Environmental Toxicology and Pharmacology 106 (2024) 104387

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap

Histological evidence of hypothyroidism in mice chronically exposed to conventional farming

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ARTICLE INFO

Edited by: Malcolm D. Tingle

Keywords:
Mus musculus
Conventional farming system
Chronic exposure
Pesticides

ABSTRACT

Worldwide, disorders of the thyroid gland are a growing concern; such can be caused by exposure to contaminants, including agrochemicals used in conventional agriculture, which act as endocrine disruptors. The purpose of this study is to evaluate whether or not exposure to an environment with conventional agriculture leads to thyroid disruption. *Mus musculus* were used as bioindicator species, captured in two sites: a farm where conventional agriculture is practiced, and a place without agriculture. Thyroid histomorphometric and morphologic data were analyzed. The impacts of the agricultural environment over the thyroid were revealed, as indications of hypothyroidism were observed in exposed mice: the area and volume of epithelial cells were much lower. Alterations in thyroid histomorphology were also observed: lower follicular sphericity, irregularly delimited epithelium and increased exfoliation into the colloid. These results highlight the need for transition from current conventional agricultural systems towards organic systems.

1. Introduction

Agricultural practices constitute some of the most important anthropogenic activities, as they play a large role in providing food, fuel and fiber to world population. With the ever-increasing global demand for food and farmland due to the rapid rise in human population numbers, the intensification of agriculture has become a pressing concern, as it is known to be one of the major drivers for environmental pollution and consequent biodiversity loss (Gabriel et al., 2013; Kuila and Ghosh, 2022; Ball and Quintart, 2023). Most of the current agricultural production methods rely heavily on the usage of agrochemicals to enhance soil fertility and to achieve the highest possible crop yield, leading to their environmental unsustainability given the extent of the damage caused (Gomiero et al., 2011; Mandal et al., 2020; Ganguly et al., 2021). As with many other anthropogenic practices, agriculture is often involved in soil and water contamination with several pollutants, such as fertilizers and pesticide residues, which severely impact ecosystems (Krami et al., 2013; Ganguly et al., 2021). Alongside all the energy and natural resources expenditure, current agricultural practices largely relying on agrochemical usage pose severe danger to human health, as they are associated with elevated cancer risks and, among other disorders, the induction of endocrine disruption in both workers and consumers (Horrigan et al., 2002; Martyniuk et al., 2020; Warner et al., 2020; Horak et al., 2021).

The thyroid is an important organ of the endocrine system which hormones control metabolism, playing a fundamental role on nervous system development, linear growth, thermogenesis, the hepatic metabolism of nutrients, and fluid balance, as well as exerting a variety of effects on the cardiovascular system. The hypothalamus-pitutary-thyroid axis (HPT-axis) involves the endocrine feedback loop responsible for thyroid function, regulating thyroid hormones (TH) – thyroxine (T4) and triiodothyronine (T3) – production through negative feedback according to their concentration circulating in the bloodstream. The hypothalamus secretes thyrotropin-releasing hormone (TRH) to stimulate the synthesis and secretion of thyrotropin (thyroid-stimulating hormone, TSH) by the anterior pituitary, which, in turn, acts on the

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[1] https://doi.org/10.54499/UIDP/00643/2020

[2] https://doi.org/10.54499/UIDB/00329/2020

https://doi.org/10.1016/j.etap.2024.104387
Received 9 October 2023; Received in revised form 31 January 2024; Accepted 7 February 2024

thyroid to promote the biosynthesis and secretion of TH (Ortiga-Carvalho et al., 2016). After binding to receptors on thyroid follicular cells, TSH stimulates the uptake of iodine by the sodium-iodine symporter. Iodine is oxidized by thyroperoxidase (TPO) and hydrogen peroxide (H_2O_2) generated by dual oxidases (DUOX) in order to be incorporated into thyroglobulin (Tg), the precursor of T4 and T3 (Leemans et al., 2019). Tg is stored in the colloid and acts as a negative feedback regulator for the expression of proteins needed in the biosynthesis of TH, such as TPO and DUOXs. For TH to be produced, Tg is reabsorbed and hydrolyzed by acidic peptidases and proteases to form T4 and T3, which are then secreted into the bloodstream (Yoshihara et al., 2012). In peripheral tissues, the expression of deiodinases, which are enzymes that catalyze the removal of iodine from TH, therefore converting lower-activity T4 to higher-activity T3, is important in increasing hormone potency. These enzymes are also capable of inactivating TH completely by further removing iodine (Sutcliffe and Harvey, 2015). TH in the bloodstream can enter cells through trans-membrane transporters (Leemans et al., 2019). In cells, the presence of TH is signaled through nuclear TH receptors, which modify gene expression by binding to specific DNA elements (Bakker, 2004).

Endocrine disruption by environmental chemicals interferes with several of the organism's systems, most evidence of which originates from studies on reproductive organs (Boas et al., 2006; Parelho et al., 2016; Zlatnik, 2016; Rodprasert et al., 2021). Agrochemicals, with particular emphasis on pesticides, can act as endocrine disruptors, i.e., exogenous substances that can act as hormones in the endocrine system, therefore, causing changes in the physiological function of endogenous hormones (Mckinlay et al., 2008; Mantovani et al., 2008; Campos and Freire, 2016; Kongtip et al., 2019). Regarding the thyroid, it is known that contaminants commonly associated with agrochemicals, such as heavy metals, can mimic the chemical structure of T4 and T3, therefore impacting the production of TSH (Kashiwagi et al., 2009; Al-Maathidy et al., 2019; Fiore et al., 2019; Babić Leko et al., 2021). According to Kongtip et al. (2019), their influence over the communication along the HPT-axis includes mechanisms such as: hindering iodine absorption, decreasing the cellular uptake of TH, increasing the clearance of TH, and even altering the expression of TH-regulated genes. Food ingestion constitutes the main route of exposure to agrochemical contaminants for the general population (Kongtip et al., 2019); however, when it comes to occupational exposure, workers are often at risk via mixing, loading and spraying activities (Shadnia *et al.*, 2005), including by frequently attending environments that are heavily polluted by agrochemicals (Saaltnik *et al.*, 2013).

