Avaliação do impacto da agricultura orgânica *vs*. convencional sobre a tiroide

Impact assessment of organic vs. conventional farming on the thyroid

Dissertação de Mestrado

Nádia Maria Pereira Coelho

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DM

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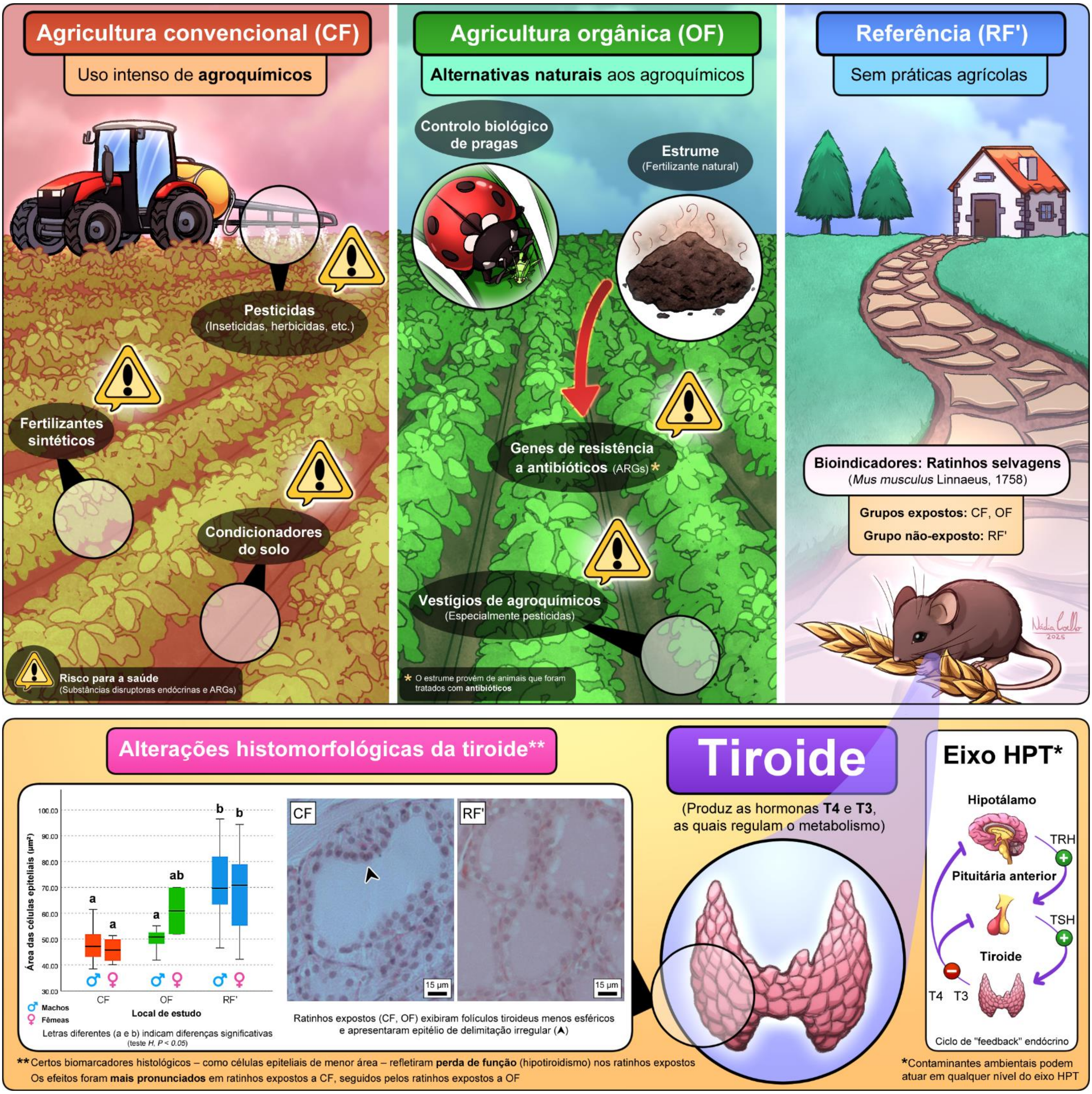
Resumo gráfico*



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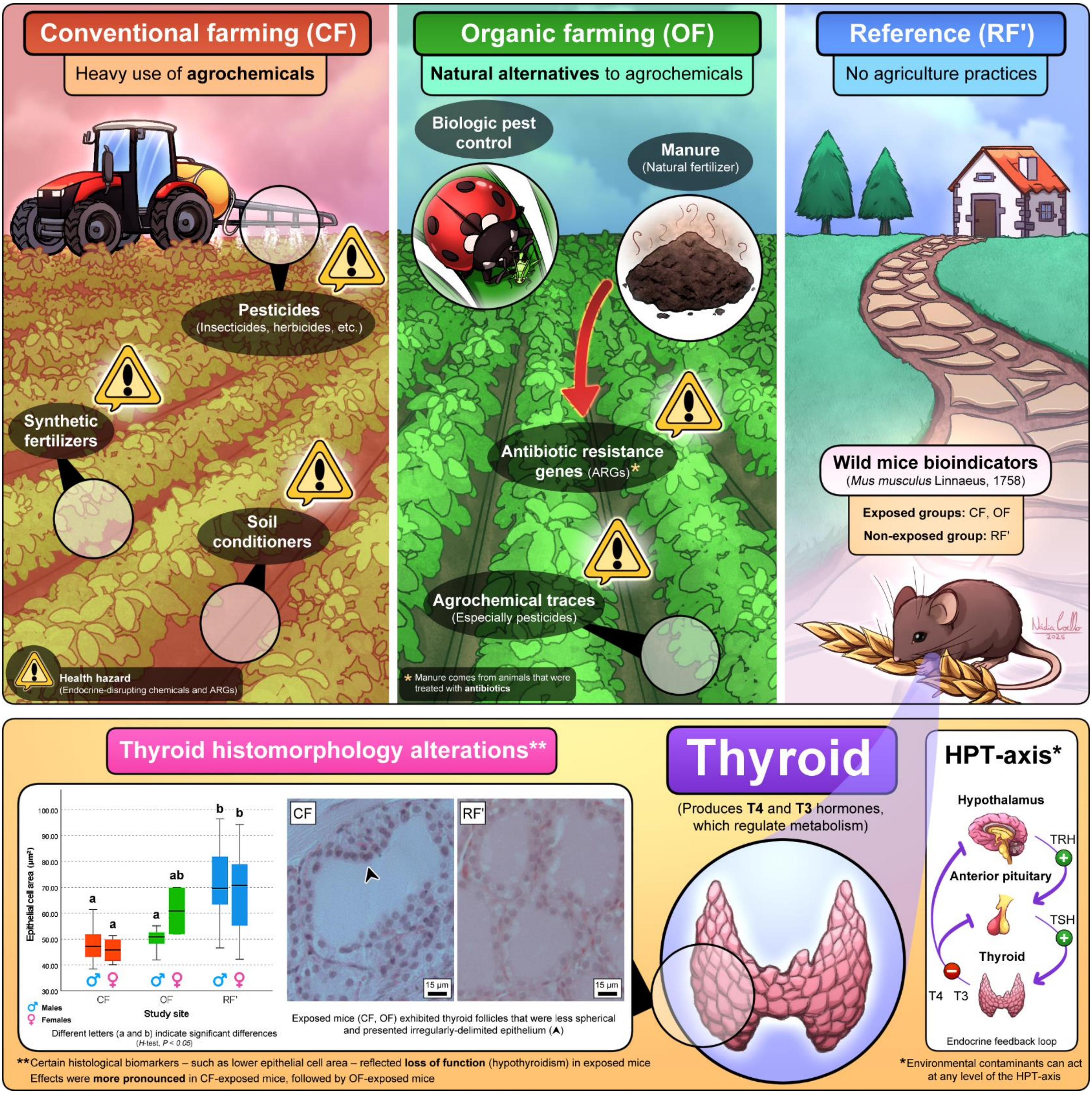
Resumo

O objetivo principal deste trabalho é avaliar e comparar qual o potencial impacto negativo da agricultura convencional e da agricultura orgânica na tiroide. As práticas de agricultura convencional utilizam agroquímicos sintéticos – muitos dos quais são disruptores endócrinos, como pesticidas – para maximizar a produtividade, enquanto as práticas de agricultura orgânica utilizam alternativas naturais para favorecer a sustentabilidade ambiental e proteger a saúde humana. Contudo, alguns estudos sugerem que os produtos de agricultura orgânica poderão apresentar contaminação residual por agroquímicos – nomeadamente pesticidas – e contribuir para a disseminação de genes de resistência a antibióticos no ambiente. A disrupção da tiroide é uma preocupação global, abrangendo algumas das patologias endócrinas mundialmente mais comuns. Estudos anteriores estabeleceram conexões entre a exposição a contaminantes de agricultura convencional com perturbações da tiroide, mas pouco se sabe sobre os efeitos da exposição a contaminantes de agricultura orgânica na tiroide. Selecionaram-se ratinhos selvagens *Mus musculus* como espécie bioindicadora, capturados (i) numa área agrícola convencional, (ii) numa área agrícola orgânica e (iii) em duas áreas sem práticas agrícolas (grupo de referência). Analisaram-se dados de medições histomorfométricas e histomorfológicas da tiroide, observando-se alterações na tiroide de ratinhos expostos a agricultura convencional e a agricultura orgânica, mais pronunciadas nos primeiros. Verificaram-se indícios de hipotiroidismo nos ratinhos expostos, traduzidos por uma menor espessura do epitélio e reduções relevantes na área e volume das células epiteliais comparativamente aos ratinhos não-expostos. As alterações histomorfológicas observadas nos ratinhos expostos incluíram menor esfericidade folicular, epitélio irregular, aumento de esfoliação para o coloide e aumento de inflamação do tecido tiroideu. Machos e fêmeas apresentaram as mesmas alterações da tiroide nos respetivos grupos, com pequenas diferenças em determinados parâmetros. Estes resultados sugerem que, embora a agricultura orgânica possa ser uma melhor alternativa à agricultura convencional, ela não é completamente isenta de riscos para a saúde. A exposição aos contaminantes da agricultura orgânica pode causar disrupção da tiroide, embora com efeitos menos pronunciados. No geral, os resultados obtidos suportam a necessidade de transição dos sistemas agrícolas convencionais atuais para sistemas agrícolas orgânicos.

Palavras-chave: Agricultura orgânica; Fertilizantes orgânicos; Agricultura convencional; Agroquímicos; Pesticidas; Exposição crónica; *Mus musculus*; Disrupção endócrina; Hipotiroidismo

Graphical abstract*



*Illustrated by the author, 2025

Abstract

The main objective of this work is assessing and comparing the potential negative impact of conventional farming and organic farming on the thyroid. Conventional farming practices deploy synthetic agrochemicals – many of which are endocrine-disrupting chemicals, like pesticides – to maximize crop yields, while organic farming practices rely on natural alternatives to promote environmental sustainability and protect human health. However, some studies suggest that organic farming yield may still contain residual contamination with agrochemicals – namely pesticides –, while contributing to the spread of antibiotic resistance genes in the environment. Thyroid disruption is a global concern, causing some of the most common endocrine pathologies worldwide. Previous studies have linked exposure to conventional farming contaminants with thyroid disruption, but little is known about the effects of exposure to organic farming contaminants on the thyroid. Wild mice *Mus musculus* were selected as bioindicator species, captured in (i) a conventional farming area, (ii) an organic farming area, and (iii) two areas without agricultural practices (reference group). Histomorphometric and histomorphological measurements of the thyroid were analyzed, revealing alterations in conventional farming- and organic farming-exposed mice. Evidence of hypothyroidism was observed in exposed mice, expressed as reduced epithelial thickness and relevant decreases in the area and volume of epithelial cells compared to non-exposed mice. Histomorphologic alterations in exposed mice included reduced follicular sphericity, irregularly-delimited epithelium, increased exfoliation into the colloid, and increased inflammation of thyroid tissue. Both male and female mice exhibited similar thyroid alterations within their groups, with only minor differences in some parameters. These results suggest that, while organic farming might be a better alternative to conventional farming, it is not completely free of health hazards. Exposure to organic farming contaminants can cause thyroid disruption, though with less pronounced effects. Overall, the results of this study support the need for a transition from current conventional farming systems towards organic farming systems.

Keywords: Organic agriculture; Organic fertilizers; Conventional agriculture; Agrochemicals; Pesticides; Chronic exposure; *Mus musculus*; Endocrine disruption; Hypothyroidism

List of abbreviations

ARB – Antibiotic-resistant bacteria

ARGs – Antibiotic resistance genes

CF – Conventional farming

DIO – Iodothyronine deiodinases

DUOX – Dual oxidases

EDCs – Endocrine-disrupting chemicals

EU – European Union

FT3 – Free triiodothyronine

GMOs – Genetically-modified organisms

H_2O_2 – Hydrogen peroxide

ha – Hectares

HPT-axis – Hypothalamus-pituitary-thyroid axis

I – Iodine

I^- – Iodide

MGEs – Mobile genetic elements

OF – Organic farming

PBDEs – Polybrominated diphenyl ethers

PCBs – Polychlorinated biphenyls

PFAS – Per- and polyfluoroalkyl substances

PM – Particulate matter

PTEs – Potentially toxic elements

RF1 – Reference group 1

RF2 – Reference group 2

RF' – New reference group (merging of RF1 and RF2 groups)

T3 – Triiodothyronine

T4 – Thyroxine, Tetraiodothyronine

Tg – Thyroglobulin

TH – Thyroid hormones

TPO – Thyroperoxidase

TRH – Thyrotropin-releasing hormone

TSH – Thyroid-stimulating hormone

1. Introduction

Agriculture is one of the most important anthropogenic activities, providing human populations with a steady supply of food, fuel, and fiber (Fanzo, 2018; Viana *et al*., 2022). In spite of its fundamental benefits, agriculture is one of the major causes of environmental pollution, posing a severe risk to ecosystem condition and human health – especially when the use of synthetic agrochemicals is involved (Sajid & Rahman, 2021; Kaur *et al*., 2024; Zhao *et al*., 2024). A lot of work has been done in searching for solutions to agrochemical contamination, promoting the rise of natural and more environmentally friendly alternatives. Nevertheless, data about current alternatives remains controversial, namely when it comes to the balance between production costs, yields, and even the presence of hazardous substances in produce (Seufert *et al*., 2012; Meng *et al*., 2017; Vigar *et al*., 2019; Thaise de Oliveira Faoro *et al*., 2024).

Thyroid disruption is a global concern, causing some of the most common endocrine pathologies worldwide (Fualal *et al*., 2016; Maniakas *et al*., 2018; Song *et al*., 2024). Although insufficient iodine (I) intake is the main cause of disorders of this gland – especially hypothyroidism –, exposure to environmental contaminants can drive thyroid disruption, thus leading to thyroid disorders (Duntas, 2015; Street *et al*., 2024; Yang *et al*., 2024). Several studies have found a connection between exposure to contaminants from conventional farming (CF) systems and thyroid disruption (Goldner *et al*., 2010; Piccoli *et al*., 2016; Suhartono *et al*., 2018; Sirikul & Sapbamrer, 2023; Coelho *et al*., 2024a). However, little is known when it comes to the effects of exposure to contaminants from organic farming (OF) systems on the thyroid.

1.1. Agriculture systems

On a global scale, agricultural land use (including cropland, and permanent meadows and pastures) covers approximately 4.8 billion hectares (ha). Given that the world total land area is around 13 billion ha (excluding Antarctica), agricultural land use alone represents about a third of the world total land area (FAO, 2023). Although agricultural land use is generally expanding, this trend is not uniform across the world. Agriculture intensification is more evident in the Global South – particularly in Asia and Latin America –, while the Global North is starting to regain forest area in detriment of agricultural land use (Pellegrini & Fernández, 2018). There are also differences between developed and developing countries, with agricultural land cultivation usually being increased in the latter. The same applies in situations of poverty, where populations turn to a more intense agricultural land use for sustenance (Zhang & Zhang, 2006). Still, with all circumstances considered, there is a global net loss of forest area, with a corresponding expansion of crop- and pastureland (Winkler *et al*., 2021).

Based on the nature of approaches towards food and livestock production, agriculture is divided into two major systems: CF, and OF. In essence, while the first heavily relies on synthetic inputs (i.e., agrochemicals) to maximize yields, the latter minimizes synthetic inputs to promote environmental sustainability (Azarbad, 2022; Vašková & Saska, 2024). Worldwide, CF is the dominant farming system (Gaitán-Cremaschi *et al*., 2019). By contrast, although growing, OF represents only about 1% of global cropland (Crowder *et al*., 2015).

1.1.1. Conventional farming (CF)

Conventional farming (also known as industrial agriculture) refers to agricultural methods heavily reliant on synthetic inputs, such as the use of agrochemicals and genetically-modified organisms (GMOs), to achieve the highest possible yield (Azarbad, 2022; Aćin *et al*., 2023). Most modern agriculture systems rely on CF practices for production, due to the rapid rise in human population numbers and consequent ever-increasing demand for food products (Brown, 1967; Pereira, 1993; Zahid *et al*., 2016; Milner *et al*., 2023). Besides an intense use of agrochemicals – pesticides (insecticides, herbicides, fungicides), fertilizers, growth regulators, soil conditioners, etc. – CF is characterized by high expenditures of energy and resources, specifically fresh water and fertile soils (Gelfand *et al*., 2010; Chmelíková *et al*., 2024). Practices of CF are involved not only in the contamination of food with agrochemical residues, but also that of soil, water, and air, rendering them a major cause of environmental pollution (Bux *et al*., 2022; Feledyn-Szewczyk & Kopiński, 2024). Because agrochemicals comprise many toxic compounds which can be harmful to living organisms beyond their targets, their diffusion through the environment is strongly linked with biodiversity loss (Bacsi *et al*., 2025; Hu *et al*., 2025).

Human exposure to CF contaminants happens both directly, and indirectly. For the general population, exposure is primarily indirect through food consumption (Lebelo *et al*., 2021; de Andrade *et al*., 2023). On the other hand, farm workers are directly and occupationally exposed to such contaminants by mixing, loading, and spraying activities, including via attending environments loaded with agrochemicals (Kesavachandran *et al*., 2009; Mamane *et al*., 2015). The presence of several cancer-promoting pesticide residues has been documented in CF food products, highlighting the serious health hazard associated with CF contaminants (Sadighara *et al*., 2023). While effects of acute exposures to CF contaminants include symptoms like skin and ocular irritation, headaches, and difficulty breathing, prolonged exposures – especially at low doses over time – have been linked to endocrine disruption, translated into increased risks of cancer, immunological disorders, reproductive problems, among other issues (Nankongnab *et al*., 2020b; Koussé *et al*., 2023; Khode *et al*., 2024).

