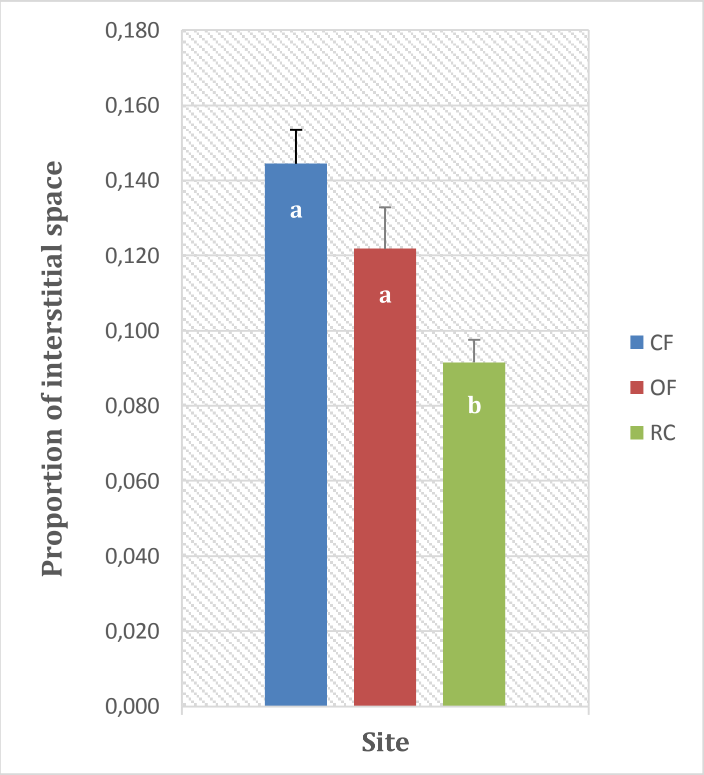


Figure 3 – Proportion (mean±SE) of testicular interstitial space of mice captured in a conventional farming (CF) site (São Roque), organic farming (OF) site (Capelas) and the control (RC) site (Pinhal da Paz). Distinct bar letters point out significant differences at P≤0,05 (Tukey-HSD tests).

In addition to interstitial space, the proportion of luminal space (mean+SE) was also affected by the site (F=3,443, df=2, P=0,044, 1-way ANOVA), accounting for a decrease in the OF group (0,183±0,014) in relation with both CF (0,207±0,019) and control groups (0,243±0,014), although the proportion only varies significantly in relation with the latter (P=0,035, Tukey-HSD test) and not with the former (P=0,537, Tukey-HSD test) (Figure 4). The CF and control site groups also do not differ significantly in their luminal space proportions (P=0,287, Tukey-HSD test).

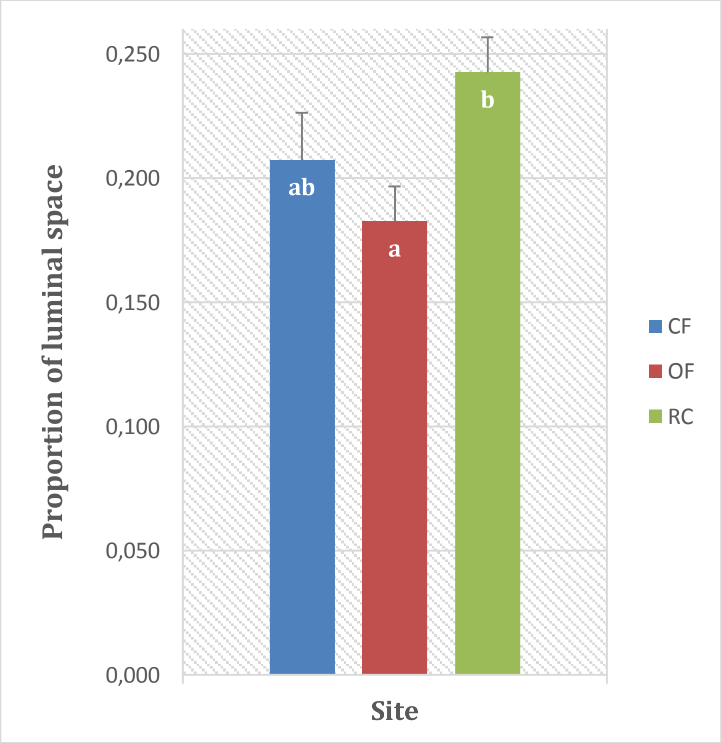


Figure 4 – Proportion (mean±SE) of tubular luminal space of mice captured in a conventional farming (CF) site (São Roque), organic farming (OF) site (Capelas) and the control (RC) site (Pinhal da Paz). Distinct bar letters point out significant differences at P≤0,05 (Tukey-HSD tests).

Finally, regarding the cellular elements, the only cellular stage of the studied germinal epithelium displaying significant variations between sites is the late spermatid-sperm cell combination (F=16,345, df=2, *P*<0,001, 1-way ANOVA). In average, mice specimens from OF site exhibit a higher combined count of late spermatids and sperm cells, resulting in a significantly greater proportion of the aforesaid combination (0,184±0,010) in relation to both CF (*P*<0,001, Tukey-HSD test) and control groups (*P*=0,028, Tukey-HSD test), that also differ significantly between each other (*P*=0,005, Tukey-HSD test), with the CF site holding the lowest proportion of late spermatids and sperm cells (0,112±0,012), while controls stand intermediate (0,149±0,004) (Figure 5).

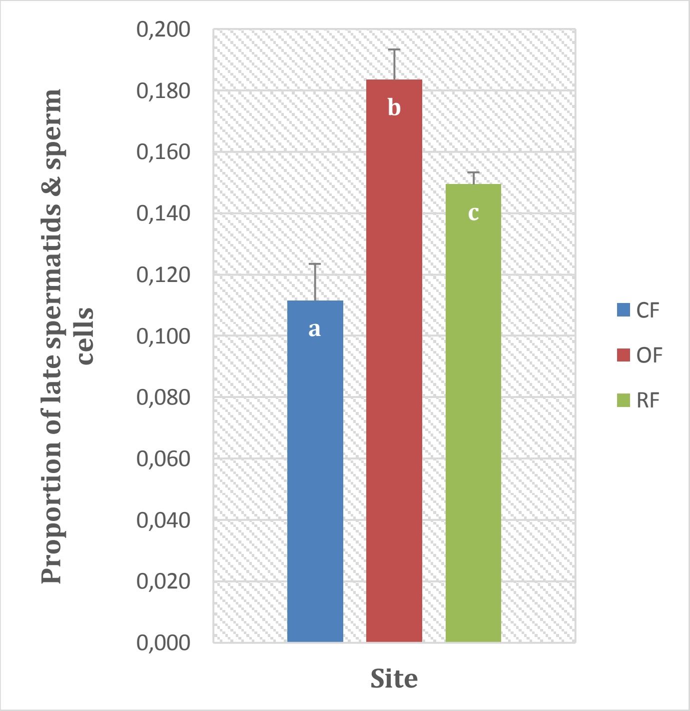


Figure 5 – Combined proportions (mean±SE) of late spermatid and sperm cells of mice captured in a conventional farming (CF) site (São Roque), an organic farming (OF) site (Capelas) and the control (RC) site (Pinhal da Paz). Distinct bar letters point out significant differences at P≤0,05 (Tukey-HSD tests).