Most research in this area corresponds to epidemiological studies that link people's exposure to different agricultural systems (conventional *vs*. organic) to the occurrence of thyroid pathologies, in which the average concentration of TH in the blood of the participating individuals is assessed (Goldner et al., 2010; Piccoli et al., 2016; Kongtip et al., 2019). Disorders of the thyroid gland fall into two general categories: endocrine disorders and neoplastic disorders. Diseases of endocrine origin lead to abnormal levels of hormones produced by the gland, constituting either hypothyroidism (insufficient amount of hormones) or hyperthyroidism (excessive amount of hormones). On the other hand, diseases of neoplastic origin, although uncommon, range from harmless adenomas to highly aggressive carcinomas (Razani et al., 2017). Among such pathologies, hypothyroidism is most often reported in workers exposed to agrochemicals (Goldner et al., 2010; Piccoli et al., 2016; Kongtip et al., 2019; Requena et al., 2019).

The thyroid gland is known for its high plasticity, which can be witnessed at a morphological level, as its structure is intimately associated with glandular activity levels. Histological alterations can be evidenced in, among others, the size of its follicles, which are the organ's functional units, and the characteristics of their epithelium (Hallgren and Darnerud, 2002). This hints at the potential offered by the evaluation of the thyroid histomorphology as an early and sensitive biomarker of glandular activity, thus as an indicator of endocrine disruption (Bowen, 1929; Baillif, 1937; Hallgren and Darnerud, 2002). Thyroid disorders are among the most common in the world, having the incidence of pathologies regarding this organ been rising swiftly among global population (Leese et al., 2008; Vanderpump, 2011; TLDE, 2013; Maniakas et al., 2018; Stahlman and Oh, 2018; Deng et al., 2020), revealing the need for the development of studies that can accurately identify what causes endocrine disruption of the thyroid and countermeasures against so.

Therefore, in this study, we intended to evaluate whether or not there are histomorphological alterations in the thyroid of individuals (using mice as surrogate species) chronically exposed to an environment where pesticides are frequently used (conventional agriculture systems) that lead to the functional disruption of the organ.

2. Materials and methods

2.1. Study sites

The study was carried out on São Miguel Island, Azores, Portugal. The two study sites were chosen depending on whether conventional agriculture was practiced or not. The mice captured in the farm in which conventional agriculture is practiced corresponded to the study group, while mice captured in Rabo de Peixe corresponded to the reference group (since it is a site without agricultural practices for over a decade). The soil of the reference site corresponds to a forest reserve of centennial Japanese cedar [*Cryptomeria japonica* (Thunb. ex L.f.) D.Don], belonging to a region with no historical records or evidence of farming activity. The conventional farming systems correspond to agricultural practices in which the use of agrochemicals (both pesticides and fertilizers) is frequent and legally framed by European and national guidelines while the organic farming systems are certified by the European Commission, being prohibited the use of synthetic agrochemicals and soil amendments are confined to organic fertilizers (compost and manure). Agrochemicals used in the study site include organic fertilizers, inorganic fertilizers and pesticides such as insecticides, herbicides, nematicides, fungicides, molluscicides and acaricides. Further details on the characteristics of the site with conventional farming can be found in Parelho et al. (2016). According to Parelho et al. (2014), agriculture is a diffuse source of metal pollution in São Miguel Island farming soils. According to these authors, the conventional farming soils in São Miguel Island are particularly contaminated with lithium (Li), potassium (K) and molybdenum (Mo) because of the intensive and repeated use of inorganic fertilizers and pesticides, while the soil of the reference site has trace metal background values significantly lower, except for Vanadium (V) and Barium (Ba), which exist in higher concentrations than in conventional farming system soils.

2.2. Mice sampling and histological slides preparation

Animal models for ecotoxicological risk assessment, particularly on terrestrial pollution, often include small mammals, such as wild mice, which can be sampled directly from the studied environments (Shore and Douben, 1994; Imholt et al., 2018). Thus, wild mice *Mus musculus* Linnaeus, 1758, were chosen as surrogate species for this study, given they fulfill the criteria for selecting bioindicators, namely: high abundance, a life expectancy wide enough for the estimation of possible long-term effects (Marcheselli et al., 2010) and a fairly small home range of, on average, 145 m^2 (Lidicker, 1966), which is much smaller than the surface area of both the study sites (farming areas of around 5000 m^2).

Live *M. musculus* were captured in the chosen conventional farm (Conventional Farming, CF) and Rabo de Peixe (Reference Site, RF) with live catch-traps, which housed them no longer than necessary before euthanasia. All mice were weighed and sexed. After euthanasia with isoflurane, the thyroids were removed *en-bloc*, then fixed in 4% buffered formaldehyde for standard histological processing. The relative age of each mouse, in days, was determined according to the methodology of

Quéré and Vincent (1989), based on dry crystalline mass. Only the males were selected for this study, to avoid the possibility that any alterations observed in the thyroid were due to the menstrual cycle of females. There were no significant differences (*t*-test, $P > 0.05$) in the weight of individuals between groups [weight (g): 13.88 ± 0.60 (CF); 15.23 ± 0.82 (RF)]. Mice from CF were, on average, significantly (*t*-test, $P < 0.05$) younger than those from RF [age (days): 101.85 ± 7.09 (CF); 227.75 ± 19.99 (RF)]. Histological slides with sections of the thyroid of 20 individuals were made (each slide corresponding to a single individual, containing several 5 μm thick sections): 8 slides of *M. musculus* captured in CF and 12 slides of *M. musculus* captured in RF. The slides were then stained with hematoxylin and eosin according to the methodology of Martoja et al. (1970). From these, a series of histomorphological data was collected, with the intent of evaluating the thyroid status of each individual.

The experimental procedures of this study were approved by the Ethics Committee of the University of Azores (REF: 10/2020). All procedures were carried out under the strict recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123), directive 2010/63EU and Portuguese legislation (DL 113/2013).

2.3. Histomorphometric analysis

A number of 5 fields of thyroid were selected per slide to take photomicrographs using the 20X and 40X objectives. The selected fields were separated by a minimum of 20 μm to ensure that the observed thyroid areas did not include the follicles present in the preceding photomicrograph. Within each field, photomicrographs were taken of the 4 largest follicles, making up a total of 20 follicles studied per individual. The criterion for selecting follicles for analysis ensured bias could be avoided, because selecting any follicles that appeared in their entirety in the field of observation could mean measuring follicles that were actually large, but which were cut on their sides (away from their equatorial section), giving the illusion of being small. The choice of the 4 largest follicles guaranteed that the follicles observed and analyzed had been cut very close to their equatorial section, actually constituting the largest follicles present in the section under observation.