1.1.2. Organic farming (OF)

Organic farming (also referred to as biological agriculture) is characterized by the exclusion of synthetic fertilizers, pesticides, and GMOs in its practices (Lombardo & Zelasco, 2016; Panday *et al*., 2024; Shen *et al*., 2025). Organic farming prioritizes quality over quantity, with the aim of producing safer and healthier food products despite relatively lower yields (Wilbois *et al*., 2019; Ondrasek *et al*., 2023). Focusing on environmental sustainability, OF employs techniques like the use of composting and manure as natural fertilizers, crop rotation, cover cropping, and biological pest control – i.e., the implementation of natural predators, parasites, and pathogens of pest populations – in its practices (Bale *et al*., 2008; Strenner *et al*., 2023; Moldavan *et al*., 2024; Panday *et al*., 2024). Because OF is more labor-intensive, time-consuming, and costly than CF, OF produce in the market tends to be more expensive (Wang *et al*., 2017; Smoluk-Sikorska, 2024). Data suggests that OF positively impacts the environment by promoting a decrease in nitrate leaching and in greenhouse gas emissions, along with increasing farmland biodiversity, and even promoting carbon sequestration via increasing soil organic matter (Leifeld & Fuhrer, 2010; Meng *et al*., 2017). According to Meng *et al*. (2017), all of these aspects compensate for the economic costs of a lower crop production.

The trend is for current CF systems to transition to OF ones, due to the perception that the latter are more beneficial for both the environment and humans. However, agrochemical residues, especially synthetic pesticide traces, are commonly detected in OF produce (Vigar *et al*., 2019; Riedo *et al*., 2021; Giampieri *et al*., 2022; Knuth *et al*., 2024; Lazarević-Pašti *et al*., 2025). The review of Lazarević-Pašti *et al*. (2025) points to the presence of pesticide residues in 28% of organic food in Southern Germany, 9% in the Swiss market, and 6% in the overall European market. The contamination of OF produce happens through several pathways, including from environmental sources such as soil, water, and air, along with handling processes during and after harvest (Chowdhury *et al*., 2021; Ramakrishnan *et al*., 2021; Guerrieri *et al*., 2024). Another problem is the fact that OF practices increase the amount and distribution of antibiotic resistance genes (ARGs) in the environment. This is because the manure used as natural fertilizer comes from livestock that was treated with antibiotics, growth factors, and other substances (Bassitta *et al*., 2022; Wu *et al*., 2023; Rosberg *et al*., 2024). Bacterial populations in the soil are exposed to these compounds, gain resistance towards them, and pass on the relevant genes both intra- (vertical gene transfer) and interspecies (horizontal gene transfer) (Urban-Chmiel *et al*., 2022; Wang *et al*., 2024; Zhuang *et al*., 2024).

1.2. Endocrine system

The endocrine system is composed of the body's tissues that produce and release hormones, mainly glands, such as the pineal, pituitary, thyroid, and adrenal glands. Hormones are chemical messengers responsible for the maintenance of several bodily functions, therefore being essential for homeostasis (Linkov *et al*., 2009; Malespin & Nassri, 2019). Whereas the nervous system allows a swift transmission of information between different organs, communication reliant on the endocrine system is slower, as it involves the production and secretion of hormones into the bloodstream. Moreover, contrasting with the short-lived electrical-chemical signals from the nervous system, endocrine signaling is often widespread and long-lasting (Hiller-Sturmhöfel & Bartke, 1998). Even if their mechanisms of action are distinct, both the nervous and endocrine systems influence the activity of one another: stimuli from the nervous system can affect the release of hormones and vice-versa (Leng, 2018; Zefferino *et al*., 2021).

Once in circulation, hormones will travel to their destination and act on specific targets. Only tissues with the correct receptors or binding sites to a given hormone will react to it, accounting to the high specificity of these messengers (Karlson, 1979). In general, hormonal effects on target cells include the modulation of gene expression, adjustment of protein activity, and regulation of cell metabolism (Perreta *et al*., 1979; Ing, 2005; Koenen *et al*., 2012).

1.2.1. Thyroid gland

The thyroid is a butterfly-shaped gland of the endocrine system, located in front of the neck, which exerts a fundamental role in metabolism (Figure 1). Its purpose is tied to the production of thyroid hormones (TH) – tetraiodothyronine (thyroxine or T4), and triiodothyronine (T3). Thyroid hormones boost metabolism by influencing the speed at which cells make use of oxygen and nutrients, which are needed for cellular activity (Harper & Brand, 1993; Yavuz *et al*., 2019; Sivertsson *et al*., 2022). These hormones regulate a plethora of processes, ranging from nervous system development, linear growth, and thermogenesis, to even the hepatic metabolism of nutrients, fluid balance, and cardiovascular system function.

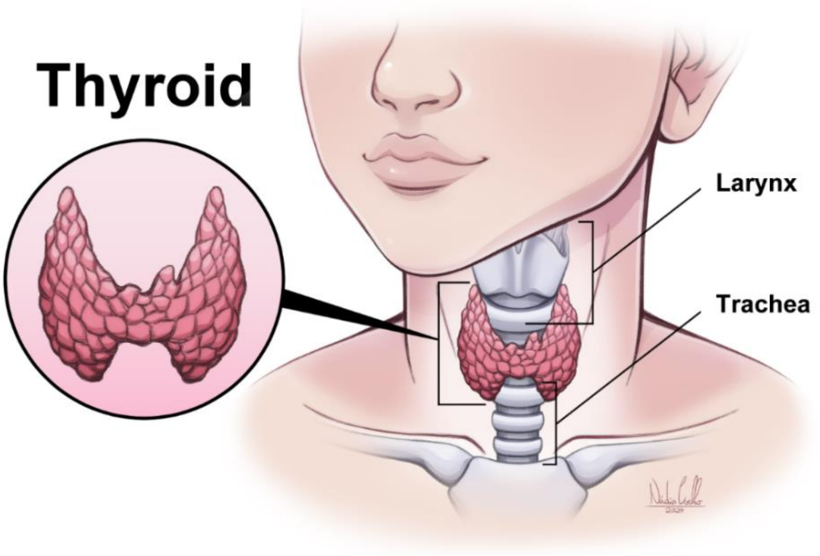


Figure 1: Diagram of the anatomical position of the thyroid. The thyroid is a butterfly-shaped gland of the endocrine system located anteriorly in the neck, in front of the trachea. Diagram illustrated by the author, 2024.

Thyrocytes (also known as thyroid follicular cells) are the cells that produce TH, which are organized in spherical structures (known as follicles) composed of a simple cuboidal epithelium, inside of which the colloid is stored (Brown *et al*., 1986; Ortiga-Carvalho *et al*., 2016; Vitale *et al*., 2017). C cells (also referred to as parafollicular cells) are present in the spaces between thyroid follicles, producing the calcitonin hormone to decrease blood calcium levels when appropriate (Cote *et al*., 2015) (Figure 2).

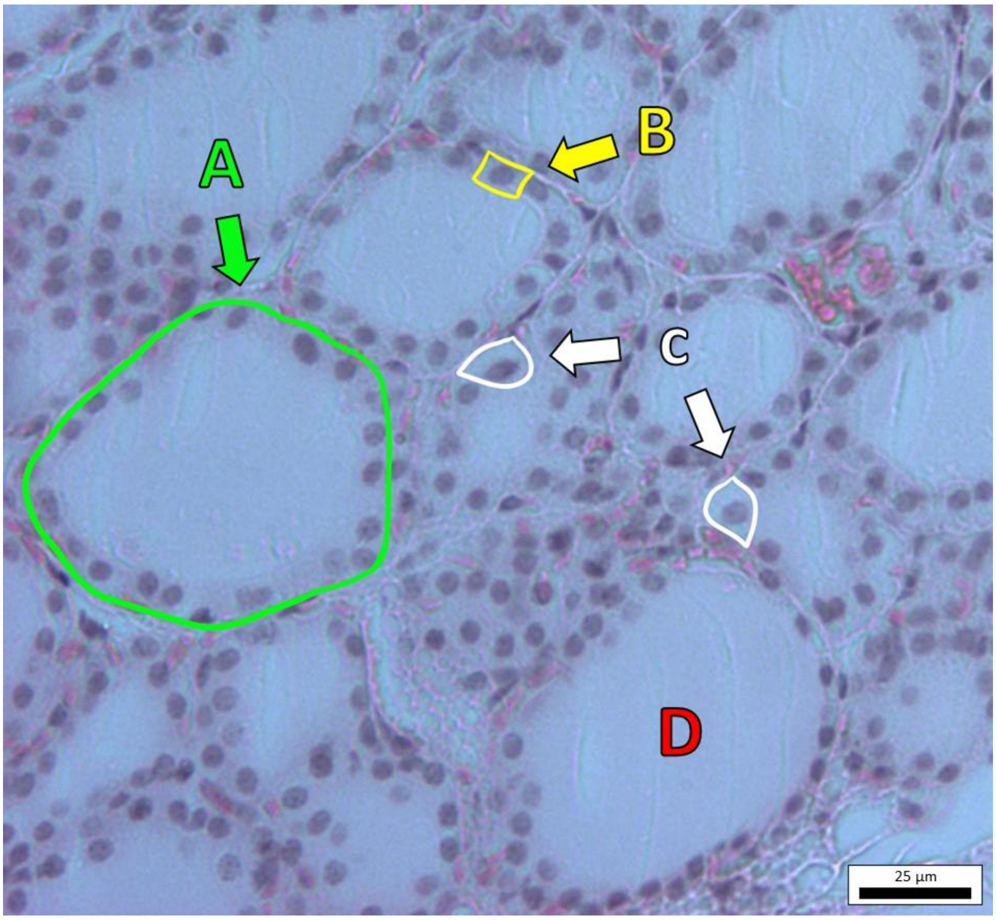


Figure 2: Histological section of the thyroid tissue of wild mice (*Mus musculus* Linnaeus, 1758). Labels: A = follicle; B = thyrocyte (or thyroid follicular cell); C = C cell (or parafollicular cell); D = colloid. Stained with hematoxylin and eosin; scale bar = 25 µm (Coelho *et al*., 2025).

Thyroid function is regulated via the endocrine feedback loop along the hypothalamus-pituitary-thyroid axis (HPT-axis, Figure 3), via which TH production is controlled by negative feedback, according to TH levels in the bloodstream. The hypothalamus secretes thyrotropin-releasing hormone (TRH) to induce the synthesis and secretion of thyrotropin (or thyroid-stimulating hormone, TSH) by the anterior pituitary. In turn, TSH acts on the thyroid to promote the biosynthesis and secretion of TH (Boelen *et al*., 2012; Ortiga-Carvalho *et al*., 2016).

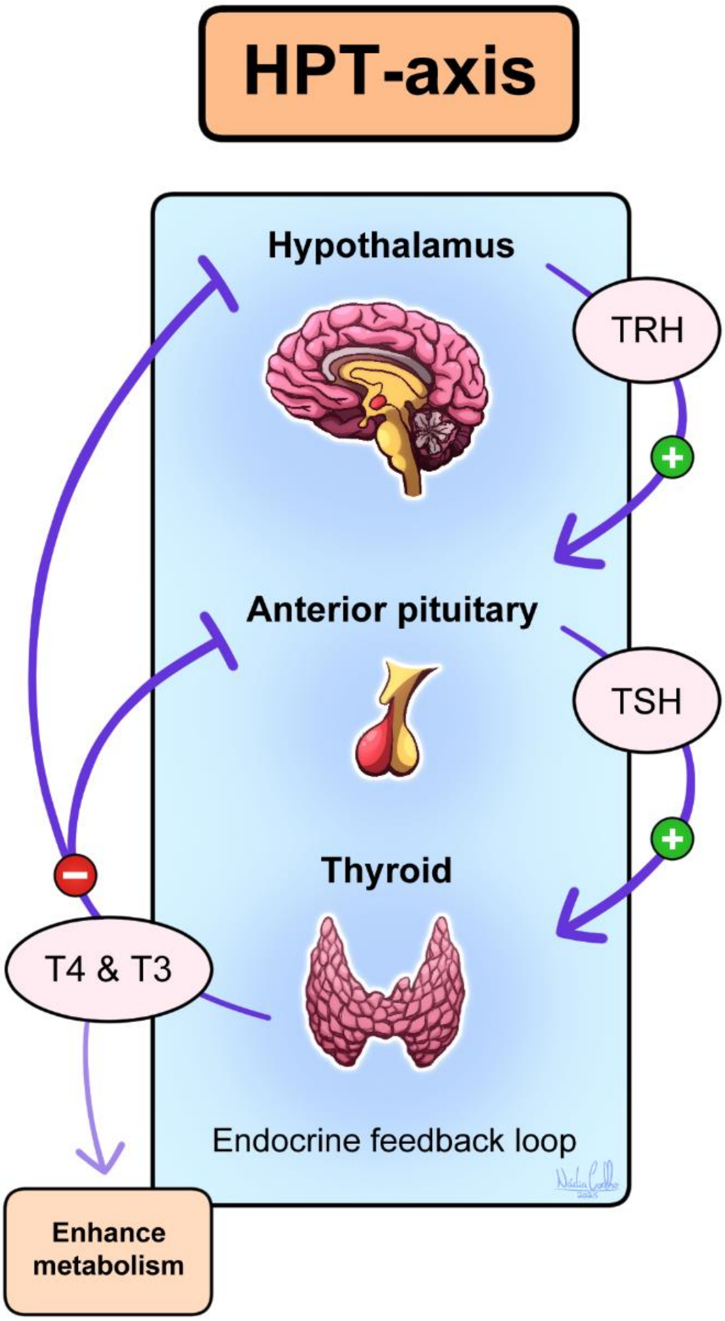


Figure 3: Hypothalamus-pituitary-thyroid axis (HPT-axis). The endocrine feedback loop spanning the HPT-axis modulates thyroid function by keeping thyroid hormone (TH) production in check through negative feedback, according to the concentration of tetraiodothyronine (thyroxine, T4) and triiodothyronine (T3) 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. In turn, TSH acts on the thyroid to promote the biosynthesis and secretion of TH (Coelho *et al*., 2025).

Upon binding to receptors on thyrocytes, TSH stimulates the uptake of iodide (I^-) from the bloodstream through the sodium-iodide symporter. In the cytoplasm, I^- is oxidized by both the thyroperoxidase (TPO) and hydrogen peroxide (H_2O_2) generated by dual oxidases (DUOX), for incorporation into thyroglobulin (Tg) (Leemans *et al*., 2019). As the precursor of TH, kept in the colloid, Tg functions as a negative feedback regulator in the expression of proteins needed for the synthesis of TH, such as TPO and DUOX. The production of TH involves the reabsorption of Tg by thyrocytes for hydrolyzation by acidic peptidases and proteases. This process generates T4 and T3, which are secreted into the bloodstream (Yoshihara *et al*., 2012; Rousset *et al*., 2015). Peripheral tissues express iodothyronine deiodinases (DIO), which are enzymes that catalyze the removal of iodine (I) from TH. The removal of I from TH converts lower-activity T4 to higher-activity T3; after that, extra I removal results in the inactivation of TH (Sutcliffe & Harvey, 2014). The entrance of TH from the bloodstream into cells is possible through trans-membrane transporters (Leemans *et al*., 2019). Once circulating, the presence of TH is signaled via nuclear TH receptors, which modify gene expression by binding to specific DNA elements (Bakker, 2004).

1.2.2. Endocrine disruption by environmental chemicals

The regulation of the output of the endocrine system is performed in a narrow window of effective hormone concentrations, yet preserving plasticity in responses according to physiological needs. A delicate balance in this network is required, as such, to maintain homeostasis (Guérineau *et al*., 2022). Because of this complexity, and the fact that hormones interact virtually with every organ in the body, interference with one hormone often has several consequences (Iatropoulos, 1994; McClure & Stupans, 1995; Campbell & Jialal, 2022).

Endocrine disruption is the process by which natural or man-made chemicals interfere with hormones, frequently by mimicking their structure or blocking their receptors (NIEHS, 2024). These exogenous chemicals are called endocrine disruptors (or endocrine-disrupting chemicals, EDCs), which lead to changes in the physiological function of endogenous hormones (Combarnous & Nguyen, 2019; Shi *et al*., 2024). Exposure to EDCs can result in fertility problems, neurological disorders, increased risks of cancer, and even transgenerational effects – when DNA mutations or changes in the methylation patterns of germ cells are involved (Patisaul *et al*., 2009; Yilmaz *et al*., 2020; Ahn & Jeung, 2023).

Some EDCs include dioxins, phtalates, perchlorate, per- and polyfluoroalkyl substances (PFAS), and polychlorinated biphenyls (PCBs) (NIEHS, 2024). These substances are diffused through the environment via pollutant discharges – especially from industrial and agricultural practices (Kumar *et al*., 2020; Ismanto *et al*., 2022). Agrochemicals, with particular emphasis on pesticides, are widespread environmental EDCs (McKinlay *et al*., 2008; Campos & Freire, 2016; Mantovani, 2016; Kongtip *et al*., 2019; Coelho *et al*., 2024a). Much of the evidence of endocrine disruption caused by environmental EDCs originates from studies on reproductive organs (Boas *et al*., 2006; Parelho *et al*., 2016; Zlatnik, 2016; Rodprasert *et al*., 2021). The term "thyroid disruption" encompasses any adverse thyroid outcomes derived of exposures to EDCs, such as hypothyroidism, hyperthyroidism, and cancer, via their endocrine-disrupting potential (He *et al*., 2024; Coelho *et al*., 2025). Several substances are known to disrupt thyroid function, namely persistent organic pollutants (POPs) [like polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs), and other halogenated flame retardants], bisphenol, particulate matter (PM, especially $PM_{2.5}$), perchlorate, phthalates, and agrochemicals (Calsolaro *et al*., 2017; Thambirajah *et al*., 2022; Coelho *et al*., 2024a; Pearce, 2024; Yang *et al*., 2024; Li *et al*., 2025).