The differences between groups concerning luminal space proportions mirror those found for the interstitial space (Figure 3 & Figure 4) and stand complementary to the combined late spermatid-sperm cell proportions (Figure 4 & Figure 5). A rise corresponding to higher counts of cells in their final stages of spermatogenesis in the specimens of the OF site is then followed by a decreased proportion of luminal space, while in the control group, the reverse happens: for control mice, lower counts of germ cells in later spermatogenic stages are followed by an increased proportion of luminal space, therefore producing the significant differences between OF and controls for the luminal space and later spermatogenic stages.

The remaining cellular elements belonging to the seminiferous epithelium, spermatogonia, spermatocytes and early spermatids, didn't display significant differences between the groups in analysis (Figure 6), with the overall proportions rising consistently from the earlier stage to the following within each group.

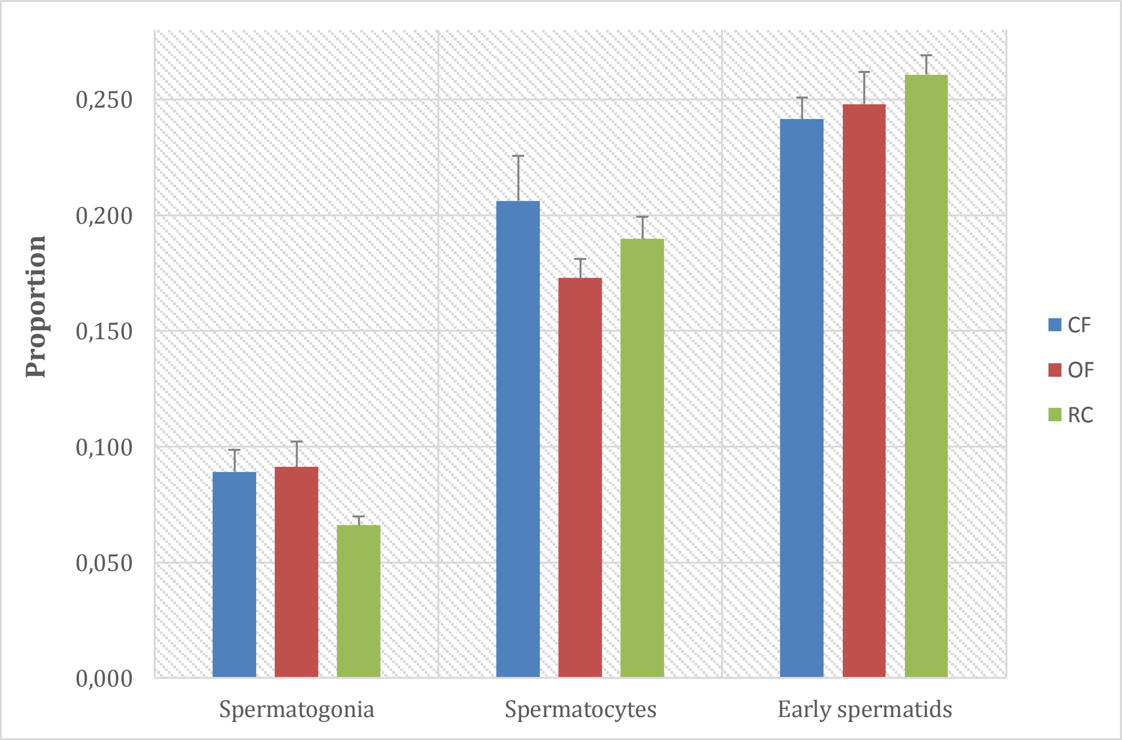


Figure 6 - Proportions of spermatogonia, spermatocytes and early spermatids of mice captured in a conventional farming (CF) site (São Roque), organic farming (OF) site (Capelas) and the control (RC) site (Pinhal da Paz).

3.3. Seminiferous injury evaluation

Significant differences were found for the distribution of seminiferous injury scores between groups (H=20,223, df=2, *P*<0,001, Kruskal-Wallis test). CF group showed a significantly higher average seminiferous injury index (the higher the value, the worst is the state of integrity of the seminiferous tubule) in relation to both OF group (H=18,133, *P*<0,001, Kruskal-Wallis 1-way ANOVA pairwise comparison test) and control group (H=14,125, *P*=0,002, Kruskal-Wallis 1-way ANOVA pairwise comparison test), while OF and control groups do not differ significantly between each other (Figure 7).

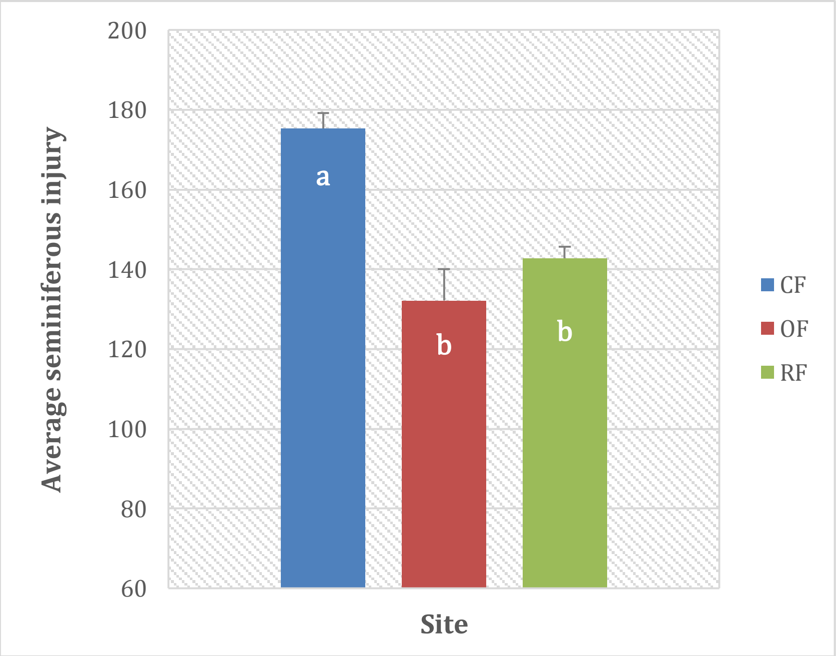
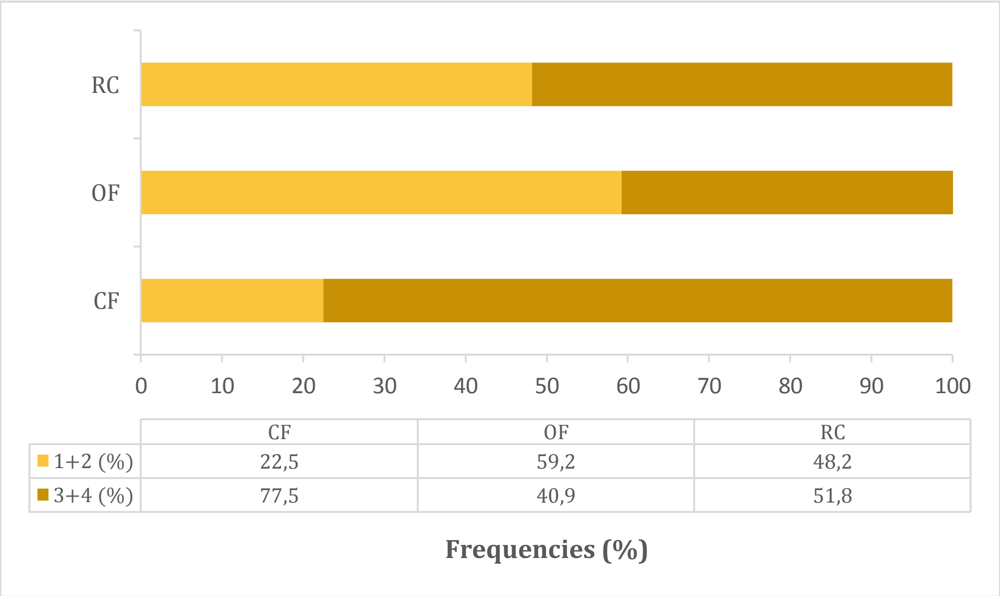


Figure 7– Average values (mean±SE) for seminiferous injury index in mice specimens from a conventional farming (CF) site (São Roque), organic farming (OF) site (Capelas) and the control (RC) site (Pinhal da Paz). Distinct bar letters point out significant differences at $P \leq 0{,}05$ (Kruskal-Wallis 1-way ANOVA pairwise comparison tests).