From the images obtained with the 20X objective, data regarding the number of follicles in an area of 30,000 μm^2 of thyroid, per individual, was collected (Appendix, Fig. A1). The referred area was chosen based on the photomicrograph with the smallest thyroid sample, having been counted all the follicles that were present entirely within that area, including cases in which at least more than half of the follicle was present.

From the images obtained with the 40X objective, data was collected regarding the following parameters: (i) the area and (ii) perimeter of the colloid, both calculated using the Image-Pro Plus 5.0 software tracing the colloid's boundary from the apical surface of the epithelial cells of the corresponding follicle (Appendix, Fig. A2); (iii) the thickness of the epithelium, given by a straight line between the basal lamina and the apical surface of the epithelial cells (perpendicular to these), measured in four regions, approximately equidistant, from each of the follicles analyzed, then registered as the average of these four regions per follicle (Appendix, Fig. A3), and (iv) the number of epithelial cells nuclei per a 50 μm transect, measured in four regions of each follicle, then registered as the average of these four regions per follicle (Appendix, Fig. A4). All the data obtained per each analyzed parameter was finally registered as the average for each individual.

The average width of epithelial cells was determined, in μm, through dividing 50 μm by the average number of nuclei, per individual. Then, the average area of epithelial cells was calculated, in μm^2, by multiplying the obtained value of the width of epithelial cells by the average thickness of the epithelium, per individual. The average volume of epithelial cells, in μm^3, was determined by calculating the squared width of epithelial cells multiplied by the average thickness of the epithelium, per individual. The average volume of epithelial cells was then considered as the main biomarker of thyroid function.

2.4. Histomorphological analysis

Based on the photomicrographs taken, essentially those obtained with the 20X objective, a set of 5 different parameters were analyzed semi-quantitatively through visual qualitative scales, per field, per individual, considering that the higher the assigned value, the greater the degree of deviation from normality, i.e., the greater the degree of thyroid disruption. The evaluated aspects were: (i) follicular sphericity, based on the overall shape of thyroid follicles, in which 1 = "spherical", 2 = "somewhat spherical" and 3 = "not spherical at all"; (ii) epithelial irregularity, based on the appearance of the luminal surface of thyroid follicles' epithelium, in which 1 = "regular", 2 = "somewhat irregular" and 3 = "very irregular"; (iii) degree of exfoliation, based on the presence of cell debris (nuclei) within the colloid, in which 1 = "none", 2 = "little", 3 = "some" and 4 = "a lot"; (iv) degree of inflammation, based on the presence of lymphocytes in the interstitial tissue, in which 1 = "none", 2 = "little", 3 = "some" and 4 = "a lot", and (v) abundance of C cells, based on the presence of such cells in the interfollicular spaces, in which 1 = "very few", 2 = "few", 3 = "some" and 4 = "a lot". The analyses were carried out independently by two observers, having the final values been registered by consensus, in a total of 40 observations regarding individuals from CF and 60 observations regarding individuals from RF.

When evaluating the degree of exfoliation, the degree of inflammation and the abundance of C cells, the category 4 was not ever registered, nor was category 3 ever registered in the abundance of C cells, since it was considered that none of the photomicrographs observed corresponded to what such categories stood for. Examples of photomicrographs to which extreme values were assigned regarding each evaluated aspect can be found in Appendix (Figs. A5, A6, A7, A8 and A9).

2.5. Statistical analysis

Statistical analysis was performed in the IBM SPSS Statistics ® v. 28.0.1 (142) software. The normality of the morphometric analysis was verified through Q-Q plots. Data regarding weight, age, area and perimeter of the colloid, the width, the area, and the epithelial cell volume showed a normal distribution, contrary to data on epithelial thickness and number of follicles per 30,000 μm^2 of thyroid. To the normal data, independent samples *t*-test were used to compare the means between the group of individuals from CF and the group of individuals from RF (preceded by a Levene's test for homogeneity of variances), while non-parametric Mann-Whitney U tests were applied with the same purpose to non-normal data. Associations between data were determined via Pearson correlations, between normal data, and via Spearman correlations, between non-normal data.

Regarding the morphological analysis, for each of the evaluated aspects, non-parametric Mann-Whitney U tests were used to compare the distributions between the values registered in the observations of the group of individuals from CF and the group of individuals from RF, having the mode been determined in each case. The relative frequency of each category was calculated, by group, to improve the perception of possible differences between their distributions per group.

3. Results

3.1. Histomorphometric analysis

Regarding normal quantitative data, the results obtained in terms of possible differences between the means of each group of individuals are shown in Table 1.

Regarding Table 1, results show that mice from CF had both an average area and volume of epithelial cells significantly (*t*-test, $P < 0.05$)

Table 1
Descriptive statistics of the registered means and respective standard errors of the histomorphometric data, referring to the individuals from Conventional Farming (CF) and Reference Site (RF).*t-test, ***U*-test, *P* values marked in bold indicate significant differences ($P < 0.05$).

Variable	Site	Mean ± standard error	*P*
Colloid area (μm^2)	CF	5796.48 ± 942.84	*0.639**
	RF	5332.55 ± 491.14	
Colloid perimeter (μm)	CF	353.04 ± 32.68	*0.057**
	RF	290.64 ± 12.68	
Epithelial cells' width (μm)	CF	11.28 ± 0.87	*0.190**
	RF	9.97 ± 0.29	
Epithelial cells' area (μm^2)	CF	48.05 ± 2.62	***0.001****
	RF	71.11 ± 4.55	
Epithelial cells' volume (μm^3)	CF	534.71 ± 41.55	***0.034****
	RF	716.07 ± 58.15	
Epithelium thickness (μm)	CF	4.60 ± 0.72	***0.003*****
	RF	7.12 ± 0.39	
Number of follicles per 30,000 μm^2	CF	5.38 ± 0.96	***0.004*****
	RF	9.10 ± 0.93	

lower than those from RF. Both the epithelium thickness and the number of follicles per 30,000 μm^2 of thyroid are significantly (*U*-test, $P < 0.05$) lower in mice from CF than in those from RF.

Results revealed a positive association between age and the number of follicles per 30,000 μm^2 of thyroid [$r_s(18) = 0.577$, $P = 0.008$]. On the other hand, there was no significant associations between age and colloid area [$r(18) = -0.120$], age and colloid perimeter [$r(18) = -0.334$, $P = 0.151$], age and epithelial cells' area [$r(18) = 0.409$, $P = 0.073$] and, age and epithelial cells' volume [$r(18) = 0.205$, $P = 0.385$].

3.2. Histomorphological analysis

Regarding each of the evaluated aspects, the results obtained in terms of possible differences in the distributions between the categories registered in each group of individuals are shown in Fig. 1.