1.2.2.1. Thyroid disruption

Thyroid disorders are the most common endocrine disorders worldwide, estimated at around 200 million people having been diagnosed (Asmelash *et al*., 2019; Zhang *et al*., 2023; Song *et al*., 2024). Estimates suggest that 1 in 10 men, and 1 in 8 women have some form of thyroid condition. It is further believed that 50% of thyroid disorders remain undiagnosed, underlining the seriousness of the topic (Bano *et al*., 2019; Hossen *et al*., 2025). In general, thyroid disorders are divided into two categories: endocrine disorders, and neoplastic disorders. Endocrine disorders are associated with abnormal levels of TH, being classed as either hypothyroidism (insufficient amount of TH), or hyperthyroidism (excessive amount of TH). Meanwhile, neoplastic disorders, although comparatively less frequent, vary between harmless adenomas to highly aggressive carcinomas (Gessl *et al*., 2012; Ghartimagar *et al*., 2020). Hypothyroidism is thought to affect approximately 5% of world population (≈ 380 million), with another 5% remaining undiagnosed (Chiovato *et al*., 2019). On the other hand, data suggests that hyperthyroidism affects roughly 2.5% of world adults (Lee & Pearce, 2023). In turn, thyroid cancer accounts for 3–4% of all cancers, placing 10^{th} in the most common cancers worldwide (Cheng *et al*., 2021; Shank *et al*., 2022). Another relevant thyroid disorder is Hashimoto's thyroiditis (also known as chronic autoimmune thyroiditis, or chronic lymphocytic thyroiditis): an autoimmune disorder where the immune system – namely lymphocytes – mistakenly attack thyroid cells, leading to their destruction via cell- and antibody-mediated responses (Klubo-Gwiezdzinska & Wartofsky, 2022; Kaur & Jialal, 2025). Globally, Hashimoto's thyroiditis has a prevalence of around 7.5% (Hu *et al*., 2022).

The thyroid retains high plasticity even in adults, reflecting its ability to adapt to stimuli by changing both its structure and function according to physiological needs. Because thyroid structure is intimately associated with glandular activity levels, its morphology is a good indicator of its functional state (Inada *et al*., 1981; Perez-Montiel & Suster, 2008; Lee *et al*., 2016; Keestra *et al*., 2020; Candanedo-Gonzalez *et al*., 2021). Thyroid follicle size and epithelium height are examples of where noteworthy histological alterations can be observed (Hallgren & Darnerud, 2002).

The majority of research linking thyroid disruption with exposure to CF and OF contaminants are epidemiological studies. In general, hypothyroidism was often reported in occupationally exposed farm workers (Goldner *et al*., 2010; Piccoli *et al*., 2016; Kongtip *et al*., 2019; Requena *et al*., 2019). However, a recent study showed that mice chronically exposed to an environment where CF is practiced had thyroid histomorphology alterations (Coelho *et al*., 2024a). Thyroid alterations in the exposed mice ranged from a much lower epithelial cell area and volume, to lower follicular sphericity, irregularly-delimited epithelium, and increased exfoliation into the colloid. Such alterations, namely when it comes to lower epithelial cell area and volume, revealed indications of hypothyroidism in the exposed mice. These results highlight the usefulness of thyroid histomorphology evaluation as an early and sensitive biomarker of glandular activity, therefore providing indications of thyroid disruption.

There are important differences in the physiology of males and females which should be taken into consideration in biomedical research, given they can affect how individuals respond to certain conditions and treatments. Such physiological differences primarily arise from chromosomal arrangement and hormones, leading to varying body composition (i.e., muscle mass and body fat distribution), bone density, organ system structures, and metabolic rates (Blair, 2007; Ngun *et al*., 2011; Massey *et al*., 2021). When it comes to the thyroid gland, it is widely observed that women are more often affected by thyroid disorders than men (Mammen *et al*., 2021; Brown *et al*., 2023). Considering the aforementioned, it would make sense to hypothesize that the thyroid of female individuals is more sensitive to the endocrine-disrupting effects of environmental contaminants. Still, studies addressing this possibility are lacking. Furthermore, there is a lack of data regarding differences between males and females in relation to thyroid histomorphology.

2. Context and relevance of the study

Agricultural practices have a fundamental role in providing food, fuel, and fiber to world population. Agriculture is a growing sector to meet the ever-increasing needs of humankind, which results in a continuous use of many agrochemicals – fertilizers, pesticides, and soil conditioners – to keep up with demand (Ball & Quintart, 2023). The fact that most agricultural practices resort to CF methods with agrochemical use for production not only means environmental unsustainability, but also represents a serious health hazard, since exposure to agrochemicals is associated with higher risks of cancer and, among other issues, the induction of endocrine disorders (Warner *et al*., 2020). The transition from CF to OF systems is being promoted, based on the perception that the latter use safer, healthier, and less environmentally impactful methods – like the use of natural fertilizers – for production. However, there are signs that OF is not as environmentally safe as believed, given that OF produce is not completely free of pesticides (Giampieri *et al*., 2022; Lazarević-Pašti *et al*., 2025). Another worrying aspect is the fact that the materials used as natural fertilizers (mainly manure) are mostly derived from animals that have been treated with antibiotics, growth factors, among other substances, which leads to an increase in the distribution and abundance of ARGs in the environment (Zhou & Yao, 2020).

Given the many biological processes and bodily functions that T4 and T3 regulate, the disruption of TH balance has consequences for the entire body. Both endocrine and neoplastic thyroid disorders can be triggered by exposure to environmental EDCs, such as agrochemicals, because these compounds mimic the chemical structure of TH (Babić Leko *et al*., 2021). Thyroid disorders are among the most common in the world, with their incidence rising swiftly among global population (Leese *et al*., 2008; Vanderpump, 2011; TLDE, 2013, Maniakas *et al*., 2018; Stahlman & Oh, 2018; Deng *et al*., 2020), revealing the need for developing studies that can accurately identify what causes thyroid disruption and countermeasures against so. The most common disorder is hypothyroidism, with estimates suggesting that approximately 5% of the world population (≈ 380 million) suffers from hypothyroidism, and that another 5% may be undiagnosed cases (Chiovato *et al*., 2019). In Portugal, the prevalence of this disorder ranges from 8–10% (≈ 1 million) (Rodrigues, 2018), with a similar scenario being the case in the Autonomous Region of the Azores.

Thyroid plasticity, closely related to its activity, can be observed at the morphological level, providing indications of endocrine disruption. Histological changes that reflect this can be evidenced in the size of the thyroid follicles – which are the functional units of the organ – and in the characteristics of the epithelium, revealing the potential of histomorphologic evaluation as an early and sensitive biomarker of glandular activity (Hallgren & Darnerud, 2002). However, most of the research in the topic corresponds to epidemiological studies that elucidate connections between human exposure to pesticides and the occurrence of thyroid disorders, in which the average concentration of TH in the blood of participants is evaluated (Kongtip *et al*., 2019; Requena *et al*., 2019). In light of this, continuing the work already developed within the context is of utmost importance (Coelho *et al*., 2024a; Coelho *et al*., 2024b).

The purpose of this thesis work is linked to the comparison of the negative impact of CF on the thyroid with the potential negative impact of OF on this organ, using histological techniques for the analysis of the thyroid of wild mice chronically exposed to these agricultural practices. The topic is especially relevant given the growing demand for agricultural products, and the tendency of regarding OF as a better alternative to CF.

As part of the Master's Degree in Biomedical Sciences, the Master's Dissertation entitled "Impact Assessment of Organic *vs*. Conventional Farming on the Thyroid: A Histological Perspective" was developed. In a previous study, conducted within the scope of the final project for a Bachelor's Degree in Biology, it was found that chronic exposure of wild mice to an environment in which CF is practiced leads to histological changes in their thyroid indicating loss of function (hypothyroidism) (Coelho *et al*., 2024a). For this reason, the aim of this work is to answer the following questions:

1. Does chronic exposure to an environment in which OF is practiced have a negative impact on the thyroid?
2. If so, is such impact less severe than that caused by CF?

For these questions, the scientific hypothesis is that chronic exposure to OF has a negative impact on the thyroid, but one less severe than that caused by CF.

On the other hand, there is a lack of histomorphological data comparing the thyroids of male and female individuals. In light of this, and considering that women tend to be affected by thyroid disorders more often than men, this work also aims to answer the question:

3. Is the impact of CF and OF contaminants more severe on the thyroid of female individuals than on the thyroid of male individuals?

The scientific hypothesis for this question is that the impact of chronic exposure to either farming system is more severe on the thyroid of female individuals than on the thyroid of male individuals.

To answer these questions and the study hypotheses, this study has the following specific objectives:

- Evaluating thyroid histomorphometric alterations, namely in regards of (i) number of follicles per 30,000 μm^2 of thyroid, (ii) the area and (iii) perimeter of the colloid, (iv) the thickness of the epithelium, (v) the number of epithelial cell nuclei per a 50 µm transect, and (vi) the average width, (vii) area, and (viii) volume of epithelial cells.
- Evaluating thyroid histomorphological alterations, namely in regards of (i) follicular sphericity, (ii) epithelium irregularity, (iii) degree of exfoliation, (iv) degree of inflammation, and (v) degree of colloid vacuolization.
- Comparing results (i) between groups CF, OF, and RF', and (ii) between males and females within groups.

3. Methodology

3.1. Study sites

This work was carried out in São Miguel Island, Azores, Portugal. Study sites were selected based on the presence or absence of agricultural practices – including the nature of those practices. A total of 4 sites was selected: 2 agricultural areas (study groups), and 2 areas without agriculture (reference groups) (Figure 4). The 2 study groups corresponded to a CF site, and an OF site. Concerning the 2 reference groups, one was a small village (Rabo de Peixe, RF1), while the other corresponded to a pristine area (Pinhal da Paz, RF2); in neither of which agricultural practices were made for at least over a decade. All sites are located in the geological complex of Picos Fissural Volcanic System, granting them the same bedrock and pedological conditions. This ensures that each site is distinguished based on the presence and nature of agricultural practices (Parelho *et al*., 2014; Parelho *et al*., 2016).

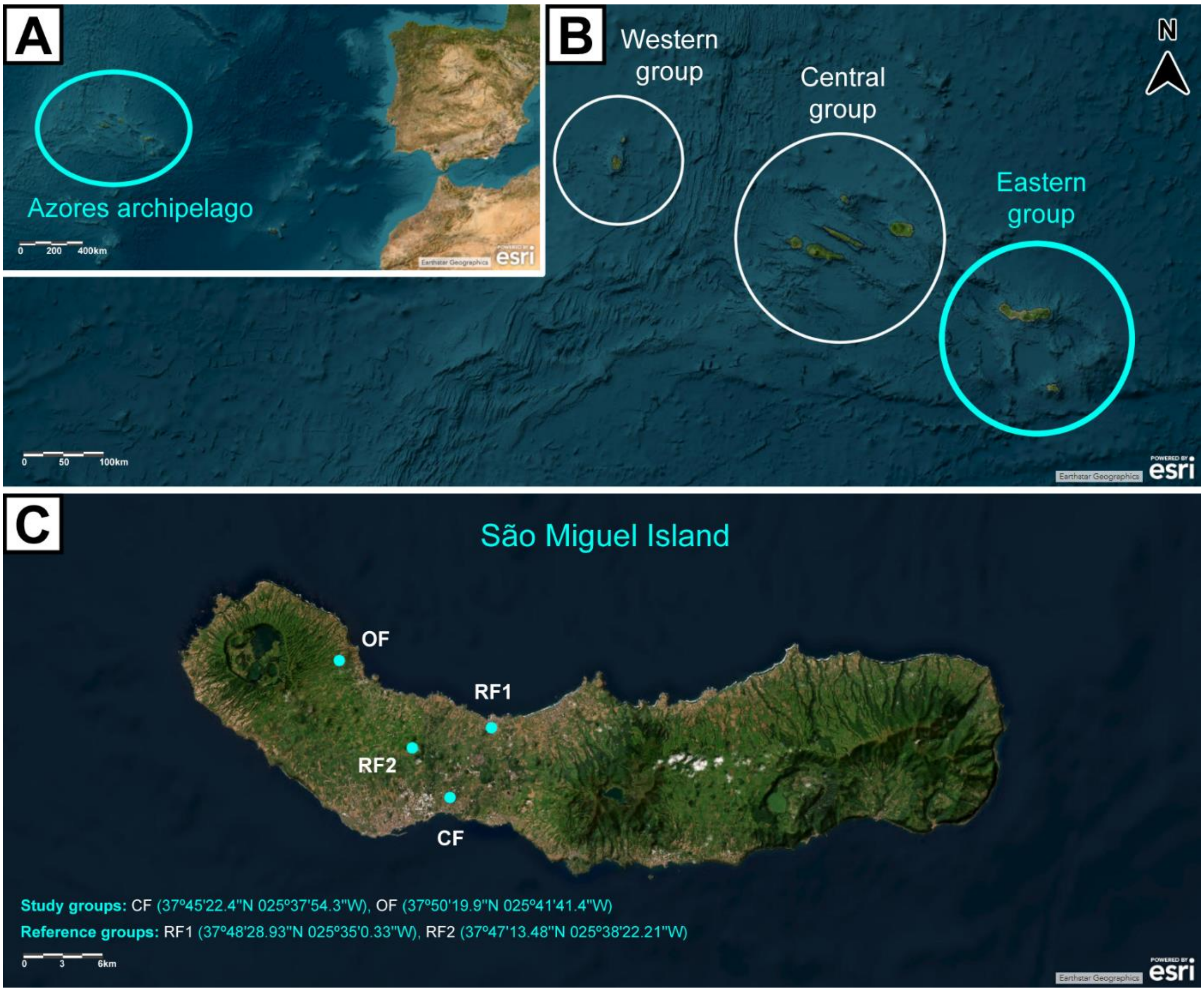


Figure 4: Map of the Azores Archipelago showcasing (A) its location in the Atlantic Ocean, (B) its geographical groups, and (C) São Miguel Island and study sites. Study groups = conventional farming (CF), and organic

farming (OF) agricultural areas; Reference groups = small village area (Rabo de Peixe, RF1), and pristine area (Pinhal da Paz, RF2), without agricultural practices. Basemap aerial view backgrounds obtained from ESRI ArcGIS online: “World Imagery” [basemap], “World Imagery Map”. Last updated on November 2024. https://www.arcgis.com/home/item.html?id=10df2279f9684e4a9f6a7f08febac2a9 (lastly consulted on 10th December 2024). Attribution to ESRI and other data providers is present in the figure.

Conventional farming systems involve agricultural practices in which the use of agrochemicals is frequent and legally framed by European and national guidelines. Agrochemicals used in the CF study site include organic fertilizers, inorganic fertilizers, and pesticides like insecticides, herbicides, nematicides, fungicides, molluscicides, and acaricides. Parelho *et al*. (2014) mention that agriculture is a diffuse source of metal pollution in farming soils of São Miguel Island. According to the authors, CF soils in São Miguel Island are particularly contaminated with lithium (Li), potassium (K), and molybdenum (Mo), due to intensive and repeated use of inorganic fertilizers and pesticides. On the other hand, OF systems are certified by the European Commission, which prohibit the use of synthetic agrochemicals. Soil amendments are confined to organic fertilizers, namely compost and manure. Both farms have been explored under the same system for at least a decade (Parelho *et al*., 2016). The RF1 site is known for being the biggest fishing village in the Azores. There are no major agricultural practices within the village, with the exception of some home gardening for a small part of its population. Meanwhile, the RF2 site is 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. According to Parelho *et al*. (2014), the soils from OF and RF2 have trace metal background values significantly lower than those found in the CF site. An exception was found for vanadium (V) and barium (Ba), which are present in higher concentrations in RF2 than in CF and OF soils. Further details on the characteristics of the study sites can be found in Parelho *et al*. (2016).

3.2. Mice sampling and histological slides preparation

Data from animals living in – or otherwise exposed to – contaminated areas is important in research, given it provides early warnings for environmental hazards and potential human health risks. The assessment of toxic effects on bioindicator species at environmentally relevant concentrations helps in understanding long-term effects of environmental stressors, monitoring contaminant flow through ecosystems and food chains, developing conservation strategies, and protecting public health. Additionally, field studies with animal models provide invaluable ecotoxicological data under the complexity of the assessed environment, which would be impossible to recreate in laboratory (Sánchez-Chardi *et al*., 2009; Chen *et al*., 2024). Animal models used for ecotoxicological risk assessment include invertebrates such as worms, and small mammals like wild mice, which can be sampled directly from the studied environments (Shore & Douben, 1994; Imholt *et al*., 2018; Gudeta *et al*., 2023). The main criteria for selecting such bioindicators are (i) high abundance, (ii) a life expectancy wide enough for the estimation of possible long-term effects (Marcheselli *et al*., 2010), and (iii) a fairly small home range of, on average, 145 m^2 (Lidicker, 1966) (which is much smaller than the surface area of the study sites – i.e., farming areas of around 5000 m^2). Given wild mice *Mus musculus* Linnaeus, 1758, fulfill all the mentioned criteria, they were selected as surrogate species for this work (Coelho *et al*., 2024a).

Following the methodology described by Coelho *et al*. (2024a), wild *M. musculus* mice were captured in each of the selected sites (CF, OF, RF1, and RF2). Live catch-traps were used, housing the mice for no longer than necessary after capture 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 based on dry crystalline mass (Quéré & Vincent, 1989). Both males and females were included in this work. Histological slides with sections of the thyroid of 64 individuals were made (each slide corresponding to a single individual, containing several 5 µm thick sections): 12 from CF, 12 from OF, 27 from RF1, and 13 from RF2. Afterwards, the slides were stained with hematoxylin and eosin following the methodology of Martoja *et al*. (1970). From these, a series of histomorphometric and histomorphologic data was collected, with the aim of evaluating the thyroid status of each individual.

Because males and females were not equally distributed between groups (χ^2-test, $P < 0.05$), their data was treated separately (Appendix). For both males and females, groups RF1 and RF2 did not differ in the majority of the evaluated parameters (*t*-test, $P > 0.05$ for normal data; *U*-test, $P > 0.05$ for non-normal data), hence they were merged into a new group, designated RF' (Appendix). A higher number of individuals in the RF' group in comparison to the CF and OF groups ensured a stronger statistical power, increasing the likelihood of detecting real differences between groups (Andrade, 2020).

The experimental procedures of this work 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).

3.3. Histomorphometric analysis

Following the methodology from Coelho *et al*. (2024a), 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 were less likely to 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 – since that 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 (Figure 5). The referred area was chosen based on the photomicrograph with the smallest thyroid sample. The follicles that were present entirely within that area were counted, including cases in which at least more than half of the follicle was present.