In fact, the combined frequency of the two highest histological scores (3 and 4) accounts for about 78% of all observations made for the CF group, whereas in the OF and control groups, that percentage drops to around 41% and 52%, respectively (Figure 8). Contrariwise, the lowest histological scores, indorsed upon the presence (2) or abundance (1) of sperm cells in the luminal space, totalizes about 23% of the observations made in the CF group, less than half to the observed in the OF group (59%) and in the control group (48%) (Figure 8).



	CF	OF	RC
■ 1+2 (%)	22,5	59,2	48,2
■ 3+4 (%)	77,5	40,9	51,8

Figure 8– Cumulative percentages of the lowest and highest two histological scores for seminiferous tubular injury in mice from a conventional farming (CF) site (São Roque), organic farming (OF) site (Capelas) and the control (RC) site (Pinhal da Paz). The histological scores (%) range from 1 to 4.

Photomicrographs of histological sections (200x) of seminiferous tubules, used in seminiferous injury evaluation, are shown in Figure 9. Each pair of photomicrographs constitutes a typical sample of each group of mice. The frequency of tubules lacking fully formed sperm cells in the luminal space, along with evidence of structural damage, such as epithelial disintegration and disorganization and macrovacuolization (A and B) in CF group, in comparison with OF (C and D) and control (E and F) groups, are amongst the main contributors to the higher average index values obtained by CF group in seminiferous injury evaluation, whose criteria are described in detail in section 2.2.4.

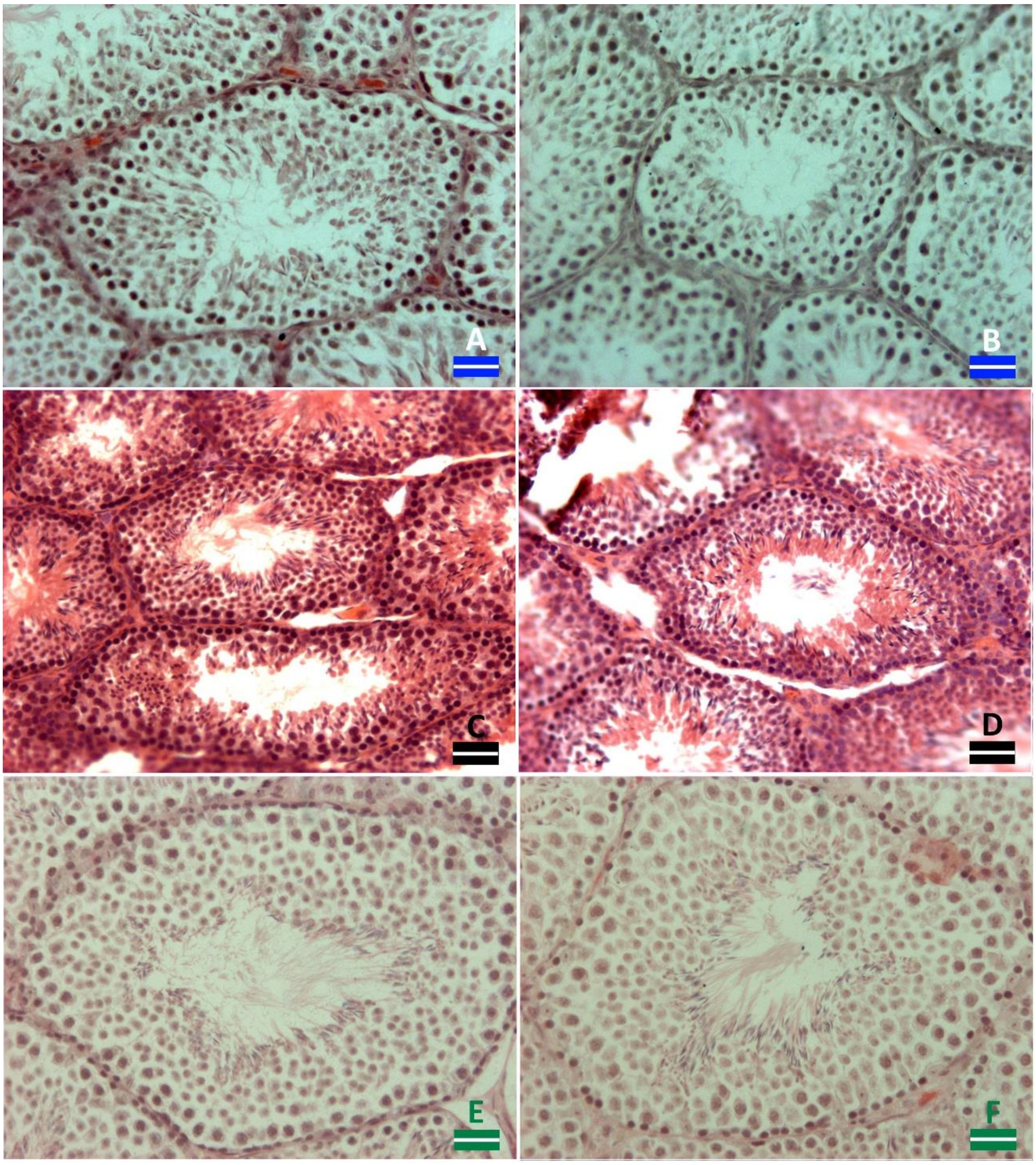


Figure 9 – Centred photomicrographs of transversally sectioned seminiferous tubules from mice captured a conventional farming (CF) site (São Roque) (A+B), organic farming (OF) site (Capelas) (C+D) and the control (RC) site (Pinhal da Paz) (E+F) (200x magnification; scale bar ≈ 25 μm).

3.4. Quantification of apoptotic cells

The number of apoptotic germ cells detected on the sampled seminiferous tubules was also greatly influenced by the site of capture, with significant differences between the 3 groups (H=18,583, df=2, *P*<0,001, Kruskal-Wallis test). The average count of detected apoptotic cells per individual in the CF group (36,75±7,95) is clearly higher than in the other two groups (OF=6,8±2,39;

control=2,92±0,98) (Figure 9). Pairwise comparison testing spotted a statistical significant difference between either OF specimens (H=13,233, *P*=0,003, Kruskal-Wallis 1-way ANOVA pairwise comparison test) or control site specimens (H=15,833, *P*<0,001, Kruskal-Wallis 1-way ANOVA pairwise comparison test) in relation to the CF specimens. The pairwise comparison between OF and control groups did not disclose any significant differences, although the average number of apoptotic cells per individual is decreased in control group. Also noteworthy is the fact that over 80% of the total amount of identified cells undergoing apoptosis was present in seminiferous tubules belonging to individuals captured on CF site.