According to Fig. 1, follicular sphericity was significantly (*U*-test, $P < 0.05$) lower in the individuals from CF, in which only 35% of the individuals had spherical follicles, while in RF the percentage was 66.67% (mode 2 *vs.* mode 1, respectively). On the other hand, epithelial irregularity was significantly (*U*-test, $P < 0.05$) higher in the individuals from CF, in which only 72.5% of the individuals had a regular epithelium, while in RF the percentage was 96.67% (mode 1, in both cases). The degree of exfoliation was significantly (*U*-test, $P < 0.05$) higher in the individuals from CF, in which only 60% of the individuals did not show exfoliation of cells into the colloid, while in RF the percentage was 78.33% (mode 1, in both cases). The degree of inflammation was significantly (*U*-test, $P < 0.05$) higher in the individuals from CF, in which only 70% of the individuals did not present inflammation in the thyroid tissue, while in RF the percentage was 91.67% (mode 1, in both cases). Finally, the abundance of C cells was significantly (*U*-test, $P < 0.05$) higher in individuals from CF, in which 22.5% of the individuals were scored as with few C cells, while in RF only 3.33% were scored as with few C cells (mode 1, in both cases).

4. Discussion

There is a number of studies which have covered the effects of exposure to environmental contaminants over the thyroid (Mukhi et al., 2005; Badiei et al., 2009; Badiei et al., 2010; Jugan et al., 2010; EFSA et al., 2019; Fiore et al., 2019; Bano and Mohanty, 2020; Babić Leko

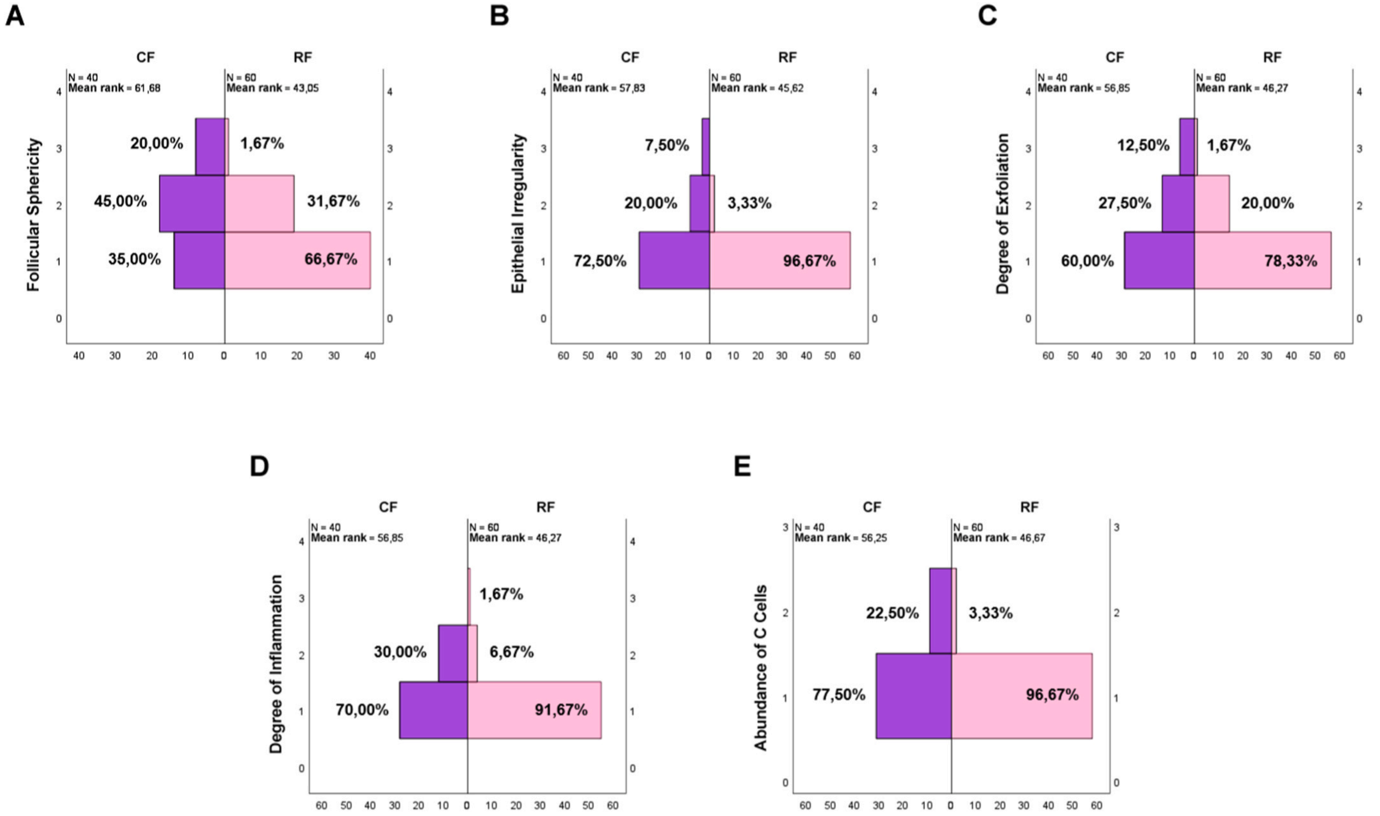

Fig. 1. Bar graphs of the distribution of the categories of: (A) follicular sphericity; (B) epithelial irregularity; (C) degree of exfoliation; (D) degree of inflammation and (E) abundance of C cells, among the individuals from Conventional Farming (CF) and Reference Site (RF). The relative frequencies of each category are represented, per group, next to the corresponding bars. Categories: (A) 1 = "spherical", 2 = "somewhat spherical", 3 = "not spherical at all"; (B) 1 = "regular", 2 = "somewhat irregular", 3 = "very irregular"; (C and D) 1 = "none", 2 = "little", 3 = "some"; (E) 1 = "very few", 2 = "few". The differences in the distribution of the categories between groups were all significant (Mann Whitney *U*-test, all $P < 0.05$).

et al., 2021; Korkmaz and Örün, 2022). In this context, several studies had also used morphological parameters to evaluate thyroid function under exposure to chemicals known to cause endocrine disruption of this organ (Al-Maathidy et al., 2019; El-Mehi and Amin, 2012; Hu et al., 2006; Khan et al., 1999; Moccia et al., 1986; Mukhi et al., 2005; Schnitzler et al., 2008). However, to our best knowledge, this was the first study to address the effect of chronic exposure to contaminants resulting from the practice of conventional agriculture on the thyroid, using wild mice *M. musculus* as bioindicators. The methodology followed revealed important connections between chronic exposure to an environment with agrochemicals from conventional agriculture and the appearance of hypothyroidism-like histological signs in exposed individuals.

