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Morphological and Functional Changes in the Thyroid Gland of Methyl Thiophanate-Injected Lizards, *Podarcis sicula*

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Abstract The thyroid has been shown to be a target organ for environmental chemicals, specifically endocrine-disrupting contaminants. Reptiles are particularly suitable as contaminant biomonitors due to their persistence in a variety of habitats, wide geographic distribution, longevity, and, in many cases, site fidelity. Methyl thiophanate is a systemic broad-spectrum fungicide used to prevent and control plant diseases caused by various fungi. The aim of this study was to develop an integrated biological model for monitoring the ecotoxic effects of thiophanate-methyl fungicide on the thyroid of the lizard *Podarcis sicula*. The results of this study indicate that both structural and functional differences in the thyroid gland of the lizard exist in the animals exposed to methyl thiophanate. Structurally, animals exposed to methyl thiophanate showed decreased epithelial cell height; the nuclei of the thyroid cells were small and elongated with dense chromatin and a greatly reduced cytoplasm. The colloid was retracted with few reabsorption vacuoles. Functionally, the same animals exhibited decreased T_4 and T_3 plasma levels compared to control animals. Methyl thiophanate administration produced statistically significant inhibition on serum thyroid-stimulating hormone levels and this is the mechanism for altering thyroid function. This study highlights how thyroid gland disruption, both structural and functional, in lizard and other nontarget organisms might also have an environmental aetiology.

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Introduction

Endocrine-disrupting chemicals are a broad group of substances that alter the functions of the endocrine systems in wildlife and humans. The endocrine disruptors are widespread in the environment and food chains and include some common environmental contaminants such as pesticides, plastic ingredients, dioxins, and biocides. The possible impact of these endocrine-disrupting chemicals needs to be considered because many of the compounds accumulate due to their persistence in the environment; moreover, the endocrine-related adverse effects can occur at lower dose levels than those causing tumorigenicity or teratogenicity (Melnick et al. 2002), with long-term consequences on health (Davis et al. 1993; Foster and McIntyre 2002).

The endocrine-disrupting contaminants (EDCs) have been shown to alter (1) hormone production at its endocrine source, (2) the release of stimulatory or inhibitory hormones from the pituitary or hypothalamus, (3) hepatic enzymatic biotransformation or hormones, and the (4) concentration or functioning of serum-binding proteins, altering free hormone concentrations in serum (Guillette et al. 2000; Guillette and Gunderson 2001).

The thyroid has also been shown to be a target organ for environmental chemicals, specifically EDCs. In fact, documented disruptions or alterations in thyroid activity, morphology or physiology, have been correlated with contaminant-induced modifications in endocrine system

functioning (Leatherland 1992, 2000). Thus, a great deal of the current focus on environmental pollution is on the potential endocrine-altering actions of various chemical contaminants (Guillette et al. 2000; Mc Lachlan 2001). Many natural and synthetic compounds in the environment change the normal functioning of the endocrine system and numerous studies have noted structural abnormalities in the thyroids of wildlife exposed to environmental contamination (Hewitt et al. 2002). The constituents of thyroid follicles (epithelial cells and colloid) are instrumental in observing morphological effects of EDCs on the thyroid. Histologically, epithelial cell height is the most frequently used method of thyroid gland assessment, as it is considered to be roughly proportional to the degree of response to thyroid-stimulating hormone (TSH) (Moccia et al. 1981).

Thiophanate methyl (MT) is a systemic broad-spectrum fungicide used to prevent and control plant diseases caused by various fungi; in comparison with other common fungicides, it has lower general toxicity (Canton 1976; Traina et al. 1998). This compounds is been found to bind to tubulins, disrupting microtubule formation and centrosome organization in human granulosa cells, in mouse oocytes, and in fungi (Barlas et al. 2002; Burland et al. 1984; Can and Albertini 1997).

Fungicides, such as MT, are widely used in agricultural and horticultural practice in the Campania region (Italy); therefore, a biological model was developed to enable "environmental health" monitoring in this region.