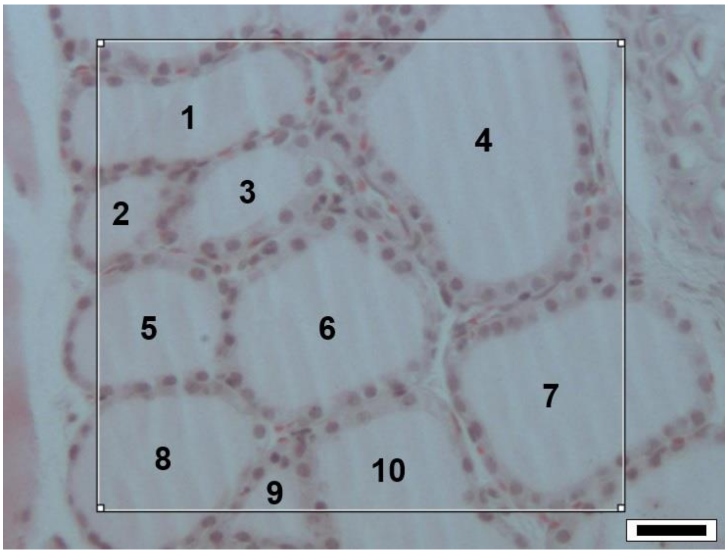


Figure 5: Photomicrograph from a thyroid histological section of a wild mouse (*Mus musculus* Linnaeus, 1758), showing the number of follicles per thyroid area. The area represented by the square is 30,000 µm^2. All follicles fully present in the area were counted, including cases in which at least more than half of the follicle was present. In this example, there was a total of 10 follicles per 30,000 µm^2 of thyroid. Stained with hematoxylin and eosin; scale bar = 25 µm.

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 boundary of the colloid from the apical surface of the epithelial cells of the corresponding follicle (Figure 6); (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 4 regions, approximately equidistant, from each of the follicles analyzed, then registered as the average of these 4 regions per follicle (Figure 7); and (iv) the number of epithelial cells nuclei per a 50 µm transect, measured in 4 regions of each follicle, then registered as the average of these 4 regions per follicle (Figure 8). All the data obtained per each analyzed parameter was finally registered as the average for each individual.

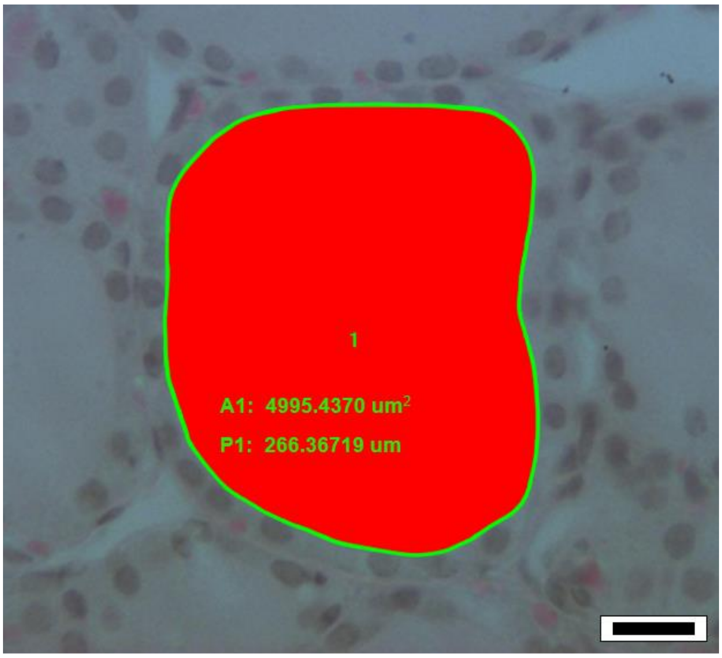


Figure 6: Photomicrograph from a thyroid histological section of a wild mouse (*Mus musculus* Linnaeus, 1758), showing the measurement of the area and perimeter of the colloid of a thyroid follicle. The area (A1) corresponds to the interior of the follicle, represented in red, in µm^2, and the perimeter (P1) corresponds to the delimitation given by the apical surface of the epithelial cells, represented in green, in µm. In this example, 4995.4370 µm^2 of area and 266.36719 µm of perimeter were registered. Stained with hematoxylin and eosin; scale bar = 15 µm.

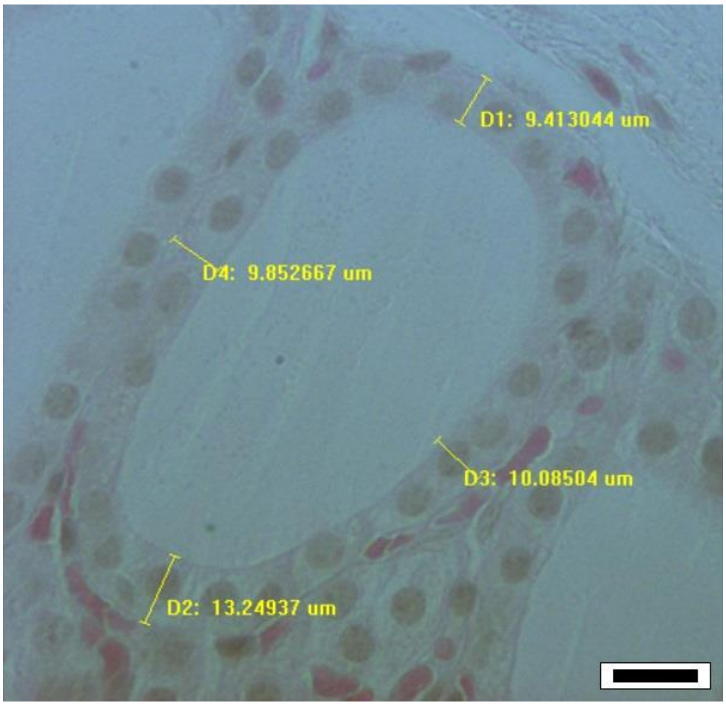


Figure 7: Photomicrograph from a thyroid histological section of a wild mouse (*Mus musculus* Linnaeus, 1758), showing the measurement of the thickness of the epithelium of a thyroid follicle. The thickness of the

epithelium was measured at 4 locations of the follicle epithelium, more or less equidistant from each other, from the basal lamina of the cells to their apical surface (in a straight line perpendicular to these). The indicated values were registered as the average per follicle, in µm. In this example, the average thickness of the follicle epithelium corresponded to 10.650030 µm. Stained with hematoxylin and eosin; scale bar = 15 µm.

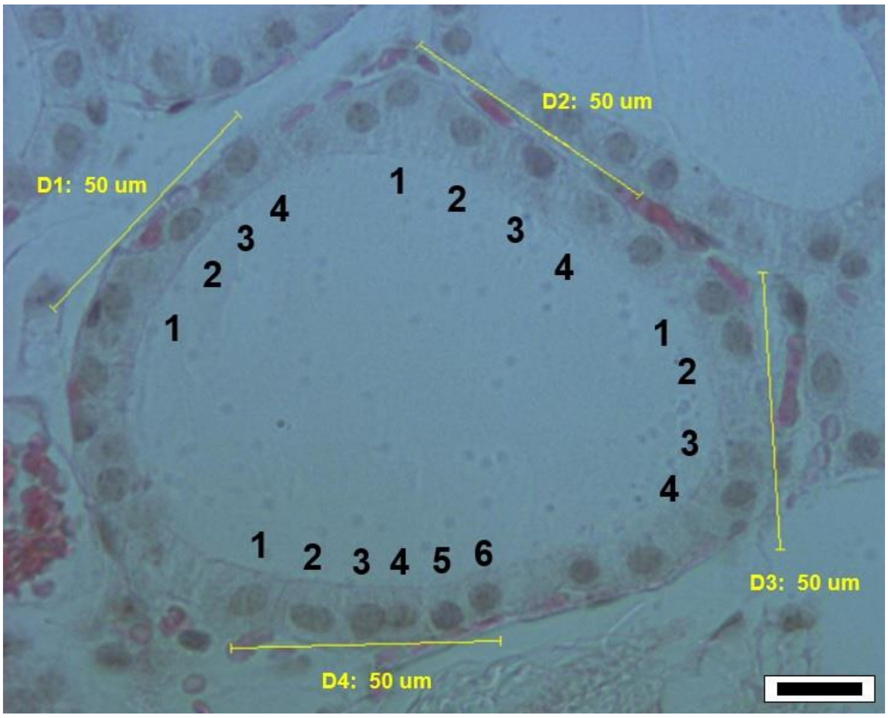


Figure 8: Photomicrograph from a thyroid histological section of a wild mouse (*Mus musculus* Linnaeus, 1758), showing the counting of the number of epithelial cell nuclei in a thyroid follicle by a 50 µm transect. The transect was applied in 4 places of the follicle epithelium, more or less equidistant from each other. 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.

The average width of epithelial cells was determined, in µm, via 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 thickness of the epithelium varies with thyroid activity, with cuboidal cells when this gland functions normally, and smaller, squamous cells when it is underactive. As such, the average epithelium thickness, and the average area and volume of epithelial cells were considered the main biomarkers of thyroid function (Takamatsu *et al*., 1992; De Felice & Di Lauro, 2010).

3.4. Histomorphological analysis

Following the criteria from Coelho *et al*. (2024a), based on the photomicrographs taken with the 20X objective, a set of 5 different parameters were analyzed semi-quantitatively through visual qualitative scales, per field, per individual. In these scales, the greater 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, "3" = not spherical at all; (ii) epithelium irregularity, based on the appearance of the luminal surface of thyroid follicles epithelium (whether it was smooth, or had folds, projections and/or depressions), in which "1" = regular, "2" = somewhat irregular, "3" = very irregular; (iii) degree of exfoliation, based on the presence of cell debris (nuclei) within the colloid, in which "1" = none, "2" = some, "3" = a lot; (iv) degree of inflammation, based on the presence of lymphocytes in the interstitial tissue, in which "1" = none, "2" = some, "3" = a lot; and (v) degree of colloid vacuolization, based on the presence of vacuoles in the colloid, in which "1" = none, "2" = some, "3" = a lot. The analyses were carried out independently by two observers, having the final values been registered by consensus, from a total of observations equivalent to: 60 for CF, 60 for OF, 135 for RF1, and 65 for RF2. The independent analyses and large number of observations ensured that the recorded abnormalities were not influenced by artifacts of histological processing. Examples of photomicrographs to which extreme values were assigned regarding each evaluated aspect can be found in Figures 9, 10, 11, 12, 13.

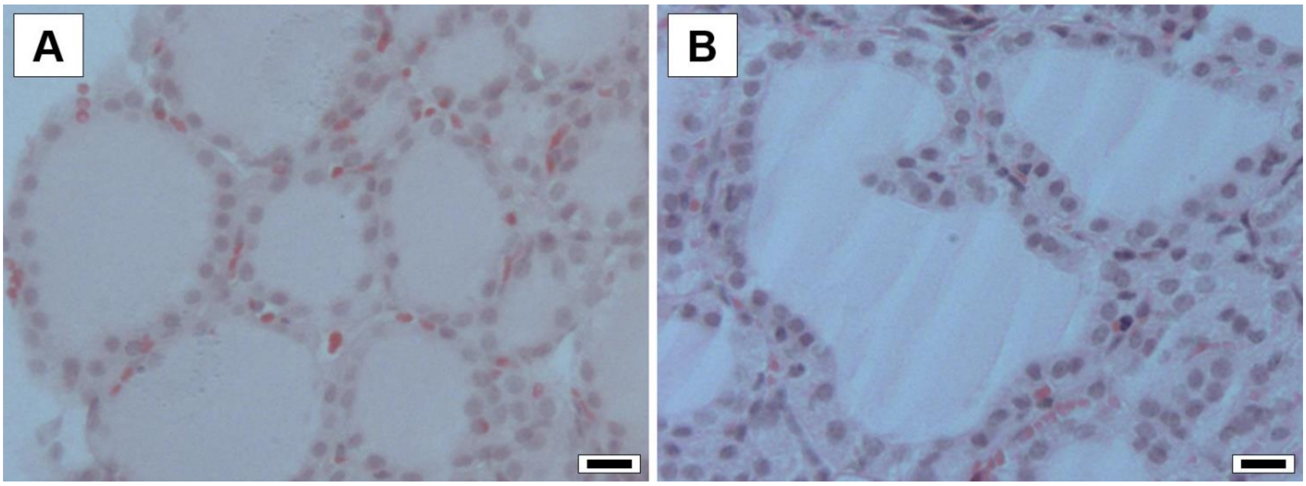


Figure 9: Photomicrographs from thyroid histological sections of wild mice (*Mus musculus* Linnaeus, 1758), showing an example of the category "spherical" (value 1, photomicrograph A) and "not spherical at all" (value 3, photomicrograph B), regarding the assessment of follicular sphericity. Stained with hematoxylin and eosin; scale bars = 25 µm (A and B).

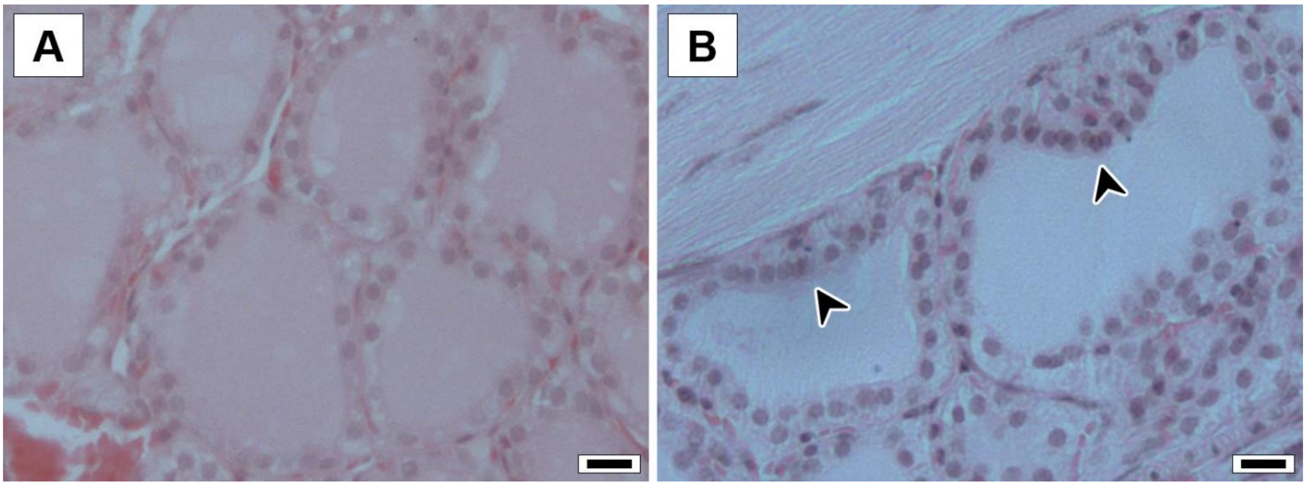


Figure 10: Photomicrographs from thyroid histological sections of wild mice (*Mus musculus* Linnaeus, 1758), regarding the assessment of epithelium irregularity, showing an example of the category “regular” (value 1, photomicrograph A) and “very irregular” (value 3, photomicrograph B). The black arrowheads in photomicrograph B indicate some areas where the epithelium delimitation is irregular. Stained with hematoxylin and eosin; scale bars = 25 µm (A and B).

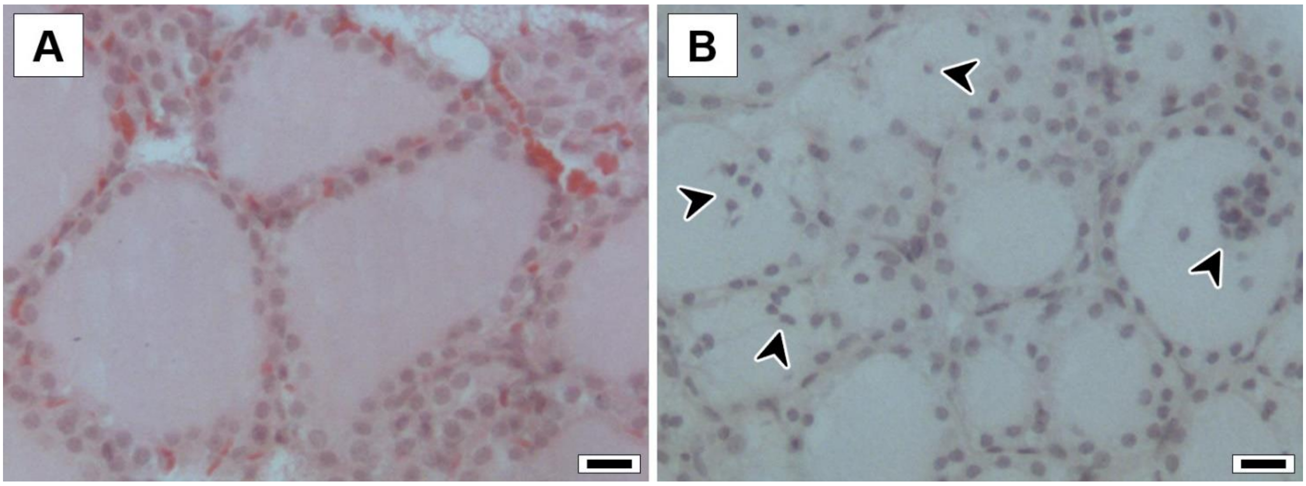


Figure 11: Photomicrographs from thyroid histological sections of wild mice (*Mus musculus* Linnaeus, 1758), regarding the assessment of the degree of exfoliation, showing an example of the category “none” (value 1, photomicrograph A) and “a lot” (value 3, photomicrograph B). The black arrowheads in 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).