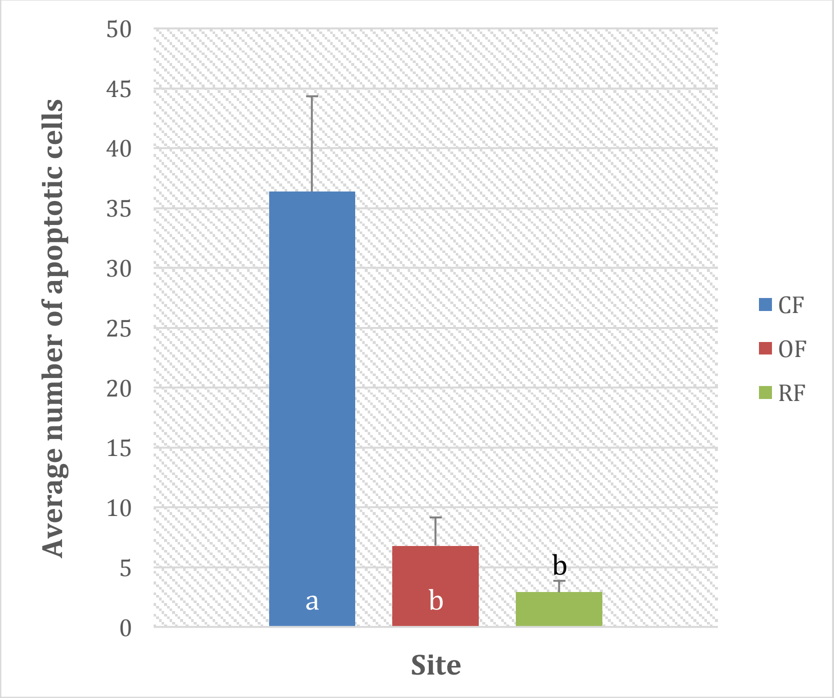


Figure 10– Average number (mean±SE) of apoptotic germ cells detected in mice specimens from a conventional farming (CF) site (São Roque), organic farming (OF) site (Capelas) and the control (RC) site (Pinhal da Paz). Distinct bar letters point out significant differences at *P*≤0,05 (Kruskal-Wallis 1-way ANOVA pairwise comparison tests).

3.5. Association between variables

3.5.1. Metal incorporation

Significant positive correlations between soil and liver contents were spotted for Pb, Cd, Se and Hg. (**Table 3**).

Table 3 – Spearman's rank coefficients for the significant correlations spotted between soil and liver concentrations of the quantified chemical elements, in mice from a conventional farming (CF) site (São Roque), an organic farming (OF) site (Capelas) and the control (RC) site (Pinhal da Paz).

Element	Spearman's correlation coefficients
Se	($\boldsymbol{\rho}$= 0,624; P< 0,001)
Cd	($\boldsymbol{\rho}$= 0,475; P= 0,003)
Pb	($\boldsymbol{\rho}$= 0,380; P= 0,022)
Hg	($\boldsymbol{\rho}$= 0,401; P= 0,015)

It should be remembered that mice captures were not evenly distributed between greenhouse soils and open field's soils, as referred in section 2.1.3. Therefore, individual liver concentrations were tested against not only the single data distribution constituted by the total mean values of all six soil samples for each site, but also with all other possible soil data distributions resulting from the combinations between greenhouse's and open field's samples. For instance, a positive correlation was detected for Cd when testing the liver data distribution against the soils' data distributions that only consider the mean value between replicate samples taken from greenhouse's soils in CF site. However, when doing the opposite or considering just the total mean value between all 6 soil samples for all sites, no significant correlation was detected. In addition to Cd, also Se does not correlate significantly with total mean values but do correlate with one or more specific data distributions, while Pb and Hg correlate in all cases.

3.5.2. Seminiferous parameters

Amongst all associations tested between seminiferous tubules parameters, a significant positive correlation was found between the average number of apoptotic cells per tubule (for each specimen) and its corresponding interstitial space proportion ($\boldsymbol{\rho}=0,604$, $P<0,001$, Spearman's correlation). In contrast, the combined proportion of early spermatids and sperm cells is negatively and significantly correlated with the average numbers of apoptotic cells ($\boldsymbol{\rho}=-0,503$, $P=0,003$, Spearman's correlation). Accordingly, the results also show that the occurrence of apoptotic cells correlate significantly and positively with seminiferous injury index ($\boldsymbol{\rho}=0,746$, $P<0,001$, Spearman's correlation).

Furthermore, not only early spermatids proportion, but also cells in later spermatogenic stages (later spermatids and sperm cells) and luminal space's proportions, all share a negative and significant correlation with the proportion of interstitial space (respectively, $R^2=-0,392$, $P=0,018$; $R^2=-0,395$, $P=0,017$; $R^2=-0,555$, $P<0,001$; Pearson's correlation). Contrarly, the first spermatogenic stages, both spermatogonia and spermatocytes, are positively with the proportion of interstitial space ($R^2=0,609$, $P<0,001$, Pearson's correlation). Moreover, these two first stages are negatively correlated with the later stages and luminal space proportions, specifically spermatogonia with earlier spermatids ($R^2=-0,447$, $P=0,006$, Pearson's correlation), spermatocytes with the combined proportion of later spermatids and sperm cells ($R^2=-0,478$, $P=0,003$, Pearson's correlation) and both of them with the luminal space (spermatogonia: $R^2=-0,533$, $P=0,001$; spermatocytes: $R^2=-0,386$, $P=0,020$; Pearson's correlation). At last, the stereological proportions of interstitial space and spermatocytes are significantly and positively correlated with seminiferous injury index (respectively: $\boldsymbol{\rho}=0,516$, $P=0,002$, Spearman's correlation; $\boldsymbol{\rho}=0,379$, $P=0,025$, Spearman's correlation), while the combined proportions of late spermatids and sperm cells are negatively and significantly correlated ($\boldsymbol{\rho}=-0,752$, $P<0,001$, Spearman's correlation).

4) Discussion

The current study consisted in the observation, analysis and quantification of testicular seminiferous parameters with the intent of statistically detecting differences between groups of mice from two different agricultural systems (conventional and organic farming) and a background scenario of a control site not associated with farming. It is important to introduce the discussion of results, as it has been noted in section 1.2 – background and pertinence, by remembering that, unlike a common dose-response laboratorial study, in a fieldwork ecotoxicological study, the integrity state of each individual, for the measured parameters, is determined by the infinite possible combinations between endogenous and environmental factors, not controlled previously to their capture.

What is certain is that each farming system contributes with different agrochemical inputs. As described in section 2.1.2 – sampling sites environmental description, soil trace metal loads quantified by Parelho *et al.*, (2014) provided an indication of what elements and in which amounts is each farming system contributing to the local soils' enrichment, an indirect measure of agrochemical application on soils. The quantified individual liver concentrations served as a biomarker of exposure, an attempt to understand how much of that environmental load could potentially be bioavailable, being incorporated by mice and eventually interfering with the established set of testicular parameters.

However, it should be kept in mind that these soil trace metal concentrations constitute a single parameter of a whole complex environment. Besides, metal toxicity in living organisms depends on several factors, such as its chemical form, level and duration of exposure, incorporation route and, host factors such as gender, age, immune status or nutritional state. In a complex and variable environment, there is an intrinsic difficulty in linearly attributing biological effects to specific chemicals. Additionally, internal doses of trace metals might even not be entirely relatable to the magnitude of the observed biological effects, since trace metals are one of many components of complex agrochemical mixtures, although the presence of metals such as lead and cadmium deserves consideration.