For years, reptiles have served as biomonitors of heavy metal (Overman and Krajicek 1995; Hewitt et al. 2002) and radionuclide contamination (Meyers-Shone et al. 1983). Reptiles are particularly suitable as contaminant biomonitors due to their persistence in a variety of habitats, wide geographic distribution, longevity, and, in many cases, site fidelity (Crain and Guillette 1998). Additionally, reptiles exhibit a sensitivity to contaminants similar to that reported for birds and mammals (Hall and Clarck 1982) and they bioaccumulate and biomagnify contaminants to levels equal to or greater than that reported for birds and mammals (Hall and Henry 1992).

Therefore, the aims of the present study were (1) to develop a biological model for monitoring the ecotoxic effects of MT in the environment of the Campania region, based on a sentinel species, *Podarcis sicula*, because it is the most abundant species living in the open country and in cultivated fields and (2) to evaluate the possible adverse effects of MT on an endocrine organ such as the thyroid gland. Therefore, we have investigated the sensitivity of the sentinel species to MT and the effects of the fungicide on the thyroid gland morphology and thyroid hormones plasma levels.

Materials and Methods

Animals and Experimental Design

Two hundred adult male lizards of *Podarcis sicula*, weighing 15 g, were live-captured in the neighborhood of Naples in June, when the thyroid gland showed clear signs of functional activity (Sciarrillo et al. 2000). After capture, the animals were housed in large soil-filled terraria containing heather and were exposed to natural temperature and photoperiod. Water dishes were present in the terraria, and the animals were fed on live fly larvae daily. Captivity lasted 20 days to reverse capture-related stress (Manzo et al. 1994). All animals have been captured with the authorization of 06/01/2000 No. SCN/2D/2000/9213 of the Italian Ministry of Environment.

Acute Study

Male lizards were divided into 6 groups (I–VI) of 20 animals each. The animals were fasted overnight before administration of test chemicals. Thiophanate methyl technical product (CAS No. 23564–05–8, 96.2%) was obtained from SIPCAM (Milano, Italy).

Methyl thiophanate suspended in physiological saline for reptiles (NaCl 0.75%) was intraperitoneally administered to lizards of groups II–VI at concentrations of 350, 500, 700, 900, and 1000 mg/kg body weight, respectively. The volume administered was 0.1 mL for all solutions. Physiological saline (0.1 mL per lizard) was given intraperitoneally to the animals of groups I and this animals were treated as the control. Animals were observed twice a day for 15 days for clinical signs of toxicity or death, if any. After 15 days, the remaining animals were necropsied for gross pathology and the LD_{50} value was calculated by the moving-average method of Well (1952) (Table 1).

Chronic Study

Male lizards were divided into 4 groups of 20 animals each. Every 2 days, the animals of the treatment groups were administered MT at doses of 5, 30, 50 mg/kg body weight/day for 30 days. All injections were in 0.1 mL. Lizards of the control group were given 0.1 mL of physiological solution per lizard every 2 days for the same period. Animals were observed daily for signs of toxicity and death, if any.

Table 1 Acute toxicity of MT in male lizards

Group	Treatment (mg/kg body weight)	Number of animals		
		Treated animals	Dead animal (% of animal mortality)	Signs of toxicity
I	Physiological solution	20	0	—
II	TM 350	20	0	—
III	TM 500	20	4 (20%)	Dyspnea, hind-limb paralysis
IV	TM 700	20	6 (30%)	Dyspnoea, hind-limb paralysis
V	TM 900	20	10 (50%)	Dyspnoea, hind-limb paralysis
VI	TM 1000	20	14 (70%)	Dyspnoea, hind-limb paralysis

Tissue Preparation

Blood samples were collected by intracardiac puncture and put into heparinized tubes. Blood collection lasted less than 3 min; plasma was obtained by centrifugation (2500 *g* for 10 min) of the blood samples and was stored at −20°C until assay. Immediately after collection of blood samples, the animals were decapitated, and the thyroid glands were removed and fixed in Bouin's fixative and processed for light microscopy. Serially cut paraffin sections (7 (m thick) were stained by Galgano I stain (Beccari and Mazzi 1966).