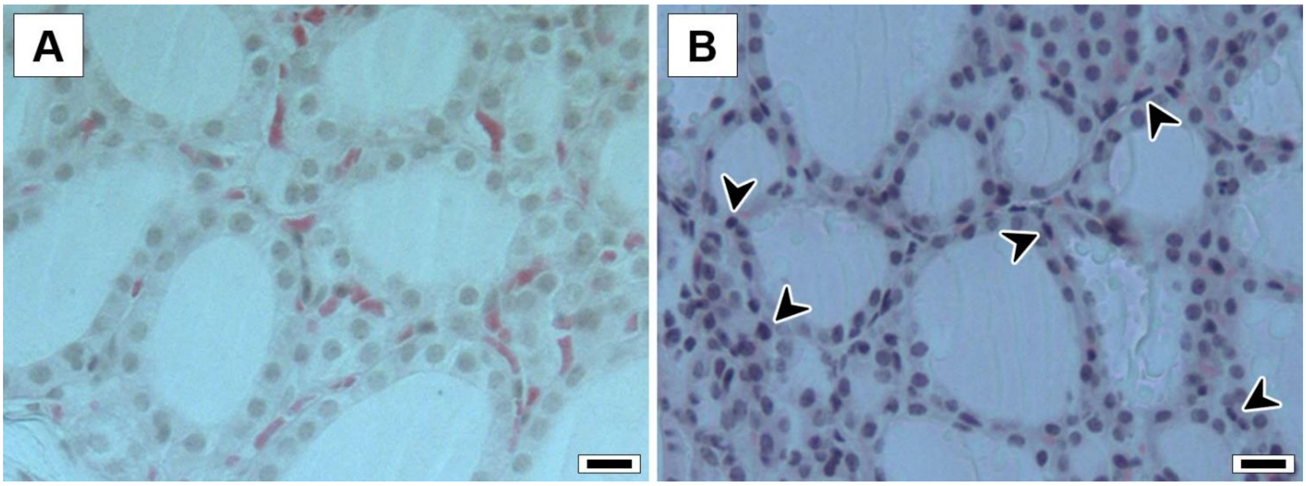


Figure 12: Photomicrographs from thyroid histological sections of wild mice (*Mus musculus* Linnaeus, 1758), regarding the assessment of the degree of inflammation, showing an example of the category “none” (value 1, photomicrograph A) and “a lot” (value 3, photomicrograph B). The black arrowheads in 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).

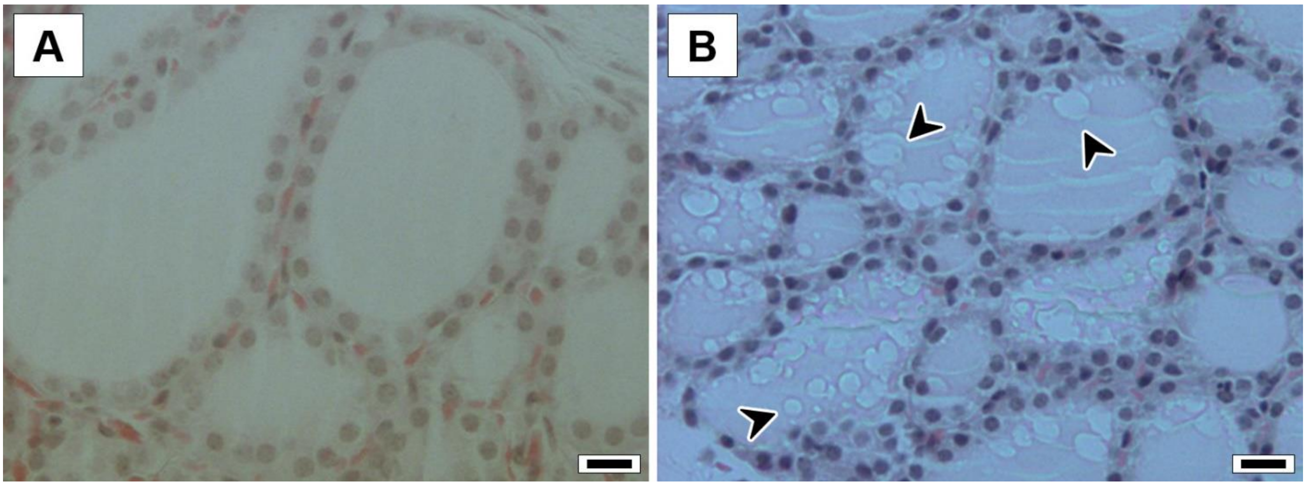


Figure 13: Photomicrographs from thyroid histological sections of wild mice (*Mus musculus* Linnaeus, 1758), regarding the assessment of the degree of colloid vacuolization, showing an example of the category “none” (value 1, photomicrograph A) and “a lot” (value 2, photomicrograph B). The black arrowheads in photomicrograph B indicate some areas where colloid vacuoles can be observed. Stained with hematoxylin and eosin; scale bars = 25 µm (A and B).

3.5. Statistical analysis

Statistical analysis was performed in the IBM SPSS Statistics ® v. 28.0.1 (142) software (IBM Corp., Armonk, NY, USA). Statistical significance was considered when $P < 0.05$.

The normality of the morphometric analysis was verified through Q-Q plots. Differences of the means between groups CF, OF, and RF' were assessed via One-way analysis of variance (ANOVA) tests [followed by post-hoc Tukey's honest significance (HSD) tests, to assess which groups differed from one another], for normal data, and non-parametric independent samples Kruskal-Wallis tests (followed by pairwise comparisons, to assess which groups differed from one another), for non-normal data. Differences of the means from males and females within groups were assessed via independent samples *t*-tests (preceded by a Levene's test for homogeneity of variances), for normal data, and non-parametric Mann-Whitney *U* tests, for non-normal data.

Box plots portraying data regarding colloid area and perimeter, epithelium thickness, and epithelial cell area and volume were made, with the aim of improving the perception of differences between their distributions per group. Much like a squamous epithelium, follicles with larger colloid area and perimeter are also indicative of an underactive thyroid. In light of this, the mentioned parameters were chosen for graphical representation due to better representing indications of thyroid disruption (Takamatsu *et al*., 1992; De Felice & Di Lauro, 2010; Lee *et al*., 2016).

Regarding the morphologic analysis, for each of the evaluated aspects, non-parametric Kruskal-Wallis tests were used to compare the distributions between the values registered in observations between groups CF, OF, and RF'. The relative frequency of each category was calculated, per group, to improve the perception of possible differences between their distributions per group.

Associations between data were determined via Spearman correlations (in all correlations assessed, at least one parameter had non-normal data).

4. Results

4.1. Histomorphometric analysis

4.1.1. Males

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

Table 1: Descriptive statistics of the registered means and respective standard errors of the histomorphometric data, referring to the male individuals from conventional farming (CF), organic farming (OF), and reference (RF') sites. Pairwise comparisons are shown. **F*-test; ***H*-test; *P* values marked in bold indicate significant differences ($P < 0.05$).

Variable	**Sites**	**Mean ± standard error**	***ANOVA/**Kruskal-Wallis**	**Tukey's HSD/Pairwise comparisons**	***P values***
Weight (g)	CF	13.8763 ± 0.60143		CF-OF	*0.964*
	OF	13.6050 ± 0.46806	*F(2,34) = 3.289, *P = 0.051*	CF-RF'	*0.165*
	RF'	15.6750 ± 0.69707		OF-RF'	*0.069*
Age (days)	CF	101.8450 ± 7.09495		CF-OF	*0.508*
	OF	119.2210 ± 12.35438	**H(2,34) = 18.742, *P* **< *0.001***	CF-RF'	**< *0.001***
	RF'	216.9650 ± 17.07903		OF-RF'	**< *0.001***
Colloid area (µm)	CF	5796.4850 ± 942.83546		CF-OF	***0.001***
	OF	2344.9890 ± 406.97965	**H(2,34) = 14.462, *P* **< *0.001***	CF-RF'	*0.674*
	RF'	5163.8750 ± 401.96417		OF-RF'	**< *0.001***
Colloid perimeter (µm)	CF	353.0425 ± 32.67886		CF-OF	**< *0.001***
	OF	212.1650 ± 20.12493	**H(2,34) = 13.292, *P* **< *0.001***	CF-RF'	*0.160*
	RF'	282.0206 ± 11.05412		OF-RF'	***0.008***
Epithelium thickness (µm)	CF	4.5988 ± 0.71935		CF-OF	***0.006***
	OF	6.1800 ± 0.12411	**H(2,34) = 15.683, *P* **< *0.001***	CF-RF'	**< *0.001***
	RF'	7.1931 ± 0.31141		OF-RF'	***0.020***
Number of epithelial cell nuclei per 50 µm	CF	4.6813 ± 0.47082		CF-OF	**< *0.001***
	OF	6.1710 ± 0.17606	**H(2,34) = 15.957, *P* **< *0.001***	CF-RF'	*0.197*
	RF'	5.0444 ± 0.11764		OF-RF'	***0.002***

Epithelial cell width (µm)	CF	11.2787 ± 0.87493	*F(2,34) = 11.654, *P* **< *0.001***	CF-OF	**< *0.001***
	OF	8.1650 ± 0.23707		CF-RF’	*0.097*
	RF’	9.9906 ± 0.21970		OF-RF’	***0.007***
Epithelial cell area (μm^2)	CF	48.0463 ± 2.62026	*F(2,34) = 18.641, *P* **< *0.001***	CF-OF	*0.898*
	OF	50.3220 ± 1.21160		CF-RF’	**< *0.001***
	RF’	71.9600 ± 3.60307		OF-RF’	**< *0.001***
Epithelial cell volume (μm^3)	CF	534.7112 ± 41.54530	*F(2,34) = 15.450, *P* **< *0.001***	CF-OF	*0.186*
	OF	412.6550 ± 19.69602		CF-RF’	***0.012***
	RF’	724,8519 ± 45.65798		OF-RF’	**< *0.001***
Number of follicles per 30,000 μm^2 of thyroid	CF	5.3750 ± 0.96395	**H(2,34) = 20.722, *P* **< *0.001***	CF-OF	**< *0.001***
	OF	17.7200 ± 2.44717		CF-RF’	***0.018***
	RF’	9.7500 ± 0.75862		OF-RF’	***0.005***

The box plots below illustrate the differences between groups (males) concerning colloid area and perimeter, epithelium thickness, and epithelial cell area and volume (Figure 14).

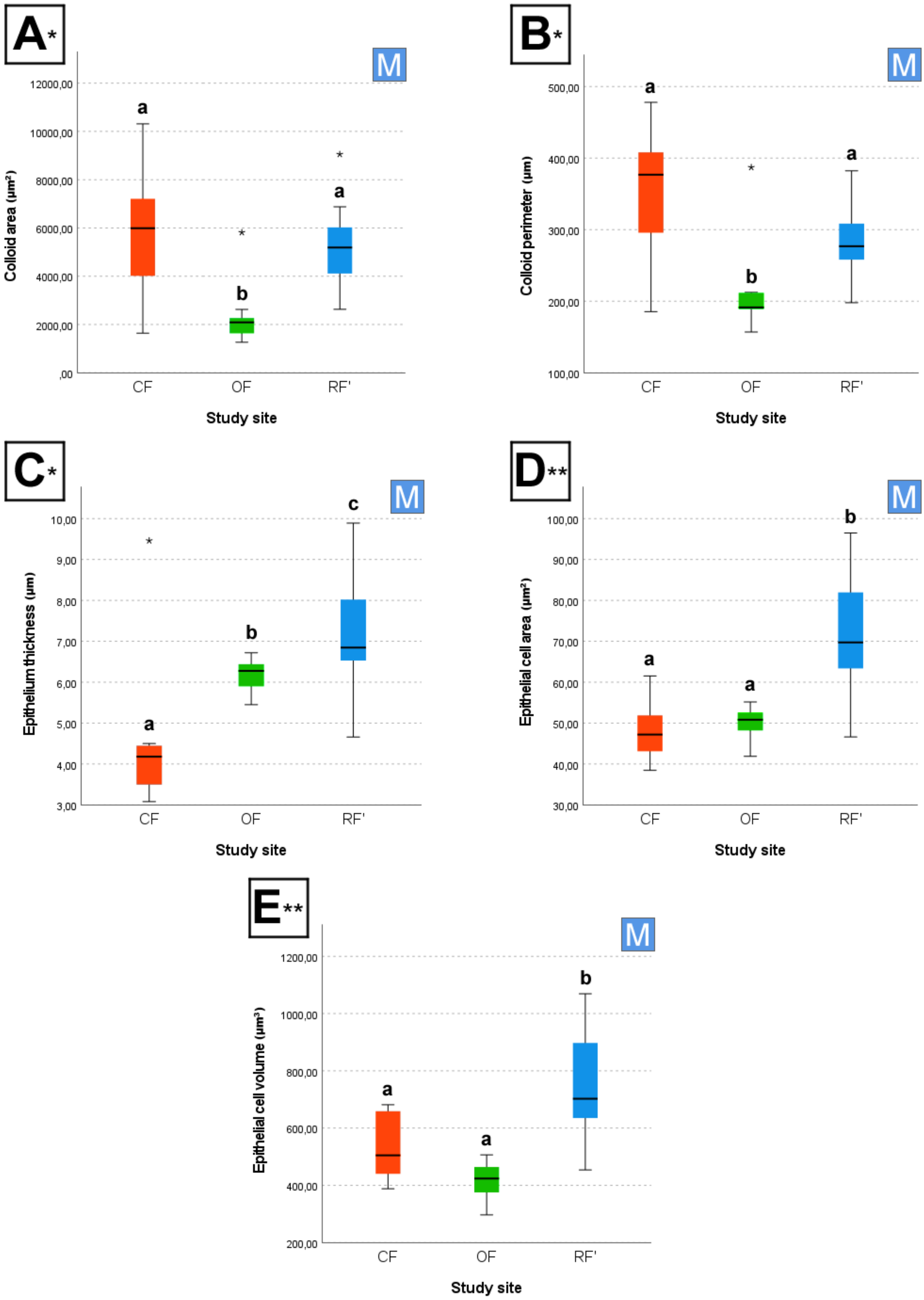


Figure 14: Box plots of the (A) colloid area, (B) colloid perimeter, (C) epithelium thickness, (D) epithelial cell area, and (E) epithelial cell volume of the male (M) individuals from conventional farming (CF), organic farming (OF), and reference (RF') sites. **H*-test; ***F*-test; different letters (a and b) indicate significant differences ($P < 0.05$). The line in the middle of each box plot represents the median of the respective group; error bars are shown; an asterisk within the graphs indicates a group outlier.

According to Table 1 and Figure 14, the groups (males) significantly differed in all parameters except weight (*F*-test, $P > 0.05$).

Between CF and OF, results show that male individuals from CF had, on average, significantly larger colloid area (*H*-test, $P < 0.05$), larger colloid perimeter (*H*-test, $P < 0.05$), lower epithelium thickness (*H*-test, $P < 0.05$), a lower number of epithelial cell nuclei per 50 µm (*H*-test, $P < 0.05$), larger epithelial cell width (*F*-test, $P < 0.05$), and a lower number of follicles per 30,000 µm^2 of thyroid (*H*-test, $P < 0.05$), when compared to male individuals from OF. However, CF and OF males did not differ significantly regarding age (*H*-test, $P > 0.05$), epithelial cell area (*F*-test, $P > 0.05$), nor epithelial cell volume (*F*-test, $P > 0.05$).

Between CF and RF', results show that male individuals from CF were, on average, significantly younger (*H*-test, $P < 0.05$), had lower epithelium thickness (*H*-test, $P < 0.05$), lower epithelial cell area (*F*-test, $P < 0.05$), lower epithelial cell volume (*F*-test, $P < 0.05$), and a lower number of follicles per 30,000 µm^2 of thyroid (*H*-test, $P < 0.05$), when compared to male individuals from RF'. However, CF and RF' males did not differ significantly regarding colloid area (*H*-test, $P > 0.05$), colloid perimeter (*H*-test, $P > 0.05$), number of epithelial cell nuclei per 50 µm (*H*-test, $P > 0.05$), and epithelial cell width (*F*-test, $P > 0.05$).

Finally, between OF and RF', results show that male individuals from OF were, on average, significantly younger (*H*-test, $P < 0.05$), had lower colloid area (*H*-test, $P < 0.05$), lower colloid perimeter (*H*-test, $P < 0.05$), lower epithelium thickness (*H*-test, $P < 0.05$), a higher number of epithelial cell nuclei per 50 µm (*H*-test, $P < 0.05$), lower epithelial cell width (*F*-test, $P < 0.05$), lower epithelial cell area (*F*-test, $P < 0.05$), lower epithelial cell volume (*F*-test, $P < 0.05$), and a higher number of follicles per 30,000 µm^2 of thyroid (*H*-test, $P < 0.05$), when compared to male individuals from RF'.

4.1.2. Females

Regarding morphometric (quantitative) data, the results obtained in terms of possible differences between the means of each group of female individuals are shown in Table 2.

Table 2: Descriptive statistics of the registered means and respective standard errors of the histomorphometric data, referring to the female individuals from conventional farming (CF), organic farming (OF), and reference (RF') sites. Pairwise comparisons are shown. *P* values marked in bold indicate significant differences (*F*-test, $P < 0.05$).

Variable	**Sites**	**Mean ± standard error**	**ANOVA**	**Tukey's HSD**	***P values***
Weight (g)	CF	14.4850 ± 0.81335	F(2,30) = 0.426, *P = 0.657*	CF-OF	*0.631*
	OF	12.6550 ± 0.19500		CF-RF'	*0.877*

	RF’	13.8813 ± 0.49159		OF-RF’	*0.749*
Age (days)	CF	116.6300 ± 5.31622		CF-OF	*0.960*
	OF	100.9650 ± 10.07500	F(2,30) = 5.115, *P* = ***0.013***	CF-RF’	***0.043***
	RF’	208.5792 ± 14.74095		OF-RF’	*0.091*
Colloid area (μm)	CF	6992.8175 ± 1685.39019		CF-OF	***0.026***
	OF	1644.2750 ± 902.89500	F(2,30) = 3.870, *P* = ***0.033***	CF-RF’	*0.219*
	RF’	4931.4629 ± 422.69850		OF-RF’	*0.131*
Colloid perimeter (μm)	CF	376.5175 ± 50.48480		CF-OF	***0.001***
	OF	154.8050 ± 46.23500	F(2,30) = 9.111, *P* **< *0.001***	CF-RF’	***0.006***
	RF’	259.9996 ± 11.66954		OF-RF’	*0.083*
Epithelium thickness (μm)	CF	3.2875 ± 0.22024		CF-OF	***0.006***
	OF	6.7950 ± 1.20500	F(2,30) = 11.102, *P* **< *0.001***	CF-RF’	**< *0.001***
	RF’	7.6388 ± 0.36998		OF-RF’	*0.783*
Number of epithelial cell nuclei per 50 μm	CF	3.9200 ± 0.15679		CF-OF	***0.007***
	OF	5.3450 ± 0.13500	F(2,30) = 11.489, *P* **< *0.001***	CF-RF’	**< *0.001***
	RF’	5.1650 ± 0.10593		OF-RF’	*0.873*
Epithelial cell width (μm)	CF	12.8225 ± 0.51286		CF-OF	**< *0.001***
	OF	9.3600 ± 0.23000	F(2,30) = 17.658, *P* **< *0.001***	CF-RF’	**< *0.001***
	RF’	9.7763 ± 0.20008		OF-RF’	*0.830*
Epithelial cell area (μm^2)	CF	42.2200 ± 3.63204		CF-OF	*0.378*
	OF	63.9000 ± 12.84000	F(2,30) = 5.451, *P* = ***0.010***	CF-RF’	***0.008***
	RF’	74.8375 ± 3.97642		OF-RF’	*0.704*
Epithelial cell volume (μm^3)	CF	545.0325 ± 63.19713		CF-OF	*0.954*
	OF	601.2350 ± 134.92500	F(2,30) = 1.553, *P* = *0.230*	CF-RF’	*0.253*
	RF’	740.1629 ± 47.59362		OF-RF’	*0.676*
Number of follicles per 30,000 μm^2 of thyroid	CF	6.8500 ± 2.02052		CF-OF	***0.004***
	OF	17.9000 ± 1.50000	F(2,30) = 6.453, *P* = ***0.005***	CF-RF’	*0.055*
	RF’	11.5917 ± 0.73519		OF-RF’	*0.062*

The box plots below illustrate the differences between groups (females) concerning colloid area and perimeter, epithelium thickness, and epithelial cell area and volume (Figure 15).