Given that i) mice from CF were significantly younger than those from RF, and ii) age was positively correlated to the number of follicles per 30,000 μm^2 of thyroid, the differences observed between both groups for this histomorphometric parameter are most probably related with the mice age and not with the environment in which they lived. These results are in line with those obtained in the studies by by Rao-Rupanagudi et al. (1992) and Lee et al. (2016). On the contrary, age was not significantly associated with the area and perimeter of the colloid, and no significant differences were observed between the groups regarding these last two parameters, although it is recognized that an increase of these traits is associated with a lower activity of the thyroid (Lee et al., 2016). However, both the area and the volume of the epithelial cells differed significantly between the two mice groups (mice from CF had smaller follicular cells) and were not associated to the age of the mice, revealing that chronic exposure to an environment in which conventional agriculture is practiced induces thyroid disruption, being the thyroids of mice from CF much less functional. It is recognized that the lowering of these two parameters can lead to hypothyroidism due to a diminished activity of the thyroid. Therefore, our results emphasize the seriousness of the danger of exposure to the agrochemicals typically used in conventional agricultural systems. Moreover, the obtained results corroborate the findings of other studies (e.g., Goldner et al., 2010; Piccoli et al., 2016; Kongtip et al., 2019) that observed an association between hypothyroidism in humans and exposure to conventional agriculture.

It is a well-known fact that the functioning of the thyroid is crucial in young individuals, since, together with other hormones, the ones produced by the thyroid contribute to the growth and development of the organism in general (Tarım, 2011). It was possible to find differences in the gland's area and volume of epithelial cells and epithelium thickness according to the environment (all of which were lower in the exposed group), regardless of differences in the age of individuals between groups, therefore hinting at the gland's lower activity in individuals belonging to the exposed group. As such, these results also highlight the usefulness of biomarkers of effect, such as the area and volume of cells, in studies to assess the effects of chronic environmental exposure to certain contaminants, especially if the studied organ plays a fundamental role in metabolism for an extended period of the organisms' life. On the other hand, having CF mice been, on average, less than half the age of RF mice, the fact that the observed differences in terms of the mentioned histomorphometric data were significant also points to the severity of the impact of chronic exposure to contaminants from the practice of conventional agriculture on the functioning of the gland, since, as CF mice are younger, their thyroid follicles would be expected to show signs of much more activity (i.e., smaller follicles, a thicker epithelium, and with cells of larger area and volume) (Rao-Rupanagudi et al., 1992; Lee et al., 2016). In fact, the discrepancy of the measured values between groups could have been even greater if the age of the mice had not differed significantly.

Other effects on the thyroid potentially linked to chronic exposure to conventional agriculture can be inferred from the results obtained regarding the treatment of histomorphological data, insofar as they seem to be associated with a lower follicular sphericity, a greater epithelial irregularity, a greater degree of exfoliation, a greater degree of inflammation and a greater abundance of C cells in mice from CF. The lower follicular sphericity, followed by greater epithelial irregularity and higher degree of exfoliation, are the parameters most likely to be associated with chronic exposure to conventional agriculture, since they portray histological characteristics that also reflect the status of the gland. On the one hand, it is known that less spherical follicles and an epithelium of irregular luminal delimitation appear in situations of thyroid injury, while exfoliation, although constituting a normal event when not exaggerated, is associated with the degeneration of follicular epithelial cells, which remains end up inside the colloid while apoptosis processes take place (Al-Maathidy et al., 2019). As such, we can assume that the disruption of the thyroid derived from chronic exposure to conventional agriculture also manifests itself from a (histological) tissue perspective, affecting, in addition to its function, the histomorphology of the gland itself.

Concerning the results of inflammation, it is uncertain whether the thyroid was one of the true focus-organs of inflammation or whether more lymphocytes were found in the bloodstream and interfollicular spaces because something present in the tissue of this organ triggered an inflammatory response, which could be the agrochemicals' present in the conventional farming system that are acting as endocrine disruptors. There are situations in which agrochemicals, namely certain pesticides, may be at the basis for triggering abnormal inflammatory responses when they interfere with the natural physiology and metabolism of cells of the immune system (Ruíz-Arias et al., 2023). There are also cases in which the immune system may actively target the thyroid, regarding autoimmune thyroid disorders, such as Hashimoto's thyroiditis. Hashimoto's thyroiditis, also known as chronic autoimmune thyroiditis or chronic lymphocytic thyroiditis, is a disorder in which the thyroid's cells are destroyed via antibody-mediated immune processes, ultimately leading to hypothyroidism or underactive thyroid (Mincer and Jialal, 2023). Although the true causes behind this remain unclear, it is also possible that certain environmental factors, such as exposure to contaminants, may play a role in it (Babić Leko et al., 2021).

It is known that agrochemicals, especially certain pesticides, can induce an increase in Ca^{2+} absorption, leading to excessive intracellular values of this ion (Costas-Ferreira and Faro, 2021). Since the function of C cells is related to the production of calcitonin, a hormone that leads to a reduction in the levels of calcium in the blood, perhaps their abundance is partially explained by the increase in the concentration of Ca^{2+} in the organism of the exposed mice. The increase in C cell abundance might indicate an attempt to reduce the excessive concentration of this ion, via having more cells available to produce the necessary hormone for such. However, the true extent to which exposure to conventional agriculture may have affected calcium metabolism in such a way that would have resulted in a greater abundance of C cells in mice from CF remains also uncertain. Again, although the semi-quantitative methodology used to evaluate the abundance of C cells is probably not the most adequate, these results pinpoint towards an impact of the environment on these last two parameters.