Observations were performed using a Zeiss Axioskop microscope; images were captured with a camera attached to an IBM computer running the Kontron Elektronik KS 300 image analysis system and Adobe Photoshop. The height of the follicular cells was measured in 30 cells every 3 slides, always on the second section of normal and treated specimens using a digital system of imaging (KS 300).

Hormones Analysis

Plasma levels of T_3 and T_4 were determined by radioimmunoassay (RIA). In the T_3 assay, a measured amount of sample serum and standards was added to a tube coated with anti-T3 rabbit antibody, with a trace (4.4 (Ci) amount of radioactively labeled T_3 ([^{125}I] T_3) (Byk-Sangtec Diagnostica, Dietzenbach, Germany) and an agent-blocking Tris-buffered saline 4 mM, ANS (8-anilino-1-naphthalenesulfonic acid) 6 mM sodium salicylate with 0.2% sodium azide as a preservative (Sigma Chemical Co., St. Louis, MO) to release T_3 from serum-binding proteins. Sensitivity was 0.1 ng/mL with an accuracy of about 97%. The range of intraassay variance in 20 assays was 1.0–2.6%, whereas the interassay variance ranged between 3.9% and 5.7% in 12 assays.

For T_4, a measured amount of sample serum and standards was added to a tube coated with anti-T_4 rabbit antibody, along with a trace amount of radioactively labeled T4 ([^{125}I] T_4), 4.4 (Ci, (Byk-Sangtec Diagnostica, Dietzenbach, Germany) and a blocking agent, Tris-buffered saline 4 mM, ANS 6 mM sodium salicylate with 0.2 % sodium azide as a preservative (Sigma Chemical Co. St. Louis, MO) to release T_4 from serum-binding proteins. Sensitivity was 0.45 ng/mL, with an accuracy close to 100%; the mean intra-assay and interassay coefficients of variance were 4.6% and 4.3%, respectively. A logit–log curve fit using a % B/Bo calculation was used. T_4 and T_3 concentrations were determined by computing the % B/Bo for each sample and then finding the results on the standard curve. Cross-reactivity for T_4 in the T_3 RIA (1.3%) was not considered for data calculations, neither was that for T_3 in the T_4 RIA (0.1%).

Plasma TSH was determined by immunoradiometric assay (IRMA). In the TSH procedure, sample serum and standards were added to antiligand-coated tubes. The Tracer/Capture Reagent, a blend of ligand-tagged TSH-specific antibody and ^{125}I-labeled TSH (10 μCi), was added to each tube. A cubic spline function with the zero standard as one of the standard points was used for calculations. The minimum detectable dose (MDD) was 0.01 μIU/mL, with an accuracy close to 100%, and the mean intra-assay and interassay coefficients of variance were 5.0% and 7.5%, respectively.

Statistics

All data are presented as means ± standard error of the mean (SEM). Statistical analyses were performed by one-way analysis of variance (ANOVA) with repeated measures followed by Duncan's multiple range test for pairwise comparisons. Differences were considered significant if $p < 0.05$.

Results

Acute Toxicity

Administration of a single intraperitoneal dose of MT in different concentrations (350, 500, 700, 900, and 1000 mg/kg body weight) to male lizards produced dose-dependent signs of toxicity as dyspnea, hind-limb paralysis, and death of some animals. There were no deaths in the control or the 350-mg MT/kg body weight treatment group. However, there was a dose-dependent mortality for animals exposed

to MT at doses of 500 (4 animals, 20% of animal mortality), 700 (6 animals, 30% of animal mortality), 900 (10 animals, 50% of animal mortality), and 1000 (14 animals, 70% of animal mortality) mg/kg body weight, respectively. The LD_{50} value was calculated as 900 mg/kg body weight and ranged between 850 and 930 mg/kg body weight (Table 1). The maximum mortality occurred during 0–7 days of exposure.