Nonetheless, this weakness, inherent to a fieldwork study is not totally incompatible with the essence of the study, which is to understand the extent to which the male reproductive ability in mice groups might be disturbed, during the course of their lifetimes, by the whole anthropogenically moulded environment around them, determined by different farming practices, allowing researchers to outline the risks implied for the fertility of farmers, occupationally subjected to the same farming environments, especially those working in greenhouses.

The results show significant differences in key aspects of mice testicular damage, which are overall consistent with each other. The distinct studied histological and cellular parameters share a transversally different response, depending and according to the farming site in question, suggesting that the ecotoxicological context present on the inhabited site is determinedly influencing the way these parameters react in each group of individuals.

The CF site group alone undisputedly, consistently and significantly underperforms in all studied seminiferous parameters, in comparison with both OF and control groups. The quantification of apoptotic cells in germinal epithelia, arguably the most sensitive and least subjective biomarker covered, shows that this group has a visibly greater amount of seminiferous epithelial cells undergoing apoptosis. Also, 78% of its seminiferous tubules are classified as 3 or 4, meaning that fully formed sperm cells were only sighted in the luminal space of less than a quarter of the sampled tubules. This lack of sperm cells is further confirmed with the stereological proportions, with only 11% of identified seminiferous elements being either late spermatids or sperm cells. Overall, results suggest that CF site's specimens indeed hold the worst state of testicular functionality out of the three groups. This is in accordance with many previous studies associating exposure to agrochemicals and evidence of decreased reproductive capacity (Astiz *et al.*, 2009; Joshi and Sharma, 2011; Martenies and Perry, 2013)

The seminiferous tubules injury index, coupled with stereological analysis, suggests that seminiferous tubules of CF mice are significantly compromised both structurally and functionally. The proliferation of interstitial fibrous tissue is commonly associated with chronic inflammation of the testicles (Meineke et al., 2000; Apa et al., 2002; Damek-Poprawa and Sawicka-Kapusta, 2003; Damek-Poprawa and Sawicka-Kapusta, 2004) 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), therefore contributing to defective testicular functionality. The results show a significant negative correlation between interstitial tissue and elements of seminiferous epithelium, such as round spermatids' proportions (which, in relative terms, share the greatest proportion across all sites), late spermatids, sperm cells and luminal space. Although no sperm load counts were performed, these parameters strongly suggest that, much likely, the fertility and consequent reproductive ability of CF mice is seriously diminished.

The severity of testicular damage described for CF mice might have been determined by extensive direct exposure to contaminated environments during their lifetime. Most of them were captured inside farming greenhouses and belonged to local mice populations living inside its soils, in small burrows and tunnels on the soft, chemically treated agricultural soils, probably seeking the greenhouses' food availability, shelter and warmth. A greenhouse creates a delimited, confined area that is favourable to contaminant accumulation (Hanke and Jurewicz, 2004) in soils, water puddles and even in the interior atmosphere, since air flow is restricted and soils are guarded from rainfall or mixture with untreated soils. Previous studies have concluded that exposure to pesticides is particularly disruptive to workers in closed areas of greenhouses (Abell et al., 2000; Petrelli and Figà-Talamanca, 2001; Slimani *et al.*, 2011).

A particular trace metal that seems to fit within this confined context is Pb. An average concentration of 1 ppm of Pb was quantified in CF group livers', which is positively correlated with the soil concentrations. Although not significantly different from controls by a thin margin, it is still twice as much. Just like with the control group (0,467±0,04 mg.kg^{-1}), background values for Pb in healthy mammals have been reported before at generally 0,5 ppm or less (Penumarthy et al., 1980), while studies performed with mice and other rodents refer to 1 ppm or below as a common range for background values (Meador, 1996), which suggests that concentrations of 1 ppm are the threshold above which incorporation from environmental sources might be considered. Occupational exposure to Pb is frequently associated with adverse effects on spermatogenesis in animals and humans, resulting in decreased sperm quality counts and abnormal sperm cell frequencies (Alexander *et al.*, 1996; Telisman *et al.*, 2000).

The fact that no significant differences were found for the hepatic concentrations of Pb, broadly associated with testicular injury and reduced fertility in the literature (Damek-Poprawa and Sawicka-Kapusta, 2003; Pillai *et al.,* 2012;) as it would be expected in a scenario of single dose administration, where significant differences are evident in terms of corresponding biological response (Batra *et al.*, 2001), such as the ones verified for the CF group in this study, further reinforces the idea that overall observed differences are the net result of the combined influence of several detrimental contaminants, rather than a single trace element in critical amounts. Much likely, by living in permanent contact with the farming greenhouses' soils, CF mice group are chronically subjected to a vast array of compounds with spermatogenic impairing properties, ever since the moment of their conception. Consequently, it is plausible that they start suffering seminiferous damage since very early age. Previous studies in humans have suggested that exposure to the same concentration of organophosphorous compounds found in pesticides produce more impact on growing infants than on adults, therefore placing them at greater vulnerability and highlighting that seemingly safe concentrations might in fact be enough to adversely affect human reproductive function, as reviewed by Peiris-John and Wickremasinghe, (2008).

Furthermore, age decline does not appear to have influence in the marked evidences of testicular damage in CF mice. In fact, this group has the lowest average age out of the three, at 109 days, a little less than 4 months, which can be considered young adults, since generally mice need between 1 and 2 months to sexually mature (Lehmann and Löwel, 2008). Besides, it is an average age much similar to OF group and significantly inferior to the control group, which might be older because they are from a natural reserve, living in the wild and not subjected to rodent control campaigns. Age is negatively correlated with all parameters, which means it can be ruled out as an interfering factor aiding to the seminiferous disruption. This further supplements the premise that the differences observed in the studied parameters are primarily determined by the peculiar ecotoxicological context prevailing on each site, during the course of the specimens' lifetime.

Wrapping up and reconstructing the whole chain of events for CF group, a compelling scenario that best fits the data at hand could be put this way. In an environment where conventional agricultural practices (known to be a major source of anthropogenic contamination) are carried out, a mice population is presumably continually exposed to synthetic compounds, which may be interfering with their male reproductive capacity. This interference is expressed not only in the atrophy and degeneration of the germinal epithelium, but also in fibrosis, expressed by the significantly greater proportions of fibrous interstitial tissue, all signs of chronic inflammation. Eventually, as result of oxidative stress (Kasahara *et al.,* 2002; Aitken and Baker, 2006; Pandya *et al.*, 2012), germ cell survival and proliferation is negatively affected, as evidenced by the amount of apoptotic cells (Wang *et al.*, 2003; Tremellen, 2008), determining a gap between earlier and later stages, given that a significantly greater percentage of early spermatogenic cells do not survive until later stages (Batra *et al.,* 2001), explaining the meagre amount of late spermatids and depletion of sperm cells in luminal space, in relation to OF and control groups. Naturally, because these cell and tissue responses happen in face of the same discriminating factor (farming practices), all correlations between seminiferous parameters are consistent with each other, namely the positive correlations between apoptotic cells, interstitial space proportions, earlier spermatogenic stages proportions, seminiferous injury scores and, inversely, the negative correlations with later spermatogenic stages' proportions.