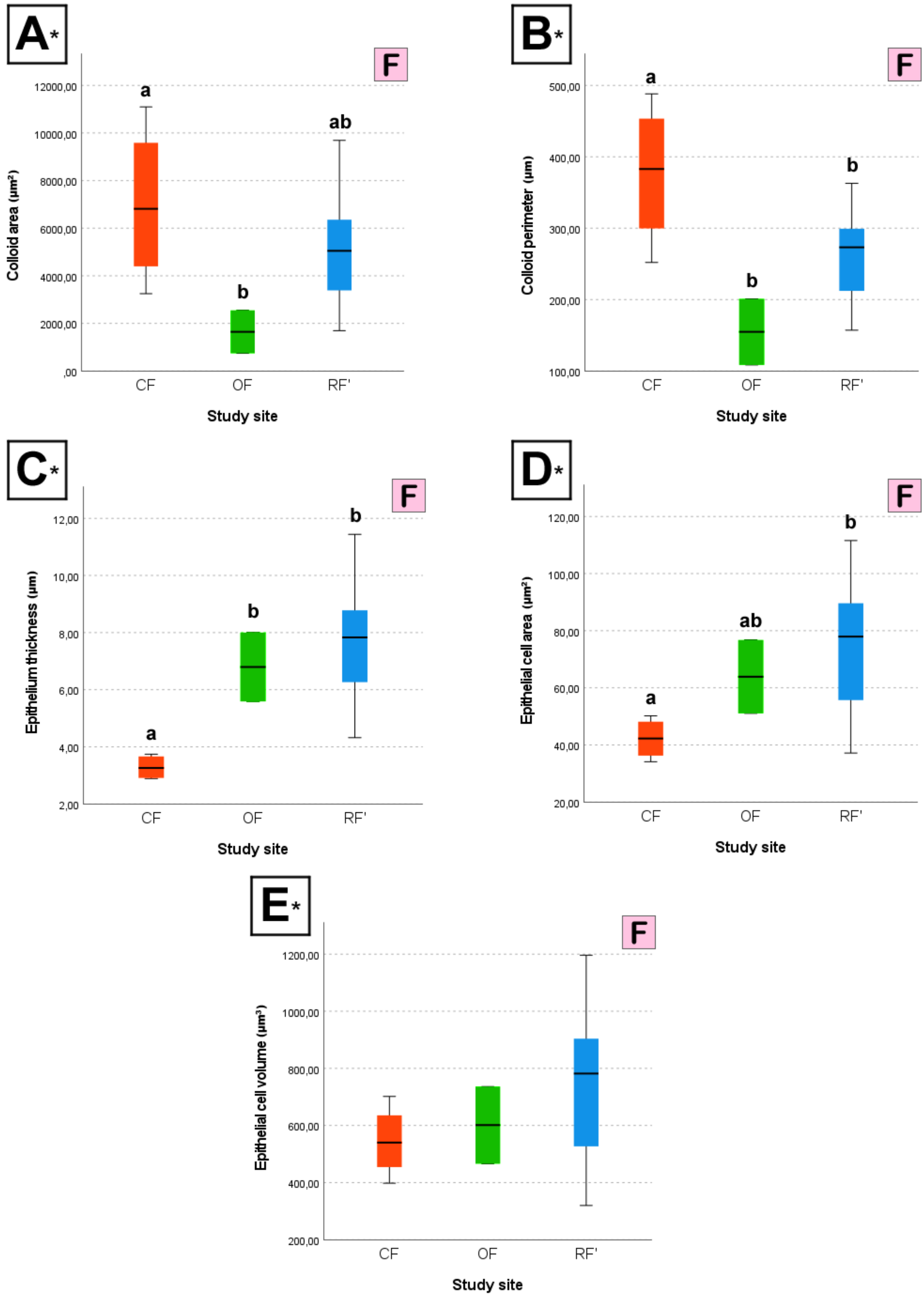


Figure 15: Box plots of the (A) colloid area, (B) colloid perimeter, (C) epithelium thickness, (D) epithelial cell area, and (E) epithelial cell volume of the female (F) individuals from conventional farming (CF), organic farming (OF), and reference (RF') sites. **F*-test; different letters (a and b) indicate significant differences ($P < 0.05$). The line in the middle of each box plot represents the median of the respective group; error bars are shown; an asterisk within the graphs indicates a group outlier.

According to Table 2 and Figure 15, the groups (females) significantly differed in all parameters except weight and epithelial cell volume (*F*-test, $P > 0.05$, both parameters).

Between CF and OF, results show that female individuals from CF had, on average, significantly larger colloid area, larger colloid perimeter, lower epithelium thickness, a lower number of epithelial cell nuclei per 50 µm, larger epithelial cell width, and a lower number of follicles per 30,000 μm^2 of thyroid (*F*-test, $P < 0.05$, all parameters), when compared to female individuals from OF. However, CF and OF females did not differ significantly regarding age, and epithelial cell area (*F*-test, $P > 0.05$, all parameters).

Between CF and RF', results show that female individuals from CF were, on average, significantly younger, had larger colloid perimeter, lower epithelium thickness, a lower number of epithelial cell nuclei per 50 µm, larger epithelial cell width, and lower epithelial cell area (*F*-test, $P < 0.05$, all parameters), when compared to female individuals from RF'. However, CF and RF' females did not differ significantly regarding colloid area, and number of follicles per 30,000 μm^2 of thyroid (*F*-test, $P > 0.05$, both parameters).

Finally, between OF and RF', results show that female individuals from OF did not differ significantly in any parameter from RF' (*F*-test, $P > 0.05$, all parameters).

4.1.3. Males vs. females

Regarding morphometric (quantitative) data, the results obtained in terms of possible differences between the means from males and females within groups are shown in Table 3.

Table 3: Descriptive statistics of the registered means and respective standard errors of the histomorphometric data, referring to the male and female individuals from conventional farming (CF), organic farming (OF), and reference (RF') sites. Differences between males and females within groups were assessed. **t*-test; ***U*-test; *P* values marked in bold indicate significant differences ($P < 0.05$).

Variable	**Sites**	**Mean ± standard error**		***P values***
		Males	**Females**	
Weight (g)	CF	13.8763 ± 0.60143	14.4850 ± 0.81335	*0.283*
	OF	13.6050 ± 0.46806	12.6550 ± 0.19500	*0.202*
	RF'	15.6750 ± 0.69707	13.8813 ± 0.49159	***0.018***
Age (days)	CF	101.8450 ± 7.09495	116.6300 ± 5.31622	**0.283*
	OF	119.2210 ± 12.35438	100.9650 ± 10.07500	**0.606*
	RF'	216.9650 ± 17.07903	208.5792 ± 14.74095	**0.557*
Colloid area (µm)	CF	5796.4850 ± 942.83546	6992.8175 ± 1685.39019	**0.808*
	OF	2344.9890 ± 406.97965	1644.2750 ± 902.89500	**0.758*

	RF’	5163.8750 ± 401.96417	4931.4629 ± 422.69850	***0.733*
Colloid perimeter (µm)	CF	353.0425 ± 32.67886	376.5175 ± 50.48480	***0.808*
	OF	212.1650 ± 20.12493	154.8050 ± 46.23500	***0.485*
	RF’	282.0206 ± 11.05412	259.9996 ± 11.66954	***0.331*
Epithelium thickness (µm)	CF	4.5988 ± 0.71935	3.2875 ± 0.22024	****0.048***
	OF	6.1800 ± 0.12411	6.7950 ± 1.20500	***0.909*
	RF’	7.1931 ± 0.31141	7.6388 ± 0.36998	***0.374*
Number of epithelial cell nuclei per 50 µm	CF	4.6813 ± 0.47082	3.9200 ± 0.15679	***0.283*
	OF	6.1710 ± 0.17606	5.3450 ± 0.13500	***0.121*
	RF’	5.0444 ± 0.11764	5.1650 ± 0.10593	***0.420*
Epithelial cell width (µm)	CF	11.2787 ± 0.87493	12.8225 ± 0.51286	**0.134*
	OF	8.1650 ± 0.23707	9.3600 ± 0.23000	***0.029***
	RF’	9.9906 ± 0.21970	9.7763 ± 0.20008	**0.242*
Epithelial cell area (μm^2)	CF	48.0463 ± 2.62026	42.2200 ± 3.63204	**0.113*
	OF	50.3220 ± 1.21160	63.9000 ± 12.84000	**0.241*
	RF’	71.9600 ± 3.60307	74.8375 ± 3.97642	**0.308*
Epithelial cell volume (μm^3)	CF	534.7112 ± 41.54530	545.0325 ± 63.19713	**0.446*
	OF	412.6550 ± 19.69602	601.2350 ± 134.92500	**0.196*
	RF’	724,8519 ± 45.65798	740.1629 ± 47.59362	**0.413*
Number of follicles per 30,000 μm^2 of thyroid	CF	5.3750 ± 0.96395	6.8500 ± 2.02052	***0.570*
	OF	17.7200 ± 2.44717	17.9000 ± 1.50000	***0.758*
	RF’	9.7500 ± 0.75862	11.5917 ± 0.73519	***0.126*

According to Table 3, the males and females (i) within CF significantly differed in epithelium thickness (males presented larger epithelium thickness, *U*-test, $P < 0.05$), (ii) within OF significantly differed in epithelial cell width (males presented lower epithelial cell width, *t*-test, $P < 0.05$), and (iii) within RF’ significantly differed in weight (males were heavier than females, *t*-test, $P < 0.05$).

4.2. Histomorphological analysis

4.2.1. Males

Regarding morphologic (qualitative) data, the results obtained in terms of possible differences between observations from each group of male individuals are shown in Table 4.

Table 4: Descriptive statistics of the registered observations regarding histomorphologic data, referring to the male individuals from conventional farming (CF), organic farming (OF), and reference (RF') sites. Pairwise comparisons are shown. *P* values marked in bold indicate significant differences (*H*-test, *P* < *0.05*). Follicular sphericity: "1" = spherical, "2" = somewhat spherical, "3" = not spherical at all; Epithelium irregularity: "1" = regular, "2" = somewhat irregular, "3" = very irregular; Degree of exfoliation: "1" = none, "2" = some, "3" = a lot; Degree of inflammation: "1" = none, "2" = some, "3" = a lot; Degree of colloid vacuolization: "1" = none, "2" = some, "3" = a lot.

Variable	Sites	Observations	Kruskal-Wallis	Pairwise comparisons	*P values*
Follicular sphericity	CF	"1" = 14 "2" = 18 "3" = 8 Total = 40	H(2,170) = 37.864, *P* **< *0.001***	CF-OF CF-RF' OF-RF'	*0.639* ***< 0.001*** ***< 0.001***
	OF	"1" = 12 "2" = 33 "3" = 5 Total = 50			
	RF'	"1" = 60 "2" = 19 "3" = 1 Total = 80			
Epithelium irregularity	CF	"1" = 29 "2" = 8 "3" = 3 Total = 40	H(2,170) = 49.335, *P* **< *0.001***	CF-OF CF-RF' OF-RF'	***< 0.001*** ***0.004*** ***< 0.001***
	OF	"1" = 20 "2" = 27 "3" = 3 Total = 50			
	RF'	"1" = 77 "2" = 3 "3" = 0 Total = 80			
	CF	"1" = 24 "2" = 11			

		"3" = 5 Total = 40			
Degree of exfoliation	OF	"1" = 9 "2" = 39 "3" = 2 Total = 50	H(2,170) = 47.923, *P* **< *0.001***	CF-OF CF-RF' OF-RF'	***< 0.001*** ***0.007*** ***< 0.001***
	RF'	"1" = 66 "2" = 13 "3" = 1 Total = 80			
	CF	"1" = 28 "2" = 12 "3" = 0 Total = 40			
Degree of inflammation	OF	"1" = 15 "2" = 32 "3" = 3 Total = 50	H(2,170) = 57.950, *P* **< *0.001***	CF-OF CF-RF' OF-RF'	***< 0.001*** ***0.011*** ***< 0.001***
	RF'	"1" = 75 "2" = 4 "3" = 1 Total = 80			
	CF	"1" = 26 "2" = 12 "3" = 2 Total = 40			
Degree of colloid vacuolization	OF	"1" = 32 "2" = 16 "3" = 2 Total = 50	H(2,170) = 0.087, *P = 0.957*	CF-OF CF-RF' OF-RF'	*0.953* *0.846* *0.781*
	RF'	"1" = 55 "2" = 17 "3" = 8 Total = 80			

The clustered bar charts below illustrate the differences between groups (males) concerning follicular sphericity, epithelium irregularity, degree of exfoliation, degree of inflammation, and degree of colloid vacuolization (Figure 16).

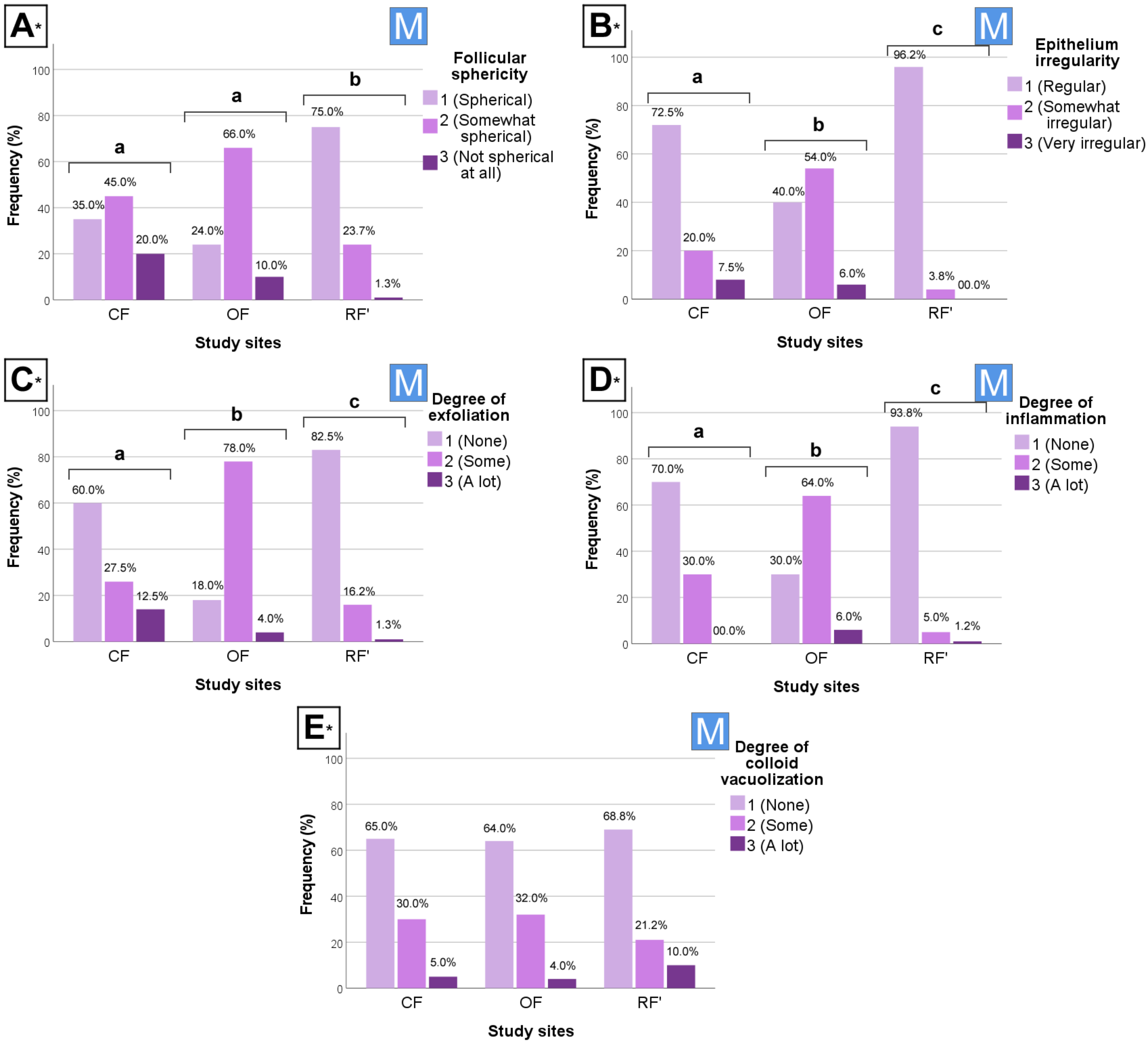


Figure 16: Clustered bar charts of the (A) follicular sphericity, (B) epithelium irregularity, (C) degree of exfoliation, (D) degree of inflammation, and (E) degree of colloid vacuolization of the male (M) individuals from conventional farming (CF), organic farming (OF), and reference (RF') sites. **H*-test; different letters (a, b, and c) indicate significant differences ($P < 0.05$); relative frequencies per category per group are shown.

According to Table 4 and Figure 16, the groups (males) significantly differed in all parameters except degree of colloid vacuolization (*H*-test, $P > 0.05$).

Between CF and OF, results show that male individuals from CF had significantly more regular epithelium, less exfoliation, and less inflammation (*H*-test, $P < 0.05$, all parameters), when compared to male individuals from OF. However, CF and OF males did not differ significantly regarding follicular sphericity (*H*-test, $P > 0.05$).

Between CF and RF’, results show that male individuals from CF had significantly less spherical follicles, more irregular epithelium, more exfoliation, and more inflammation (*H*-test, $P < 0.05$, all parameters), when compared to male individuals from RF’.

Finally, between OF and RF’, results show that male individuals from OF had significantly less spherical follicles, more irregular epithelium, more exfoliation, and more inflammation (*H*-test, $P < 0.05$, all parameters), when compared to male individuals from RF’.