Chronic Toxicity

Administration every 2 days of MT (5, 30, and 50 mg/kg body weight/day) to male lizards by intraperitoneal injections for 30 days produced signs of toxicity such as dyspnea, hind-limb paralysis, and death of some animals during the course of the experiment. There were two (10% of animal mortality) and four (20% of animal mortality) deaths in animals at MT doses of 30 and 50 mg/kg body weight/day, respectively. Interestingly, maximum mortality occurred during 0–7 days of exposure (Table 2).

Thyroid Histology

The thyroid gland of the lizard *P. sicula* control specimens is a single discrete ribbonlike structure that transversely crosses the middle of the trachea. It is formed by follicles that are connected by an interfollicular connective tissue that contains blood vessels. A superficial connective tissue capsule envelops the gland and sends branches that form a network that surrounds the follicles. These thyroids showed cuboidal follicular epithelial cells with medium-sized colloidal mass (Fig. 1a). Animals treated with MT exhibited dose-dependent morphological changes in the thyroid gland. In fact, after chronic treatment, the follicular epithelium was low and the nuclei of the thyrocytes were small and elongated with dense chromatin and a greatly reduced cytoplasm. The colloid was retracted with few reabsorption vacuoles (Figs. 1b–1d). Data about the height of follicular epithelium after chronic treatment are shown in Table 3.

In the acute treatment, the thyroid gland showed very evident signs of poor functional activity. The follicular epithelium was very low and the thyrocyte nuclei were small and elongated with dense chromatin and greatly reduced cytoplasm. The colloid showed rare reabsorption vacuoles (Fig. 1e). Data about the height of follicular epithelium after acute treatment are shown in Table 4.

T_4 and T_3 Plasma Levels

Plasma levels of thyroid hormones in the lizard *P. sicula* were affected by the different doses of MT after 30 days of treatment. In fact, the level of circulating T_4 and T_3 were found to be dose-dependent decreased in all treatment groups. Plasma T_4 decreased ($p < 0.05$) from 5.16 ± 0.04 ng/mL in the control specimens to 4.41 ± 0.03 ng/mL in animals exposed to 5 mg MT/kg body weight/day and to 4.10 ± 0.02 ng/mL in animals exposed to 30 mg MT/kg body weight/day and reached its minimum value ($p < 0.05$) (3.89 ± 0.02 ng/mL) in animals exposed to 50 mg MT/kg body weight/day. Plasma T_3 decreased ($p < 0.05$) from 3.15 ± 0.02 ng/mL in the control specimens to 2.01 ± 0.04 ng/mL in animals exposed to 5 mg MT/kg body weight/day and 1.79 ± 0.05 ng/mL in animals exposed to 30 mg MT/kg body weight/day and reached its minimum value ($p < 0.05$) (1.50 ± 0.02 ng/mL) in animals exposed to 50 mg MT/kg body weight/day (Fig. 2).

TSH Plasma Levels

Plasma concentrations of TSH decreased in all treatment groups. Exposure of lizards to 5, 30, and 50 mg MT/kg body weight/day resulted in a mild to highly significant inhibition ($p < 0.05$) in the levels of TSH at 30 days. A MT dose of 5 mg/kg body weight/d produced a slight

Table 2 Chronic toxicity of MT in male lizards

Group	Treatment (mg/kg body weight/day)	Number of animals		
		Treated animals	Dead animals (% of animal mortality)	Signs of toxicity
Control	Physiological solution	20	0	—
A	TM 5	20	0	—
B	TM 30	20	2 (10%)	Dyspnoea, hind-limb paralysis
C	TM 50	20	4 (20%)	Dyspnoea, hind-limb paralysis