Considering that the agrochemicals used in the study site include inorganic fertilizers, which often have nitrate in their composition, the observed histological effects in the thyroid of CF mice were expected, since exposure to nitrate is known as a cause for hypothyroidism. This is because nitrate "inhibits thyroid uptake of iodide by binding to the sodium-iodide symporter on the surface of thyroid follicles", therefore hindering the production of TH (Ward et al., 2010). It is also acknowledged that some pesticides, even after being metabolized within the body, can bioaccumulate, mainly in energy reserve tissues, as is body fat (Devi et al., 2022). Pesticides interfere with thyroid homeostasis in that they impair "TH production and its control, displacement from distributor proteins and liver metabolism" (Leemans et al., 2019). Pesticides such as organochlorine insecticides (Goldner et al., 2010), fungicides, herbicides and dithiocarbamates (Piccoli et al., 2016) are known to induce thyroid endocrine disruption, leading to hypothyroidism and hypothyroidism-like manifestations as they lead to a lower functioning of the organ. In this sense, mechanisms of interference with the HPT-axis by specific pesticides include “displacement from distributor proteins, increased hepatic metabolism, and indirect effects on thyroid function” by organochlorines, reduction of TSH production resulting in lower production of TH by organophosphates, reduction of TPO activity by carbamates, and antagonistic action on the TH receptors by pyrethroids (due to their similar chemical structure to TH). Neonicotinoids and phenylpyrazoles are also described as potential thyroid-disrupting pesticides, although further evidence on such is lacking in epidemiological studies (Leemans et al., 2019). All of this information contributes to a better understanding of the underlying endocrine-disrupting effects over the thyroid of the mice exposed to conventional agriculture, translated into the differences observed in comparison to the reference group.

The obtained results, in general, highlight the danger arising from chronic exposure to contaminants from conventional agriculture, given the notorious disruption of the thyroid in mice from CF, marked by all the significant changes observed at the histological level (that are reflected at a functional level). Thus, emphasis is put on the need to change predominantly conventional agricultural systems towards organic systems, as a way of safeguarding the population from harmful effects due to an agricultural system that uses agrochemicals, which is the most common. However, and in order to more assertively prove these harmful effects of conventional agriculture on the thyroid, it would be useful to carry out further studies in this area with the analysis of an additional group of mice from organic farming systems, with the objective of ascertaining whether, in fact, the potential disruption of the thyroid gland by such is less than what happens with chronic exposure to conventional agriculture; in this case, a greater similarity between the thyroid characteristics of this new group and the ones exhibited by mice not exposed to agriculture, i.e., the reference group, is expected.

Data collected from animals that live in or are exposed to contaminated areas is crucial for human and environmental health risk assessment, as such provide valuable information on hazardous effects towards the environment and along food chains, including on living beings themselves. In this context, field studies that use animal models provide significant ecotoxicological data under the complexity of the assessed environment that would otherwise be inaccessible, as such conditions are hard to mimic in full within laboratory (Sánchez-Chardi et al., 2009). Thus, no less important was the fundamental role carried by wild mice *M. musculus* as bioindicators, since it was the analysis of histological sections of their thyroids that made it possible to obtain all the conclusions described in the present study, including their extrapolation into a broader perspective, proving the usefulness of these living beings in future studies of a similar nature. In fact, even though mice in this study are playing the role of bioindicators for occupational exposure to agrochemicals (i.e., chronic exposure of workers, such as farmers, to conventional agriculture), it is known that the general population is also exposed to these contaminants via food consumption (Horrigan et al., 2002), hence, the meaning of the results obtained extends to this level, reinforcing, in this sense, the relevance of this study. The efforts in monitoring the presence of pesticide residues in food have been great in the EU (EFSA, 2023; EFSA et al., 2023). Sadly, the same still can’t be said in regards of other regions of the world.

5. Conclusion

Disorders of the thyroid gland are an important concern worldwide, specifically when it comes to hypothyroidism, as it is known that it affects up to 5% of the general world population, and it is estimated that another 5% comprise undiagnosed cases (Chiovato et al., 2019).

The results obtained indicate disruption of the thyroid gland in mice from the conventional agriculture site, derived from the action of the agrochemicals used in this agricultural system, including pesticides, that may act as endocrine disruptors, leading to a lower function of the thyroid. Such disruption is revealed by the histological alterations in the thyroids of these mice, namely the reduction of the area and the volume of thyroid epithelial cells, a lower follicular sphericity, an epithelium with irregular luminal delimitation and an increased exfoliation of cells towards the interior of the colloid.

The need for transition from current conventional agricultural systems towards organic systems is emphasized, although, at present, results are still lacking regarding the potential disruption of the thyroid by the latter; as such, further studies in this context are encouraged.

CRediT author statement

Nádia Coelho: methodology, conceptualization, investigation, writing original draft; Ricardo Camarinho: corresponding author, conceptualization, software, investigation, writing-reviewing and editing; Patricia Garcia: conceptualization, software, validation, data curation and writing-reviewing and editing; Armindo Rodrigues: conceptualization, methodology, validation, writing-reviewing and editing.

Funding

This work and Ricardo Camarinho were financially supported by IVAR project UIDP/00643/2020, from FCT – Fundação para a Ciência e Tecnologia – Portugal.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors declare that there were no generative AI and AI-associated technologies in the writing process.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Data availability

Data will be available upon request.

Acknowledgements

The authors of the present study would like to thank Paulo Melo for his support in fieldwork.

Appendix

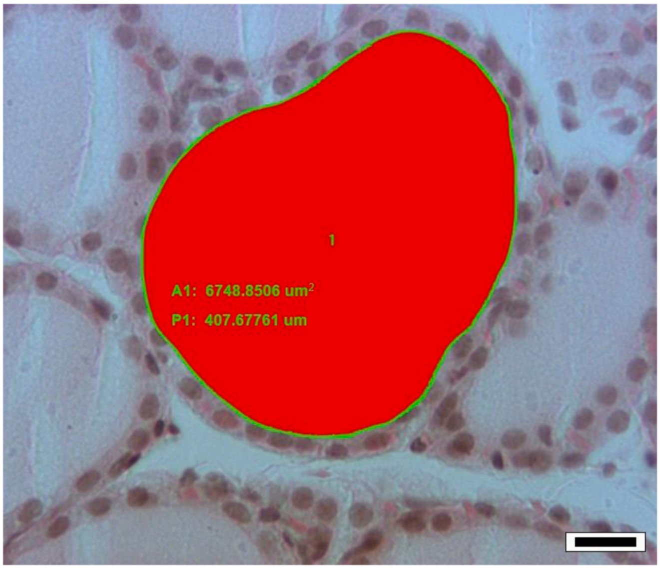

Fig. A1. **:** Photomicrograph of a histological section of *M. musculus* thyroid showing the count of the number of follicles per thyroid area. The area represented by the square is equivalent to 30,000 μm^2, within which all follicles present in full were counted, including cases in which at least more than half of the follicle was present. In this example, there was a total of 18 follicles per 30,000 μm^2 of thyroid. Stained with hematoxylin and eosin; scale bar = 25 μm.