4.2.2. Females

Regarding morphologic (qualitative) data, the results obtained in terms of possible differences between observations from each group of female individuals are shown in Table 5.

Table 5: Descriptive statistics of the registered observations regarding histomorphologic data, referring to the female individuals from conventional farming (CF), organic farming (OF), and reference (RF’) sites. Pairwise comparisons are shown. *P* values marked in bold indicate significant differences (*H*-test, $P < 0.05$). Follicular sphericity: “1” = spherical, “2” = somewhat spherical, “3” = not spherical at all; Epithelium irregularity: “1” = regular, “2” = somewhat irregular, “3” = very irregular; Degree of exfoliation: “1” = none, “2” = some, “3” = a lot; Degree of inflammation: “1” = none, “2” = some, “3” = a lot; Degree of colloid vacuolization: “1” = none, “2” = some, “3” = a lot.

Variable	Sites	Observations	Kruskal-Wallis	Pairwise comparisons	*P values*
Follicular sphericity	CF	“1” = 5 “2” = 9 “3” = 6 Total = 20	H(2, 150) = 68.835, *P* **< *0.001***	CF-OF CF-RF’ OF-RF’	***0.048*** ***< 0.001*** ***< 0.001***
	OF	“1” = 5 “2” = 5 “3” = 0 Total = 10			
	RF’	“1” = 114 “2” = 6 “3” = 0 Total = 120			
Epithelium irregularity	CF	“1” = 11 “2” = 9 “3” = 0 Total = 20	H(2, 150) = 38.123, *P* **< *0.001***	CF-OF CF-RF’	*0.685* ***< 0.001***
	OF	“1” = 6 “2” = 4			

		"3" = 0 Total = 10		OF-RF'	**< *0.001***
	RF'	"1" = 116 "2" = 4 "3" = 0 Total = 120			
Degree of exfoliation	CF	"1" = 8 "2" = 7 "3" = 5 Total = 20			
	OF	"1" = 8 "2" = 2 "3" = 0 Total = 10	H(2, 150) = 15.137, *P* **< *0.001***	CF-OF CF-RF' OF-RF'	***0.010*** **< *0.001*** *0.819*
	RF'	"1" = 92 "2" = 27 "3" = 1 Total = 120			
Degree of inflammation	CF	"1" = 8 "2" = 5 "3" = 7 Total = 20			
	OF	"1" = 8 "2" = 2 "3" = 0 Total = 10	H(2, 150) = 42.566, *P* **< *0.001***	CF-OF CF-RF' OF-RF'	***0.002*** **< *0.001*** *0.278*
	RF'	"1" = 112 "2" = 8 "3" = 0 Total = 120			
Degree of colloid vacuolization	CF	"1" = 13 "2" = 3 "3" = 4 Total = 20			
	OF	"1" = 4 "2" = 2 "3" = 4 Total = 10	H(2, 150) = 2.172, *P* = *0.338*	CF-OF CF-RF' OF-RF'	*0.146* *0.562* *0.198*
	RF'	"1" = 65 "2" = 35 "3" = 20 Total = 120			

The clustered bar charts below illustrate the differences between groups (females) concerning follicular sphericity, epithelium irregularity, degree of exfoliation, degree of inflammation, and degree of colloid vacuolization (Figure 17).

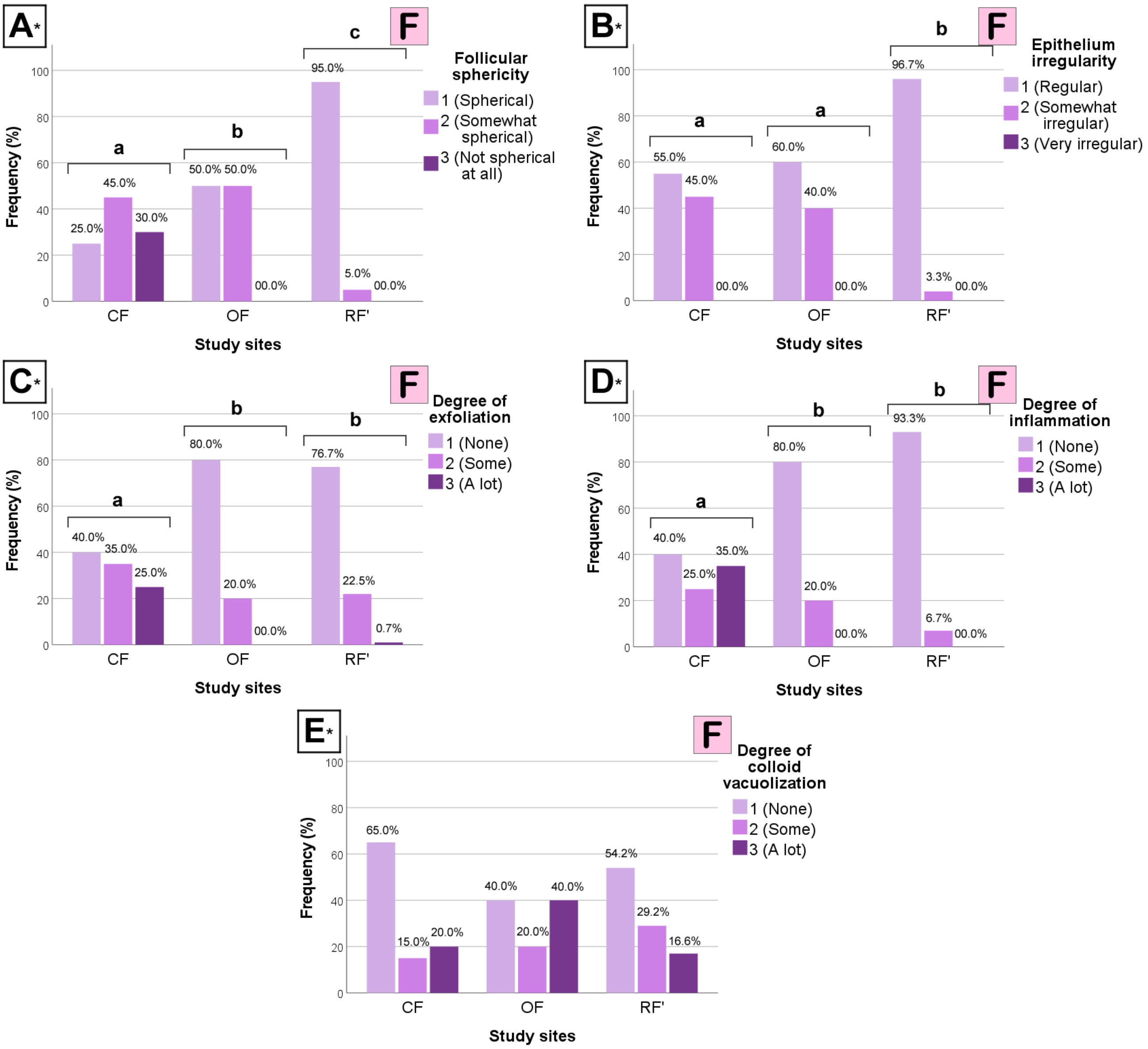


Figure 17: Clustered bar charts of the (A) follicular sphericity, (B) epithelium irregularity, (C) degree of exfoliation, (D) degree of inflammation, and (E) degree of colloid vacuolization of the female (F) individuals from conventional farming (CF), organic farming (OF), and reference (RF') sites. **H*-test; different letters (a, b, and c) indicate significant differences ($P < 0.05$); relative frequencies per category per group are shown.

According to Table 5 and Figure 17, the groups (females) significantly differed in all parameters except degree of colloid vacuolization (*H*-test, $P > 0.05$).

Between CF and OF, results show that female individuals from CF had significantly less spherical follicles, more exfoliation, and more inflammation (*H*-test, $P < 0.05$, all parameters), when compared to female individuals from OF. However, CF and OF females did not differ significantly regarding epithelium irregularity (*H*-test, $P > 0.05$).

Between CF and RF', results show that female individuals from CF had significantly less spherical follicles, more irregular epithelium, more exfoliation, and more inflammation (*H*-test, $P < 0.05$, all parameters), when compared to female individuals from RF'.

Finally, between OF and RF', results show that female individuals from CF had significantly less spherical follicles, and more irregular epithelium (*H*-test, $P < 0.05$, all parameters), when compared to female individuals from RF'. However, OF and RF' females did not differ significantly regarding exfoliation, and inflammation (*H*-test, $P > 0.05$, both parameters).

4.2.3. Males vs. females

Regarding morphologic (qualitative) data, the results obtained in terms of possible differences between observations from males and females within groups are shown in Table 6.

Table 6: Descriptive statistics of the registered observations regarding histomorphologic data, referring to the male and female individuals from conventional farming (CF), organic farming (OF), and reference (RF') sites. Differences between males and females within groups were assessed. *P* values marked in bold indicate significant differences (*U*-test, $P < 0.05$). Follicular sphericity: "1" = spherical, "2" = somewhat spherical, "3" = not spherical at all; Epithelium irregularity: "1" = regular, "2" = somewhat irregular, "3" = very irregular; Degree of exfoliation: "1" = none, "2" = some, "3" = a lot; Degree of inflammation: "1" = none, "2" = some, "3" = a lot; Degree of colloid vacuolization: "1" = none, "2" = some, "3" = a lot.

Variable	Sites	Observations		*P values*
		Males	Females	
Follicular sphericity	CF	"1" = 14 "2" = 18 "3" = 8 Total = 40	"1" = 5 "2" = 9 "3" = 6 Total = 20	*0.328*
	OF	"1" = 12 "2" = 33 "3" = 5 Total = 50	"1" = 5 "2" = 5 "3" = 0 Total = 10	*0.071*
	RF'	"1" = 60 "2" = 19 "3" = 1	"1" = 114 "2" = 6 "3" = 0	***< 0.001***

		Total = 80	Total = 120	
Epithelium irregularity	CF	“1” = 29 “2” = 8 “3” = 3 Total = 40	“1” = 11 “2” = 9 “3” = 0 Total = 20	*0.283*
	OF	“1” = 20 “2” = 27 “3” = 3 Total = 50	“1” = 6 “2” = 4 “3” = 0 Total = 10	*0.209*
	RF’	“1” = 77 “2” = 3 “3” = 0 Total = 80	“1” = 116 “2” = 4 “3” = 0 Total = 120	*0.875*
Degree of exfoliation	CF	“1” = 24 “2” = 11 “3” = 5 Total = 40	“1” = 8 “2” = 7 “3” = 5 Total = 20	*0.118*
	OF	“1” = 9 “2” = 39 “3” = 2 Total = 50	“1” = 8 “2” = 2 “3” = 0 Total = 10	***< 0.001***
	RF’	“1” = 66 “2” = 13 “3” = 1 Total = 80	“1” = 92 “2” = 27 “3” = 1 Total = 120	*0.335*
Degree of inflammation	CF	“1” = 28 “2” = 12 “3” = 0 Total = 40	“1” = 8 “2” = 5 “3” = 7 Total = 20	***0.004***
	OF	“1” = 15 “2” = 32 “3” = 3 Total = 50	“1” = 8 “2” = 2 “3” = 0 Total = 10	***0.004***
	RF’	“1” = 75 “2” = 4 “3” = 1 Total = 80	“1” = 112 “2” = 8 “3” = 0 Total = 120	*0.926*
Degree of colloid vacuolization	CF	“1” = 26 “2” = 12 “3” = 2 Total = 40	“1” = 13 “2” = 3 “3” = 4 Total = 20	*0.696*
	OF	“1” = 32 “2” = 16 “3” = 2	“1” = 4 “2” = 2 “3” = 4	***0.040***

	Total = 50	Total = 10	
RF’	“1” = 55 “2” = 17 “3” = 8 Total = 80	“1” = 65 “2” = 35 “3” = 20 Total = 120	***0.037***

According to Table 6, the males and females (i) within CF significantly differed in degree of inflammation (males had less inflammation, *U*-test, $P < 0.05$), (ii) within OF significantly differed in degree of exfoliation (males had more exfoliation, *U*-test, $P < 0.05$), degree of inflammation (males had more inflammation, *U*-test, $P < 0.05$), and degree of colloid vacuolization (males had more vacuolization, *U*-test, $P < 0.05$), and (iii) within RF’ significantly differed in follicular sphericity (males had less spherical follicles, *U*-test, $P < 0.05$), and degree of colloid vacuolization (males had less vacuolization, *U*-test, $P < 0.05$).

4.3. Data correlations

Regarding morphometric data, results showed a significant ($P < 0.05$) positive association between (i) weight and colloid area [rs(64) = 0.274, $P = 0.028$], (ii) weight and colloid perimeter [rs(64) = 0.257, $P = 0.040$], (iii) age and epithelium thickness [rs(64) = 0.429, $P < 0.001$], (iv) age and epithelial cell area [rs(64) = 0.480, $P < 0.001$], and (v) age and epithelial cell volume [rs(64) = 0.389, $P = 0.001$]. On the other hand, there was a significant ($P < 0.05$) negative association between weight and number of follicles per 30,000 μm^2 of thyroid [rs(64) = -0.312, $P = 0.012$].

5. Discussion

Several studies have addressed the effects of exposure to environmental EDCs on the thyroid (Badiei *et al*., 2010; Jugan *et al*., 2010; Ferrari *et al*., 2017; EFSA *et al*., 2019; Yao *et al*., 2020; Korkmaz & Örün, 2022; Coelho *et al*., 2025). Within the scope, some works also involved the use of morphological parameters to evaluate thyroid function, under exposure to chemicals known to cause thyroid endocrine disruption (Khan *et al*., 1999; Barlas *et al*., 2002; Mukhi *et al*., 2005; Hu *et al*., 2006; Schnitzler *et al*., 2008; Sonne *et al*., 2010; El-Mehi & Amin, 2012; Andrade *et al*., 2018; Hartmann *et al*., 2024). In Coelho *et al*. (2024a), connections were found between chronic exposure to an environment in which CF is practiced and thyroid disruption, particularly the appearance of hypothyroidism-like histological signs in exposed wild mice. The methodology followed in this work corroborated those results, while also revealing the same – albeit less strikingly, in certain parameters – for chronic exposure to an environment in which OF is practiced. The obtained results are in accordance with those of *in vivo* studies linking hypothyroidism with exposure to CF contaminants, with emphasis on pesticides (Van den Berg *et al*., 1991; Kackar *et al*., 1997; Satar *et al*., 2005; Jeong *et al*., 2006; Liu *et al*., 2006; Mallem *et al*., 2006; Stoker *et al*., 2007; Taxvid *et al*., 2007; Axelstad *et al*., 2011; Bhanu, 2016). Results also support the findings of other studies indicating an association between hypothyroidism in humans and exposure to CF contaminants (Goldner *et al*., 2010; Goldner *et al*., 2013; Farokhi *et al*., 2014; Piccoli *et al*., 2016; Shrestha *et al*., 2018; Kongtip *et al*., 2019; Nankongnab *et al*., 2020a; Sirikul & Sapbamrer, 2023) and OF contaminants (Kongtip *et al*., 2019).

In research, it is important that study populations are equally distributed, namely when it comes to parameters such as number of subjects per group, age, and sex. This ensures representative samples which support the validity and generalization of results towards larger, more diverse populations, while minimizing the risk of bias (Haas *et al*., 2012; Suresh & Chandrashekara, 2012). Confounding factors potentially influencing the results of this work were the weight, age, and sex of the mice, which should be taken into account when interpreting results (Karp & Reavey, 2019; Wallach *et al*., 2020; Mäkinen & Ala-Korpela, 2024; Kim *et al*., 2025). As seen by data correlations, weight could have influenced colloid area, colloid perimeter (heavier mice had larger colloid area and perimeter), and number of follicles per 30,000 μm^2 of thyroid (heavier mice had a lower number of follicles per 30,000 μm^2 of thyroid). Even so, the influence of weight on results was negligible, due to the lack of statistically significant differences in weight between groups. On the other hand, age may have influenced epithelium thickness, epithelial cell area, and epithelial cell volume (younger mice presented lower epithelium thickness, and lower epithelial cell area and volume). This is an important aspect, given that both CF and OF mice were significantly younger than RF' mice. Finally, sex may have influenced epithelial cell area, epithelial cell volume (males had lower epithelial cell area and volume than females), follicular sphericity, epithelium irregularity, degree of exfoliation, degree of inflammation (males presented a higher degree of thyroid disruption in these parameters than females), and degree of colloid vacuolization (males presented a lower degree of colloid vacuolization than females). Another factor was that data from a low number of females in the OF group was compared to that of groups CF and RF', which female population numbers were greater. As such, a lower variability in data from OF females could be seen as a limitation in this work. Nevertheless, for the most part, the same variations between males and females within groups were observed.

Collectively, indications of a higher degree of thyroid disruption in CF, followed by OF, in comparison to RF' were found for both males and females – especially when it comes to lower epithelium thickness, lower epithelial cell area and volume, and a higher degree of thyroid disruption registered in the majority of the evaluated histomorphologic parameters. A noteworthy observation across several of these parameters was that, while CF and OF males presented a similar level of thyroid disruption (no significant differences between them, yet both significantly differing from RF'), indications of thyroid disruption in OF females were of an intermediate level (OF females tended to not differ significantly from CF nor RF', while the latter significantly differed from each other).

Most studies solely address male populations to avoid the introduction of sex bias into results, namely due to the complexity of hormonal fluctuations in females. The cyclical hormonal changes females experience thanks to their menstrual cycle have long been thought to be a source of unpredictable variability – which can be avoided if only males are considered (Schmalenberger *et al*., 2021; Allegra *et al*., 2023). However, this not only leads to a severe underrepresentation of females in research, but also to data that, ultimately, is not applicable to the entire population (Plevkova *et al*., 2020; Merone *et al*., 2022; Nunamaker *et al*., 2023). Findings indicating important differences between sexes – or lack thereof – could provide valuable insights to fill in existing knowledge gaps, and give direction towards personalized treatments (Hamberg, 2008; Barlek *et al*., 2022). This is especially relevant when it comes to conditions to which female individuals are more susceptible, which is the case for thyroid disorders: women are affected by thyroid disorders more often than men (Mulder, 1998; Mammen *et al*., 2021; Brown *et al*., 2023). Recent works have been challenging the notion that female cohorts are inherently more variable than male ones, with demonstrations of variability between sexes occurring within comparable levels (Prendergast *et al*., 2014; Becker *et al*., 2016; Zajitschek *et al*., 2020; Kaluve *et al*., 2022; Graham *et al*., 2023; Levy *et al*., 2023). In this work, males and females did not differ significantly in the majority of the studied parameters within groups, with the presentation of similar thyroid histological changes in exposed mice. These findings further corroborate evidence showing that variability between sexes can be comparable. Still, more research within the scope is needed, particularly because it is not known to what extent the menstrual (estrous) cycle of female mice might affect thyroid histomorphology.