Meanwhile, the general performance of the OF group in most parameters appears closer to that of the control group. This might not disconnected from the fact that most mice were not captured in the same confined conditions of a greenhouse. The level of exposure of these mice to any contaminants derived from organic practices is presumably lower than the verified for CF site's specimens. However, the significantly greater proportion of interstitial fibrous tissue (Bomhard *et al.,* 1987; Gouveia, 1988), in relation to controls, should not be overlooked and neither should be the significantly higher hepatic concentration of cadmium and its correlation with the soil concentrations of cadmium (table 3). As indicated by Parelho *et al.*, 2014, organic fertilizers are a considerable source of cadmium and organic farms rely solely on soil amendments based on compost and manure. Kramárová et al., (2005) measured the accumulation of cadmium in the liver and kidneys of wild mammals, in comparison with farm mammals and found similar greater average concentrations (0,48 ppm) of hepatic cadmium in farm hares. Blood-testis barrier and spermatogenic cells have been shown to be extremely sensitive to cadmium toxicity (Benoff *et al.*, 2000; Thompson and Banningan, 2008), even at considerably low doses (Siu *et al.*, 2009). Cd is equally known to accumulate in biological tissues for a long period of time (Siu *et al.*, 2009).

Many of the sampled tubules of OF mice did indeed reveal some signs of damage associated by other authors with Cd toxicity, particularly frequent leakage of erythrocytes into the interstitial space, caused by the amply described disruption of tight junctions, sometimes coupled with a very thick fibrous capsule and generally an evident state of testis atrophy and weight loss (Siu *et al.*, 2009). It's expected that these evidences of testicular injury did not translate so well into parameters other than interstitial space proportion because the supreme criteria for seminiferous injury evaluation was the abundance of sperm cells in luminal space, even when structural damage was simultaneously present, and also because the interstitial space was not taken into account in that evaluation, but only the seminiferous tubule itself. Future seminiferous tubules injury assessments should be more sensitive to extra tubular structural damage.

The high concentrations of hepatic Hg in control mice are more pronounced in a few exemplars, whose concentrations surpassed the 4 ppm, as revealed by the standard error of the mean group concentration in ppm (0,71±0,36). Still, it's a high average value in comparison with the other two groups. Parelho *et al.*, 2014 analysis did not identify Hg as an anthropogenic input in agricultural volcanic soils but rather as a trace metal derived from volcanic parent materials. Table 3 shows there is a significant positive correlation between soil and liver concentrations of Hg, suggesting bioavailability. Being older in average, it is reasonable to assume that the control group have had a longer record of incorporating naturally occurring Hg from soils. Also, pristine soils should have concentrations of Hg that closely resemble the natural background inherent to volcanic soils, since they have not been altered by farming practices.

The decreased proportion of late spermatids and sperm cells, in comparison with the OF group, might be partially attributable to Hg, since Hg has been associated with spermatogenesis disruption (Homma-Takeda *et al.*, 2001; Boujbiha *et al.*, 2011). But apart from that, no other signs of testicular damage are apparent in results. Previous studies have reported that the formation of a complex Se-Hg is common in soft tissues of mammals, such as liver, resulting in a high correlation between the occurrences of both elements (Endo *et al.*, 2002). As a matter of fact, table 2 shows a significantly increased hepatic concentration of Se in controls as well, twice as much than OF group, suggesting the Se-Hg association. Also, the ability of Se to complex with Hg and reduce its toxicity has been extensively investigated (Agarwal and Behari, 2007). The presence of Se reduces Hg accumulation in kidney but increases it in other tissues, especially in the liver (Cuvin-Aralar and Furness, 1991), while reducing its urinary and fecal excretion, the main pathway for elimination of inorganic Hg (Agarwal and Behari, 2007; Fang, 1977).

In accordance with this, it is possible that not only some mice from the control group have been incorporating the geogenically occurring Hg from volcanic soils, but also have been accumulating it in the liver due to the formation of the Se-Hg complex, which counteracts Hg toxicity, at least in modest doses (Agarwal and Behari, 2007) and therefore, might lessen expectably worse detrimental effects upon seminiferous germ cells, usually associated with Hg. Similar protective roles have been described for zinc as well in protecting against testicular damage induced by Hg (Orisakwe *et al.*, 2001). So in short, if this is the case, Hg accumulation might be involved in the decreased amount of late spermatids and sperm cells, although the formation of a complex with Se is suggested to preventing more severe damage.

In addition, the decreased proportion of later spermatogenic stages in control mice might also be explained by their apparently poorer nutritional state, as shown in table 1. Most control mice have their livers parasitized and their average weight/age ratio is the lowest. While this is a very gross indication, much likely the harsher wild conditions of the natural reserve provide less food availability, in comparison with OF specimens, which coupled with liver parasitization, might lead to a worse nutritional condition that is not favourable to cell proliferation.

However, since no other major signs of damage are evident in control group, which has the lowest mean value of apoptotic cells, less than 0,10 of interstitial space proportion and an average value for seminiferous injury that is significantly lower than CF group and does not differ significantly from OF group, results seem to clearly support the hypothesis that farming, particularly conventional farming, enhances testicular damage, potentially decreasing male reproductive capacity.

5) Main conclusions

In face of the significant deteriorated state of testicular integrity in mice captured on a conventional farm, it is possible to conclude, first and foremost, that *Mus musculus* performed well as an adequate bioindicator species of the hypothesised potential for testicular damage and ultimately decreased male fertility following chronic exposure to contaminated farming environments, by means of stereological analysis, quantification of apoptotic cells and scoring assessment of seminiferous injury encompassing structural aspects and functional impairment.

Relative volumetric density determination revealed significantly increased proportions of interstitial space in mice from both farming sites. Mice from the conventional farm had the greatest amount of interstitial space, followed by mice from the organic farm, each one significantly greater than controls, suggesting that the proliferation of fibrous tissue and parenchymal reduction in testicles is a consequence of chronic exposure to contaminants derived from either agricultural practices, mostly conventional farming.

Meanwhile, mice from the organic farm have a significantly higher combined proportion of late spermatids and sperm cells than CF group and even than controls themselves. The quantification of apoptotic cells adequately fits stereological data, since a significantly greater average number of epithelial cells were undergoing apoptosis in CF group's seminiferous tubules. The negative association between late spermatids and sperm cell proportions and the amount of apoptotic cells suggests that the environmental context of the conventional farm is compromising the viability of spermatogenesis and sperm cell formation, by promoting an increased cellular death rate of the pre-meiotic stages, which in turn results in decreased amounts of post-meiotic stages.

To reinforce this suggestion, seminiferous injury confirms the previous cellular assessments, with CF group presenting a significantly higher frequency of sampled seminiferous tubules depleted of sperm cells and overall significantly higher seminiferous injury score, which is negatively correlated with the stereological proportions of post-meiotic stages and positively correlated with the average amount of apoptotic cells.

While data is consistent in suggesting that testicular differences in studied groups are mainly attributable to the ecotoxicological context of each site, determined by anthropogenic influence of farming practices, nevertheless, it should be noted that the level and duration of exposure of CF mice, in general, was intense and permanent, probably since fetal development. The same could not be verified for OF mice. Therefore, CF mice might provide a better ideal representation of testicular damage effects, in face of continued exposure to farming environments, than OF mice. If CF mice were as old as the control group mice, probably their degree of testicular damage would be even greater.

In future studies, it should be interesting to focus on the Hg incorporation from pristine volcanic soils and determine if they also pose risks to male fertility.

In general, this study further highlights the described potential damaging effects on male reproductive system and consequent risks involved for the fertility of farmers that are continually exposed to agrochemicals and trace metal enriched farming environments, especially those that deal directly with the application of agrochemicals.