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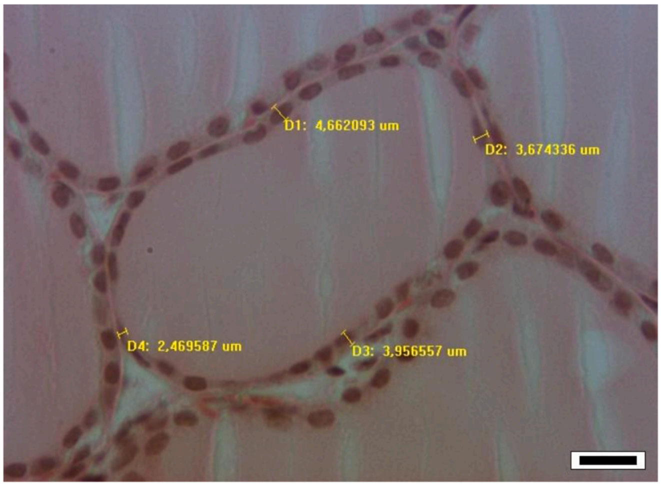

Fig. A2. **:** Photomicrograph of a histological section of *M. musculus* thyroid showing the measurement of the area and perimeter of the colloid of a thyroid follicle. The area (A1) corresponds to the interior of the follicle, represented in red, in μm^2, and the perimeter (P1) corresponds to the delimitation given by the apical surface of the epithelial cells, represented in green, in μm. In this example, 6748.8506 μm^2 of area and 407.67761 μm of perimeter were registered. Stained with hematoxylin and eosin; scale bar = 15 μm.

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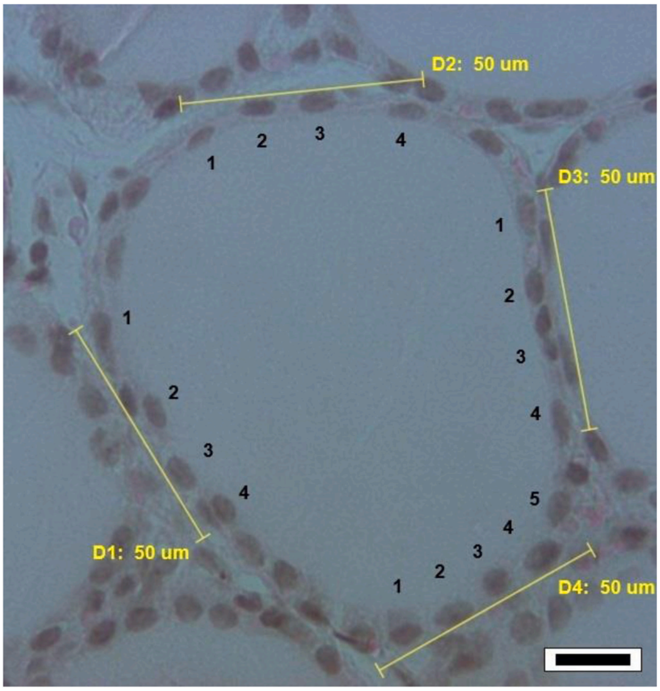

Fig. A3. **:** Photomicrograph of a histological section of *M. musculus* thyroid showing the measurement of the thickness of the epithelium of a thyroid follicle. The thickness of the epithelium was measured at four locations, more or less equidistant from the follicle's epithelium, from the basal lamina of the cells to their apical surface (in a straight line perpendicular to these), and the indicated values were registered as the average per follicle, in μm. In this example, the average thickness of the follicle epithelium corresponded to 3.690643 μm. Stained with hematoxylin and eosin; scale bar = 15 μm.

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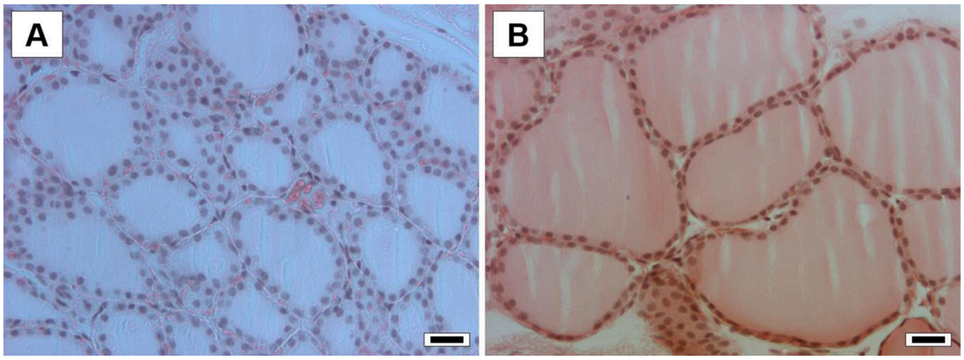

Fig. A4. **:** Photomicrograph of a histological section of *M. musculus* thyroid showing the counting of the number of epithelial cell nuclei in a thyroid follicle by a 50 μm transect. The transect was applied in four places, more or less equidistant, from the follicle epithelium, and the number of nuclei indicated in each one was registered as the average per follicle. In this example, the average number of nuclei per 50 μm was 4.25. Stained with hematoxylin and eosin; scale bar = 15 μm.

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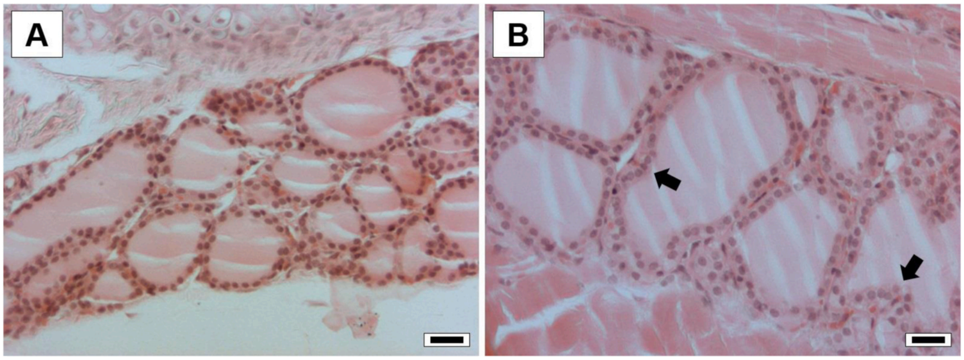

Fig. A5. **:** Photomicrographs of histological sections of *M. musculus* thyroid showing an example of the category "spherical" (value 1, photomicrograph A) and "not spherical at all" (value 3, photomicrograph B), regarding the assessment of follicular sphericity. Stained with hematoxylin and eosin; scale bars = 25 μm (A and B).