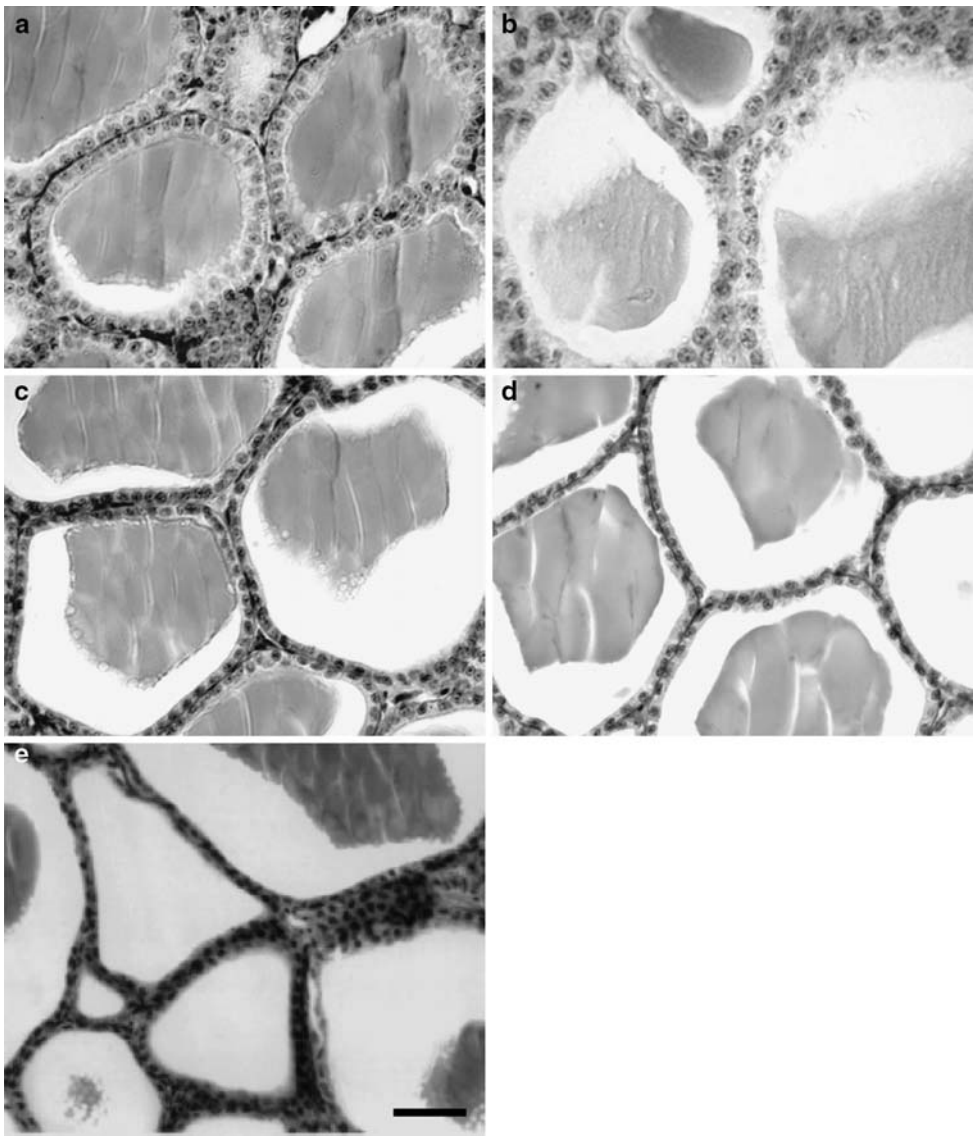

Fig. 1 Normal and MT-treated thyroids of exposed lizards *P. sicula* (stain Galgano I; scale bar: 20 (m). **a** Normal specimen; note the cuboidal follicular epithelial cells; **b** specimen treated with 5 mg/kg body weight/day of MT; the follicular epithelium is lower than in normal specimen; **c** specimen treated with 30 mg/kg body weight/day of MT; the follicular epithelium is very low; **d** specimen treated with 50 mg/kg body weight/day of MT; note the colloid is retracted with few reabsorption vacuoles; **e** specimen treated with 1000 mg/kg body weight/day of MT; note the follicular epithelium very low and no reabsorbing vacuoles in the colloid

Table 3 Variations of epithelium height of the follicular cells of the thyroid gland in *P. sicula* subjected to chronic treatment (see Materials and Methods section)

Group	Treatment (mg/kg body weight/day)	Height of follicular epithelium (μm)
Control	Physiological solution	15.1 ± 0.02
A	TM 5	8.32 ± 0.05*
B	TM 30	5.10 ± 0.04*
C	TM 50	3.02 ± 0.02*