6) References

Abell, A., Ernst, E., and Bonde, J.P. (2000). Semen quality and sexual hormones in greenhouse workers. *Scandinavian Journal of Work, Environment & Health*, *26*, 492–500. doi: 10.5271/sjweh.573

Adamo, P., Denaix, L., Terribile, F., and Zampella, M. (2003). Characterization of heavy metals in contaminated volcanic soils of the Solofrana river valley (southern Italy). *Geoderma 117*, 347–366. doi: 10.1016/S0016-7061(03)00133-2

Agarwal, R., and Behari, J.R. (2007). Role of selenium in mercury intoxication in mice. *Industrial Health*, *45*, 388–395. doi: 10.2486/indhealth.45.388

Aitken, R.J., and Baker, M.A. (2006). Oxidative stress, sperm survival and fertility control. *Molecular and Cellular Endocrinology*, 250, 66–69.
doi: 10.1016/j.mce.2005.12.026

Alexander, B.H., Checkoway, H., van Netten, C., Muller, C.H., Ewers, T.G., Kaufman, J.D., Mueller, B.A., Vaughan, T.L., and Faustman, E.M. (1996). Semen quality of men employed at a lead smelter. *Occupational and Environmental Medicine*, *53*, 411–416. doi: 10.1136/oem.53.6.411

Apa, D. d., Çayan, S., Polat, A., and Akbay, E. (2002). Mast Cells and Fibrosis on Testicular Biopsies in Male Infertility. *Systems biology in reproductive medicine*, *48*, 337–344. doi: 10.1080/01485010290099183

Astiz, M., de Alaniz, M.J.T., and Marra, C.A. (2009). The impact of simultaneous intoxication with agrochemicals on the antioxidant defense system in rat. *Pesticide Biochemistry and Physiology*, *94*, 93 – 99. doi: 10.1016/j.pestbp.2009.03.005

Barth, A.D., Alisio, L., Avilés, M., Arteaga, A., Campbell, J.R., and Hendrick, S.H. (2008). Fibrotic lesions in the testis of bulls and relationship to semen quality. *Animal Reproduction Science,106*, 274–288.
doi: 10.1016/j.anireprosci.2007.05.002

Batra, N., Nehru, B., and Bansal, M.P. (2001). Influence of lead and zinc on rat male reproduction at 'biochemical and histopathological levels'. *Journal of Applied Toxicology*, *21*, 507–512. doi: 10.1002/jat.796

Benoff, S., Jacob, A., and Hurley, I.R. (2000). Male infertility and environmental exposure to lead and cadmium. *Human Reproduction Update*, *6*, 107–121.
doi: 10.1093/humupd/6.2.107

Bian, Q., Xu, L.C., Wang, S.L., Xia, Y.K., Tan, L.F., Chen, J.F., Song, L., Chang, H.C., and Wang, X.R. (2004). Study on the relation between occupational fenvalerate exposure and spermatozoa DNA damage of pesticide factory workers. *Occupational and Environmental Medicine*, *61*, 999–1005.
doi: 10.1136/oem.2004.014597

Bomhard, E., Vogel, O., and Löser, E. (1987). Chronic effects on single and multiple oral and subcutaneous cadmium administrations on the testes of Wistar rats. *Cancer Letters*, *36*, 307–315. doi: 10.1016/0304-3835(87)90024-3

Boujbiha, M.A.M., Hamden, K., Guermazi, F., Bouslama, A., Omezzine, A., and Feki, A.E. (2011). Impairment of Spermatogenesis in Rats by Mercuric Chloride: Involvement of Low 17β-Estradiol Level in Induction of Acute Oxidative Stress. *Biological Trace Element Research*, *142*, 598–610.
doi: 10.1007/s12011-010-8774-2

Bustos-Obregón, E. (2001). Adverse effects of exposure to agropesticides on male reproduction. Acta Pathologica Microbiologica et Immunologica Scandinavica (APMIS), *109*, S233–S242. doi: 10.1111/j.1600-0463.2001.tb05772.x

Bustos-Obregón, E., and Hartley, B. (2008). Ecotoxicology and testicular damage (Environmental chemical pollution). A review. International Journal of Morphology, 26(4), 833-840. doi: 10.4067/S0717-95022008000400009

Cheng, C.Y., Wong, E.W., Lie, P.P., Li, M.W., Su, L., Siu, E.R., Yan, H.H., Mannu, J., Mathur, P.P., Bonanomi, M., Silvestrini, B., and Mruk, D. (2011). Environmental toxicants and male reproductive function. *Spermatogenesis,* 1, 2–13.
doi: 10.4161/spmg.1.1.13971

Creasy, D., Bube, A., Rijk, E. de, Kandori, H., Kuwahara, M., Masson, R., Nolte, T., Reams, R., Regan, K., Rehm, S., et al. (2012). Proliferative and Nonproliferative Lesions of the Rat and Mouse Male Reproductive System. *Toxicologic Pathology*, *40*, 40S – 121S. doi: 10.1177/0192623312454337

Cuvin-Aralar, M.L., and Furness, R.W. (1991). Mercury and selenium interaction: a review. *Ecotoxicology and Environmental Safety*, *21*, 348–364.

Damek-Poprawa, M., and Sawicka-Kapusta, K. (2003). Damage to the liver, kidney, and testis with reference to burden of heavy metals in yellow-necked mice from areas around steelworks and zinc smelters in Poland. *Toxicology*, *186*, 1–10.
doi: 10.1016/S0300-483X(02)00595-4

Damek-Poprawa, M., and Sawicka-Kapusta, K. (2004). Histopathological changes in the liver, kidneys, and testes of bank voles environmentally exposed to heavy metal emissions from the steelworks and zinc smelter in Poland. *Environmental Research*, *96*, 72–78. doi: 10.1016/j.envres.2004.02.003

Endo, T., Haraguchi, K., and Sakata, M. (2002). Mercury and selenium concentrations in the internal organs of toothed whales and dolphins marketed for human consumption in Japan. *Science of The Total Environment*,*300*, 15–22.
doi: 10.1016/S0048-9697(02)00137-7

Fang, S.C. (1977). Interaction of selenium and mercury in the rat. *Chemico-Biological Interactions, 17*, 25–40. doi: 10.1016/0009-2797(77)90069-2

Gouveia, M.A. (1988). The testes in cadmium intoxication: morphological and vascular aspects. *Andrologia*, *20*, 225–231.
doi: 10.1111/j.1439-0272.1988.tb01061.x

Hanke, W., and Jurewicz, J. (2004). The risk of adverse reproductive and developmental disorders due to occupational pesticide exposure: an overview of current epidemiological evidence. *International Journal of Occupational Medicine and Environmental Health*, *17*, 223–243.

Homma-Takeda, S., Kugenuma, Y., Iwamuro, T., Kumagai, Y., and Shimojo, N. (2001). Impairment of spermatogenesis in rats by methylmercury: involvement of stage- and cell- specific germ cell apoptosis. *Toxicology*, *169*, 25–35. doi: 10.1016/S0300-483X(01)00487-5

Joshi, S.C., and Sharma, P. (2011). Male reproductive toxicity of organophosphorous compounds: a review*. Toxicological & Environmental Chemistry, 93*, 1486–1507.
doi: 10.1080/02772248.2011.581874

Kasahara, E., Sato, E., Miyoshi, M., Konaka, R., Hiramoto, K., Sasaki, J., Tokuda, M., Nakano, Y., and Inoue, M. (2002). Role of oxidative stress in germ cell apoptosis induced by di(2-ethylhexyl)phthalate. *Biochemical Journal*, 365, 849-856.
doi: 10.1042/BJ20020254

Kramárová, M., Massanyi, P., Jancova, A., Toman, R., Slamecka, J., Tataruch, F., Kovacik, J., Gasparik, J., Nad, P., Skalická, M., Koréneková, B., Jurcik, R., Cubon, J., and Hascik, P. (2005). Concentration of cadmium in the liver and kidneys of some wild and farm animals. *Bulletin of the Veterinary Institute in Pulawy*, *49*, 465.