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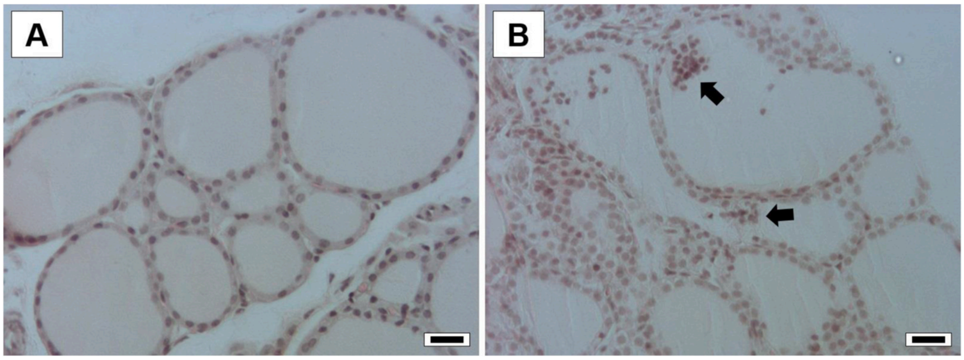

Fig. A6. **:** Photomicrographs of histological sections of *M. musculus* thyroid showing an example of the category “regular” (value 1, photomicrograph A) and “very irregular” (value 3, photomicrograph B), regarding the assessment of epithelial irregularity. The black arrows in photomicrograph B indicate some areas where the epithelium delimitation is irregular. Stained with hematoxylin and eosin; scale bars = 25 μm (A and B).

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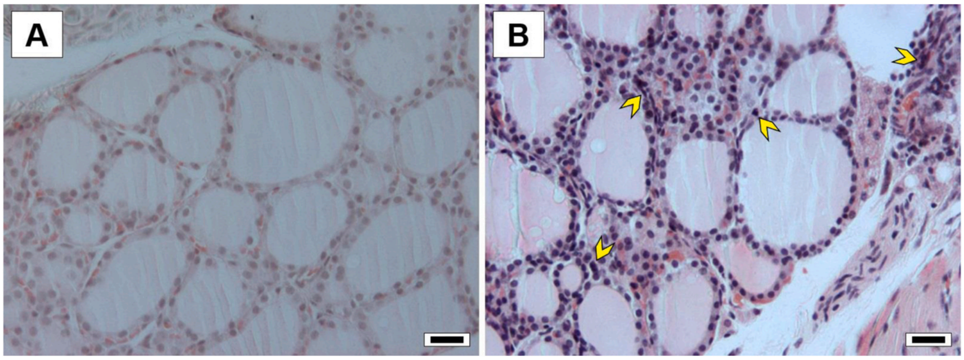

Fig. A7. **:** Photomicrographs of histological sections of *M. musculus* thyroid showing an example of the category “none” (value 1, photomicrograph A) and “some” (value 3, photomicrograph B), regarding the assessment of the degree of exfoliation. The black arrows in photomicrograph B indicate some areas where there is exfoliation (i.e., cell debris within the colloid of thyroid follicles). Stained with hematoxylin and eosin; scale bars = 25 μm (A and B).

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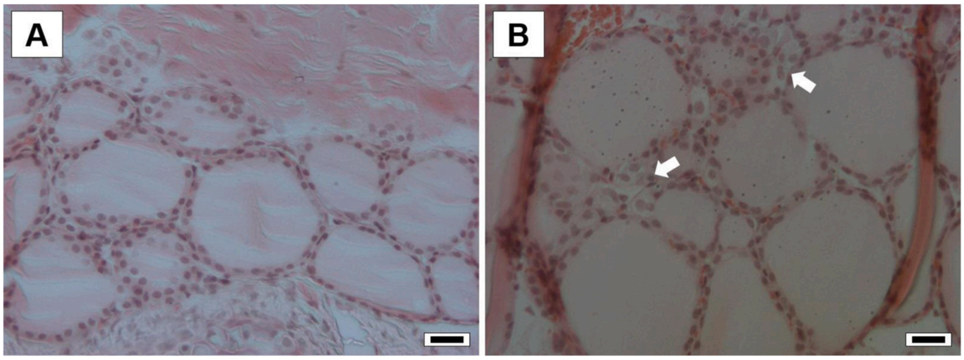

Fig. A8. **:** Photomicrographs of histological sections of *M. musculus* thyroid showing an example of the category “none” (value 1, photomicrograph A) and “some” (value 3, photomicrograph B), regarding the assessment of the degree of inflammation. The yellow arrowheads in photomicrograph B indicate some areas where lymphocyte nuclei are observed (i.e., smaller and more basophilic nuclei, compared to the nuclei of epithelial cells in thyroid follicles). Stained with hematoxylin and eosin; scale bars = 25 μm (A and B).

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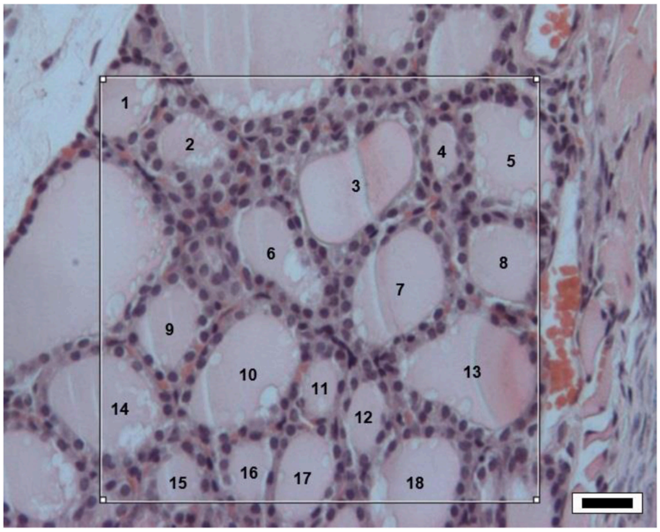

Fig. A9. **:** Photomicrographs of histological sections of *M. musculus* thyroid showing an example of the category “very few” (value 1, photomicrograph A) and “few” (value 2, photomicrograph B), regarding the assessment of the abundance of C cells. The white arrows in photomicrograph B indicate some areas where an abundance of C cells is observed. Stained with hematoxylin and eosin; scale bars = 25 µm (A and B).

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