Note: Values are shown as means ± SEM

*Significant at $p < 0.05$

Table 4 Variations of epithelium height of the follicular cells of the thyroid gland in *Podarcis sicula* subjected to acute treatment (see Materials and Methods section)

Group	Treatment (mg/kg body weight)	Height of follicular epithelium (μm)
I	Physiological solution	15.1 ± 0.02
II	TM 350	8.12 ± 0.05*
III	TM 500	6.37 ± 0.03*
IV	TM 700	4.25 ± 0.04*
V	TM 900	2.15 ± 0.05*
VI	TM 1000	1.01 ± 0.02*

Note: Values are shown as means ± SEM

*Significant at $p < 0.05$

decrease in the level of TSH (2.30 ± 0.03 (IU/mL) with respect to the control group (2.89 ± 0.03 (IU/mL). A mild to significant ($p < 0.05$) inhibition in the plasma levels of TSH was observed in lizards exposed to 30 mg/kg body weight/day (2.10 ± 0.04 (IU/mL) and 50 mg/kg body weight/day (1.90 ± 0.04 (IU/mL) (Fig. 3).

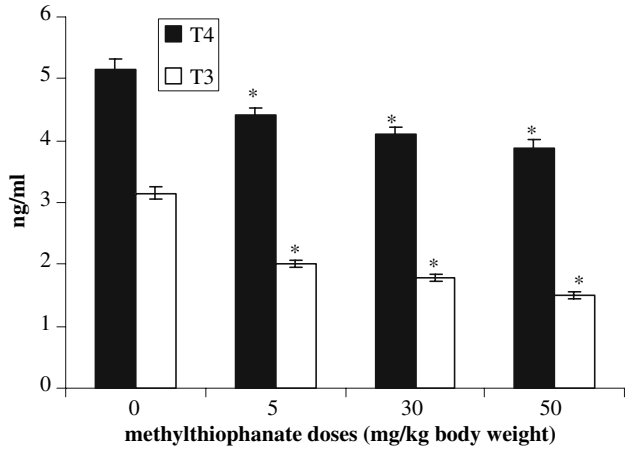

Fig. 2 Variations of T_3 and T_4 levels in the plasma of *P. sicula* subjected to different experimental treatments (see Materials and Methods section). Values are shown as means ± SEM. *Significant at $p < 0.05$

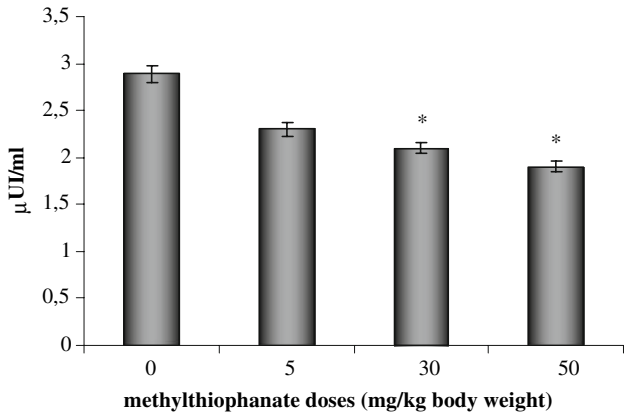

Fig. 3 Variations of TSH levels in the plasma of *P. sicula* subjected to different experimental treatment (see Materials and Methods section). Values are shown as means ± SEM. *Significant at $p < 0.05$

Discussion

The present study is the first report dealing with the effects of MT on the thyroid gland of the lizard *P. sicula*. MT, a major member of the thiophanate derivatives, is widely used to control important fungal diseases of crops because it possesses a broader range of activity than most other available fungicides (Maranghi et al. 2003; Traina et al. 1998). MT is well absorbed by oral administration and distributed throughout the organism. It is metabolized by animals into benzimidazole compounds, including methyl-2-benzimidazole carbamate (carbendazim), through a cyclizing cleavage of the side chains (Maranghi et al. 2003; Traina et al. 1998).