Lehmann, K., and Löwel, S. (2008). Age-Dependent Ocular Dominance Plasticity in Adult Mice. *PLoS ONE*, *3(9)*, e3120. doi: 10.1371/journal.pone.0003120

Mantovani, A., Maranghi, F., La Rocca, C., Tiboni, G., and Clementi, M. (2008). The role of toxicology to characterize biomarkers for agrochemicals with potential endocrine activities. *Reproductive Toxicology*, *26*, 1–7.
doi: 10.1016/j.reprotox.2008.05.063

Martenies, S.E., and Perry, M.J. (2013). Environmental and occupational pesticide exposure and human sperm parameters: A systematic review. *Toxicology*, *307*, 66 – 73.
doi: 10.1016/j.tox.2013.02.005

Meador, J.P. (1996). *Environmental Contaminants in Wildlife: Interpreting Tissue Concentrations*. Boca Raton, Florida: Lewis Publishers (494 pp).
ISBN: 156670071X
Accessed from: books.google.pt/books?isbn=156670071X

Mehrpour, O., Karrari, P., Zamani, N., Tsatsakis, A.M., and Abdollahi, M. (2014). Occupational exposure to pesticides and consequences on male semen and fertility: A review. *Toxicology Letters, 230(2)*, 146-156.
doi: 10.1016/j.toxlet.2014.01.029

Meineke, V., Frungieri, M.B., Jessberger, B., Vogt, H. J., and Mayerhofer, A. (2000). Human testicular mast cells contain tryptase: increased mast cell number and altered distribution in the testes of infertile men. *Fertility and Sterility*, *74*, 239–244.
doi: 10.1016/S0015-0282(00)00626-9

Mnif, W., Hassine, A., Bouaziz, A., Bartegi, A., Thomas, O., and Roig, B. (2011). Effect of endocrine disruptor pesticides: a review. *International Journal of Environmental Research and Public Health*, *8*, 2265–2303. doi: 10.3390/ijerph8062265

Monsees, T.K., Franz, M., Gebhardt, S., Winterstein, U., Schill, W.-B., and Hayatpour, J. (2000). Sertoli cells as a target for reproductive hazards. *Andrologia*, *32*, 239–246.
doi: 10.1046/j.1439-0272.2000.00391.x

Oliva, A., Spira, A., and Multigner, L. (2001). Contribution of environmental factors to the risk of male infertility. *Human Reproduction*. *16*, 1768–1776.
doi: 10.1093/humrep/16.8.1768

Orisakwe, O.E., Afonne, O.J., Nwobodo, E., Asomugha, L., and Dioka, C.E. (2001). Low-dose mercury induces testicular damage protected by zinc in mice. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, *95*, 92–96.
doi: 10.1016/S0301-2115(00)00374-2

Pandya, C., Pillai, P., Nampoothiri, L., Bhatt, N., Gupta, S., and Gupta, S. (2012). Effect of lead and cadmium co-exposure on testicular steroid metabolism and antioxidant system of adult male rats. *Andrologia*, *44*, 813 – 822.
doi: 10.1111/j.1439-0272.2010.01137.x

Parelho, C., Rodrigues, A.S., Cruz, J.V., and Garcia, P. (2014). Linking trace metals and agricultural land use in volcanic soils — A multivariate approach. *Science of the Total Environment*, *496*, 241–247. doi: 10.1016/j.scitotenv.2014.07.053

Peiris-John, R.J., and Wickremasinghe, R. (2008). Impact of low-level exposure to organophosphates on human reproduction and survival. *Transactions of the Royal Society of Tropical Medicine & Hygiene*, *102*, 239–245.
doi: 10.1016/j.trstmh.2007.11.012

Penumarthy, L., Oehme, D.F.W., and Hayes, R.H. (1980). Lead, cadmium, and mercury tissue residues in healthy swine, cattle, dogs, and horses from the midwestern United States. *Archives of Environmental Contamination and Toxicology*, *9*, 193–206. doi: 10.1007/BF01055374

Perry, M.J. (2008). Effects of environmental and occupational pesticide exposure on human sperm: a systematic review. *Human Reproduction Update*, *14*, 233–242. doi: 10.1093/humupd/dmm039

Petrelli, G., and Figà-Talamanca, I. (2001). Reduction in fertility in male greenhouse workers exposed to pesticides. *European Journal of Epidemiology*, *17*, 675–677. doi: 10.1023/A:1015511625099

Pillai, P., Pandya, C., Bhatt, N., and Gupta, S. (2012). Biochemical and reproductive effects of gestational/lactational exposure to lead and cadmium with respect to testicular steroidogenesis, antioxidant system, endogenous sex steroid and cauda-epididymal functions. *Andrologia*, *44*, 92–101.
doi: 10.1111/j.1439-0272.2010.01109.x

Pizent, A., Tariba, B., and Živković, T. (2012). Reproductive toxicity of metals in men. *Archives of Industrial Hygiene and Toxicology*, *63*, 35–46.
doi: 10.2478/10004-1254-63-2012-2151

Rowe, F.P., Bradfield, A., Quy, R.J., and Swinney, T. (1985). Relationship Between Eye Lens Weight and Age in the Wild House Mouse (Mus musculus). *The Journal of Applied Ecology*, *22*, 55-61. doi: 10.2307/2403326

Siu, E., Mruk, D., Porto, C., and Cheng, C. (2009). Cadmium-induced testicular injury. *Toxicology and Applied Pharmacology*, *238*, 240–249.
doi: 10.1016/j.taap.2009.01.028

Slimani, S., Boulakoud, M.S., and Abdennour, C. (2011). Pesticide exposure and reproductive biomarkers among male farmers from north-east Algeria. *Annals of Biological Research*, *2*, 290-297.

Telisman, S., Cvitkovic, P., Jurasovic, J., Pizent, A., Gavella, M., and Rocic, B. (2000). Semen quality and reproductive endocrine function in relation to biomarkers of lead, cadmium, zinc, and copper in men. *Environmental Health Perspectives*, *108*, 45–53.

Thompson, J., and Bannigan, J. (2008). Cadmium: toxic effects on the reproductive system and the embryo. *Reproductive Toxicology*, *25*, 304–315.
doi: 10.1016/j.reprotox.2008.02.001

Tremellen, K. (2008). Oxidative stress and male infertility—a clinical perspective. *Human Reproductive Update*, *14*, 243–258. doi: 10.1093/humupd/dmn004

Wang, X., Sharma, R.K., Sikka, S.C., Thomas Jr., A.J., Falcone, T., and Agarwal, A. (2003). Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. *Fertility and Sterility*, *80*, 531–535. doi: 10.1016/S0015-0282(03)00756-8

van der Schalie, H.S.G. (1999). Animals as sentinels of human health hazards of environmental chemicals. *Environmental Health Perspectives*, *107*, 309–315.