A healthy thyroid is crucial for young individuals, given that TH contribute to the growth, development, and proper functioning of every organ system in the body (Tarım, 2011). These hormones play a critical role in brain development, to the point where even minor TH deficiencies in youth can cause irreversible brain damage (Weber *et al*., 2003; Segni, 2017; Wanjari *et al*., 2022). However, thyroid function is known to decline with age, marked by a reduction in TH secretion, and a decline in T3 levels due to decreased peripheral conversion of T4. The HPT-axis also loses response plasticity to shifts in TH production, which can be seen, for instance, in a less pronounced TSH response to TH deficiency (Bégin *et al*., 2008; Taylor *et al*., 2023). In light of this, the fact that the thyroids of exposed mice presented more indications of disruption, even while CF and OF mice were significantly younger than non-exposed RF' mice (practically half their age), is very alarming. While it would have been expected for the thyroid of younger mice to show signs of much more activity (i.e., smaller follicles, a thicker epithelium, and epithelial cells of larger area and volume), they instead presented lower epithelium thickness, lower epithelial cell area, and lower epithelial cell volume, all of which indicate loss of function (hypothyroidism) (Rao-Rupanagudi *et al*., 1992; Lee *et al*., 2016). The presence of significant differences between groups regardless of age highlights the severity of the impact of chronic exposure to CF and OF contaminants on thyroid function. Moreover, it is plausible that the discrepancy of the measured values between groups could have been even greater if the age of the mice had not differed significantly. Increased colloid area and perimeter are also often associated with hypothyroidism (Lee *et al*., 2016); nevertheless, in this work, differences between groups were mostly not significant in such a way. Therefore, epithelium thickness, along with epithelial cell area and volume, might be a better alternative to assess thyroid disruption than colloid area and perimeter alone. The usefulness of biomarkers of effect, like the thickness of epithelia, and the area and volume of cells, for studies to assess chronic exposures to contaminants is also highlighted. These parameters can be especially relevant for organs that play a fundamental role in metabolism for an extended period of life.

In addition to decreasing the ability of the thyroid to produce hormones – as seen through a thinner epithelium, and epithelial cells of smaller area and volume –, chronic exposure to CF and OF contaminants seemed to alter the histomorphology of the gland. Lower follicular sphericity, irregularly-delimited epithelium, a higher degree of exfoliation, and a higher degree of inflammation were observed in the thyroids of CF-exposed mice; the same alterations were the case for OF-exposed mice, though less pronouncedly. For male mice in the OF group, noteworthy exceptions include a more irregular epithelium and a higher degree of exfoliation than the CF group. Moreover, for male mice, the CF group presented a significantly lower degree of inflammation than the OF group (with both groups significantly differing from RF'). This may be because CF mice were slightly younger than OF mice – inflammation is known to increase with age (Singh *et al*., 2024) –; furthermore, such age difference also implies that CF mice experienced a shorter period of contaminant exposure. These findings suggest that perhaps there are differences in how certain contaminants affect the thyroid histomorphology of male and female individuals. Finally, no differences were found between groups when it comes to degree of vacuolization [when in abundance, colloid vacuoles tend to be a sign of thyroid epithelial cell hyperactivity (Nilsson *et al*., 1988; Andrade *et al*., 2018; Pirahanchi *et al*., 2023)].

Lower follicular sphericity, irregularly-delimited epithelium, and higher degree of exfoliation are alterations plausibly connected with exposure to CF and OF contaminants, as they reflect thyroid function status. Less spherical follicles and an irregular epithelium are often associated with situations of thyroid injury (Mense & Boorman, 2018; Al-Maathidy *et al*., 2019). In turn, exfoliation, while being a part of normal thyroid function, is tied to the degeneration of epithelial cells, which remains are shed into the colloid – where apoptosis (cell death) processes take place (Al-Maathidy *et al*., 2019; Zhang *et al*., 2021). In light of this, results suggest that thyroid disruption arising from exposure to CF and OF contaminants not only involves indications of loss of function, but also observable alterations to tissue histomorphology. On the other hand, although a higher abundance of lymphocytes was observed in the thyroid tissue of CF- and OF-exposed mice, it is uncertain whether the thyroid was one of the true focus-organs of inflammation. It is possible that more of these immune cells were found in the bloodstream and interfollicular spaces because something in thyroid tissue triggered an inflammatory response. If so, such might be due to agrochemical residues acting as endocrine disruptors in exposed mice. In fact, agrochemicals have been reported to trigger abnormal inflammatory responses, given they can interfere with the natural physiology and metabolism of immune system cells (Lopes-Ferreira *et al*., 2023; Ruíz-Arias *et al*., 2023). In Hashimoto's thyroiditis, the immune system actively attacks the thyroid, destroying thyroid cells via cell- and antibody-mediated processes, which can lead to hypothyroidism or underactive thyroid (Zaghlol *et al*., 2017; Erge *et al*., 2023; Mincer & Jialal, 2025). The causes for Hashimoto's thyroiditis are still unclear; however, it is plausible that environmental factors, such as exposures to contaminants, may contribute to its onset (Babić Leko *et al*., 2021; Cyna *et al*., 2024).

It has been shown that EDCs can act at any level of the HPT-axis communication, including by interfering with tissue-specific factors such as DIO enzymes. Some mechanisms of EDCs interference with thyroid metabolism and signaling pathways are (i) mimicking TH structure, (ii) binding to TH receptors, (iii) binding to TH transport proteins, (iv) inhibiting or stimulating DIO activity, (v) inhibiting or stimulating TPO activity, and (vi) inhibiting I^- transport into thyrocytes (Van den Berg *et al*., 1991; Kackar *et al*., 1997; Thambirajah *et al*., 2022). The heavy metals (also designated potentially toxic elements, PTEs) present in the composition of agrochemicals – with special emphasis on pesticides – often mimic the chemical structure of TH (Kashiwagi *et al*., 2009; Al-Maathidy *et al*., 2019; Fiore *et al*., 2019; Babić Leko *et al*., 2021; Zhang *et al*., 2022; Sirikul & Sapbamrer, 2023; Chandramohan *et al*., 2024). It has been described that their influence on the HPT-axis includes (i) inhibiting I^- absorption, (ii) decreasing the uptake of TH, (iii) increasing the clearance of TH, (iv) altering TH displacement from transport proteins, and (v) altering the expression of TH-regulated genes (Van den Berg *et al*., 1991; Kackar *et al*., 1997; Kongtip *et al*., 2019; Leemans *et al*., 2019). Nitrates in particular are important components of inorganic fertilizers, providing immediate nutrition for plants. The problem is that nitrates are highly leachable: they easily infiltrate groundwater upon excess rainfall, and disseminate through the environment (Wang & Li, 2019; Wang *et al*., 2020). Moreover, nitrate is known to disrupt thyroid metabolism by interfering with TH synthesis, specifically via inhibiting the uptake of I^- by binding to the sodium-iodide symporter on the surface of thyroid follicles (Ward *et al*., 2010). Another important factor is that some pesticides can bioaccumulate in energy reserve tissues, like body fat, even after being metabolized (Devi *et al*., 2022). Exposures to organochlorine insecticides (Goldner *et al*., 2010), fungicides, herbicides, and dithiocarbamates (Piccoli *et al*., 2016) have been reported to induce hypothyroidism and hypothyroidism-like manifestations. Associated mechanisms of interference with the HPT-axis include (i) displacement from distributor proteins, increased hepatic metabolism, along with indirect effects on thyroid function by organochlorines; (ii) reduction of TSH production, resulting in lower production of TH by organophosphates; (iii) reduction of TPO activity by carbamates, and (iv) antagonistic action on TH receptors by pyrethroids, due to their similar chemical structure to TH (Leemans *et al*., 2019; Coelho *et al*., 2024a). In view of the aforementioned, because inorganic fertilizers and several pesticides were used in the CF study site, the observed histological effects on CF-exposed mice were expected.

Contrasting studies about CF contaminants, fewer works have addressed the impact of OF contaminants on health (Ramakrishnan *et al*., 2021; Zhuang *et al*., 2024; Kongtip *et al*., 2019; Nankongnab *et al*., 2020a, 2020b). In the study of Zhuang *et al*. (2024), 8 types of major ARGs and 10 mobile genetic elements (MGEs) were detected in the gut microbiome of mice exposed to a diet consisting of organic foods, including tetracycline, multidrug, sulfonamide, aminoglycoside, beta-lactamase, chloramphenicol, MLSB, and vancomycin resistance genes. It was also found that the abundance and diversity of ARGs, MGEs, and potential antibiotic-resistant bacteria (ARB) in the gut increased with time after organic food consumption. ARGs are not known to directly disrupt the thyroid; however, environmental antibiotic-related contaminants can cause thyroid disruption via TH synthesis inhibition or by causing chemical thyroiditis. It has been documented that long-term exposures to certain antibiotics – like tetracyclines – present in contaminated food and water can cause thyroid disruption (Yu *et al*., 2020). The immune system, which is dysregulated in thyroid autoimmune disorders, can also be influenced by the microbiome (Pollock *et al*., 2016). The works of Kongtip *et al*. (2019) and Nankongnab *et al*. (2020a) were the only ones to directly study thyroid effects on OF farmers. Their works involved the same population of CF and OF farmers in Thailand. In both studies, the authors found that the TSH, free T3 (FT3), T3, and T4 levels were significantly higher in CF farmers than OF farmers. In both groups, several individuals suffered from hypothyroidism, though there was a higher proportion of hypothyroid individuals in the OF group. The authors suggested the higher number of females in the OF group – some of which were nearing or in postmenopausal age – as a likely explanation for a higher proportion of hypothyroid individuals. Another relevant factor was that many individuals in the OF group had handled agrochemicals, particularly pesticides, in the past. The studies lacked a population non-exposed to farming environments to constitute a reference group, which makes it difficult to establish the true extent of the impact of OF on the thyroid. Still, their results align with those of the present work in that thyroid effects were overall more pronounced in the CF-exposed group than the OF-exposed group.

Supporting the first hypothesis, the results obtained in this work underline the risks of chronic exposure to CF contaminants, but also – though less pronouncedly – those of chronic exposure to OF contaminants: the thyroids of exposed mice showed several histological signs of disruption (particularly indicating hypothyroidism), when compared to the RF' group. This means that, while OF might be a better alternative to CF, it is not completely free of health hazards, mainly because OF products can be indirectly contaminated by the agrochemicals used in CF practices. Considering this, organic certification must guarantee total exemption of agrochemical contamination in OF products. Moreover, the consequences of exposures to OF contaminants are still barely understood, and could even differ between male and female individuals. Contrary to the second hypothesis, the impact of chronic exposure to either farming system was not more severe on the thyroid of female individuals than on the thyroid of male individuals. In fact, it was equal to – or, in select parameters, less severe than – what was registered for males. Overall, seeing as contaminants present in the OF environment seemed to have a less severe impact on the thyroid, the need for transition from current CF systems towards OF systems is supported. Petit *et al*. (2024) alert to the fact that thyroid disorders among farmers "likely arise from the combined effects of various causal agents and triggering factors (agricultural exposome)". In light of this, it is important to keep researching about what exactly causes thyroid disruption in both CF and, especially, OF farming practices, so that countermeasures can be taken against so. Filling in these knowledge gaps is of utmost importance, knowing that not only are farmers occupationally exposed to such hazards, but also the general population – via food consumption. Efforts made towards monitoring the presence of pesticide residues in food products have been substantial in the European Union (EU) (EFSA, 2023, 2024, 2025). Unfortunately, the same still cannot be said about other regions of the world.

6. Conclusion

Conventional farming – the dominant agriculture system – heavily relies on synthetic inputs to maximize yields, while organic farming minimizes synthetic inputs to promote environmental sustainability and human health. There is debate whether OF is an overall better alternative to CF, due to, among other reasons, evidence of pesticide residues in OF produce. Thyroid disorders are a pressing concern worldwide, the onset of which can be influenced by exposures to environmental contaminants – including those from agricultural practices.

The results from this work suggest that chronic exposure to contaminants from CF and OF (less pronouncedly) causes thyroid disruption, due to observable histological alterations in exposed wild mice, namely (i) lower epithelium thickness, (ii) lower epithelial cell area and (iii) volume, (iv) lower follicular sphericity, (v) irregularly-delimited epithelium, (vi) increased exfoliation into the colloid, and (vii) increased inflammation of thyroid tissue. Lower epithelium thickness, along with lower epithelial cell area and volume, are indications commonly associated with hypothyroidism. The same alterations were observed within groups for males and females, with minor differences in select parameters.

Because the OF environment seemed to have a less severe impact on the thyroid than the CF environment, the transition from the globally-predominant CF systems to OF ones is supported. Still, knowledge gaps remain in regards of whether CF and OF contaminants affect male and female individuals differently, and the general health impact of OF. More research is needed about what exactly causes thyroid disruption in both CF and, especially, OF farms, so that countermeasures can be applied. The topic is of utmost importance, given that not only are farmers occupationally exposed to such hazards, but also the general population is at risk through food consumption.

7. References

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Appendix

The distribution of males and females between groups (CF, OF, RF1, and RF2) was assessed via Pearson's Chi-square test (χ^2). The distribution of males and females between groups (CF, OF, RF1, and RF2) was significantly different: $\chi^2(3,64) = 8.707$, $P = 0.033$ ($P < 0.05$). As such, data from males and females was treated separately. Table A1 shows which groups differed from one another in terms of the distribution of males and females.

Table A1: Distribution of males and females from conventional farming (CF), organic farming (OF), and reference (RF1, RF2) sites. Pairwise comparisons are shown. *P* values marked in bold indicate significant differences (χ^2-test, $P < 0.05$).

Study sites	**Males**	**Females**	**Pairwise comparisons**	**χ^2 test**
CF	8	4	CF-OF	$\chi^2(1,24) = 0.889$, $P = 0.346$
OF	10	2	CF-RF1	$\chi^2(1,39) = 1.642$, $P = 0.200$
RF1	12	15	CF-RF2	$\chi^2(1,25) = 3.222$, $P = 0.073$
RF2	4	9	OF-RF1	$\chi^2(1,39) = 5.110$, $\mathbf{P = 0.024}$
			OF-RF2	$\chi^2(1,25) = 6.997$, $\mathbf{P = 0.008}$
			RF1-RF2	$\chi^2(1,40) = 0.684$, $P = 0.408$

According to Table A1, the distribution of males and females significantly differed between OF and RF1, and between OF and RF2 (χ^2-test, $P < 0.05$, for both pairs).

For males, data regarding weight, epithelial cell width, epithelial cell area, and epithelial cell volume showed a normal distribution, contrary to data regarding age, colloid area, colloid perimeter, epithelium thickness, number of epithelial cell nuclei per a 50 µm transect, and number of follicles in 30,000 μm^2 of thyroid. For females, all morphometric data showed a normal distribution.

Differences of the means between groups RF1 and RF2 were assessed via independent samples *t*-tests (preceded by a Levene's test for homogeneity of variances), for normal data, and non-parametric Mann-Whitney *U* tests for non-normal data. Because there were no differences between these groups when it comes to all data (*t*-test, $P > 0.05$ for normal data; *U*-test, $P > 0.05$ for non-normal data) except the number of follicles in 30,000 μm^2 of thyroid (*U*-test, $P < 0.05$) between males, and weight (*t*-test, $P < 0.05$) between females, groups RF1 and RF2 were merged into one, designated RF'.

The distribution of males and females between groups (CF, OF, RF') was still significantly different: $\chi^2(2,64) = 8.048$, $P = 0.18$ ($P < 0.05$). In this case, Table A2 shows which groups differed from one another in terms of the distribution of males and females.

Table A2: Distribution of males and females from conventional farming (CF), organic farming (OF), and reference (RF') sites. Pairwise comparisons are shown. *P* values marked in bold indicate significant differences (χ^2-test, $P < 0.05$).

Study sites	Males	Females	Pairwise comparisons	χ^2 test
CF	8	4	CF-OF	$\chi^2(1,24) = 0.889$, $P = 0.346$
OF	10	2	CF-RF'	$\chi^2(1,52) = 2.641$, $P = 0.104$
RF'	16	24	OF-RF'	$\chi^2(1,52) = 6.933$, $\mathbf{P = 0.008}$

According to Table A2, the distribution of males and females significantly differed between OF and RF', (χ^2-test, $P < 0.05$). Figure A1 illustrates the distribution of males and females between groups CF, OF, and RF'.

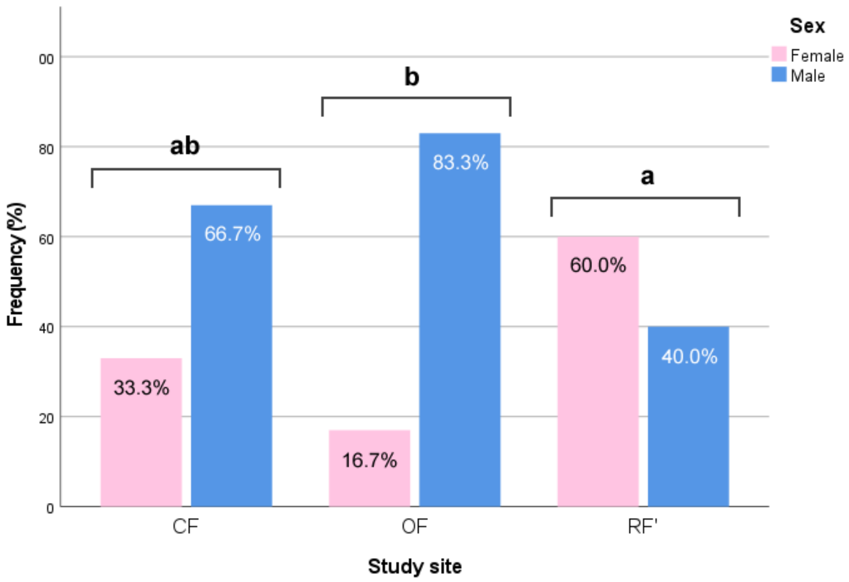

Figure A1: Distribution of males and females from conventional farming (CF), organic farming (OF), and reference (RF') sites. Different letters (a and b) indicate significant differences (χ^2-test, $P < 0.05$). Relative frequencies are shown.