The results of our acute toxicity study indicate that *P. sicula* has a higher sensitivity to the acute effects of the fungicide with respect to other vertebrate species. The LD_{50} value of 850 and 930 mg/kg body weight found for MT is largely higher than the values found in other vertebrates. Therefore, our finding shows that reptiles are more enduring MT than other vertebrates (Hall and Henry 1992; Maranghi et al. 2003; Thomas and Schein 1974). In fact, our results show that mortality was higher at a high dose (70% of animal mortality, 14 deaths of 20 lizards dosed at 1000 mg/kg body weight) with respect to the lower dose of 500 mg/kg body weight (20% of animal mortality, 4 deaths of 20). Additionally, administration of different doses of MT has produced noticeable signs of poisoning (dyspnea, hind-limb paralysis, and death) similar to the findings of Ivanova-Chemishanska (1982) and of Kackar et al. (1997) for another fungicide (mancozeb). In fact, we find that MT-induced paralysis typically begins in lizards treated with 500 mg/kg body weight/day after 4 days of injection, and all lizards show some degree of paralysis by day 5. Paralysis is initially mild with decreased hind-limb movement, but as to the increase of the doses of MT, all animals were severely paralyzed and unable to move their hind limbs. After 7 days, more than 70% of treated lizards had died. The pathogenesis of this hind-limb paralysis is not completely understood; therefore, this paralysis can be due to a central block of neuromuscular transmission or to neuronal damage.

Although MT is not considered a specific developmental and reproductive toxicant, several studies on rodents showed that this fungicide is able to induce histopathological damages in the thyroid and adrenal glands, which have a pivotal role in both processes (Barlas et al. 2002). MT has been studied in order to find its effects on the structural and functional changes of the thyroid gland in the lizard.

The results of this study indicate that both structural and functional differences in the thyroid gland of the lizard *P. sicula* exist in the animals exposed to MT. Structurally, animals exposed to MT showed decreased epithelial cell height and the nuclei of the thyrocytes were small and elongated with dense chromatin and a greatly reduced cytoplasm. The colloid was retracted with few reabsorption vacuoles.

Functionally, the same animals exhibited decreased T_4 and T_3 plasma levels compared to control animals. Both histological and hormonal data have been used to indicate thyroid endocrine disruption. Additionally, MT administration produced a significant inhibition on serum TSH levels. This result might have caused hypothyroidism induced by MT exposure; therefore, the authors think that the mechanism of the MT action is at the level of the

pituitary (decreased TSH) and that is the cause of the low thyroid function.

Another possible interpretation is that this fungicide has general toxic effects on many tissues types; in fact, considering the list of cellular effects of the fungicide presented in this article, one might expect the function of many tissues to be altered.

Our results confirm the ability of MT to influence the function of the endocrine system; however, they show that the endocrine-related adverse effects might also occur at many levels.

The endocrine effects of MT have been investigated prevalently in mammals and are, to date, poorly understood. Maranghi et al. (2003) considered MT a weak endocrine disruptor, showing effects at high dose levels (560 mg/kg body weight). The authors found histological and histomorphometric alterations in thyroid and adrenals of rat pups exposed *in utero* to the fungicide.

Finally, in rats exposed to carbendazim (300 and 600 mg/kg body weight/day for 15 weeks), an active fungicide and a metabolite of MT, an increase in serum T_3 levels and histopathological changes in thyroid and parathyroid glands were observed.

The present study suggest that abnormalities in thyroid histology and circulating thyroid hormone concentration exist in lizards exposed to an environmental chemical. Thus, thyroid gland disruption, structural and functional, in lizards appear to have an environmental etiology.

Additionally, there exists a need to select and validate models to delayed developmental effects for a more comprehensive and accurate risk assessment of EDCs.

In conclusion, our findings show that MT acts as endocrine disruptor, able to influence both the morphology and the functionality of the lizard thyroid gland at different doses. Therefore, our results indicate that MT might be toxic for nontarget organisms, such as lizards. Moreover, it has been recently demonstrated that several pesticides can be detected in fruits and vegetables (Aysal et al. 2004; Rawn et al. 2006a, 2006b) and so enter the human food chain.

Moreover, although the majority of research in the field of endocrine toxicology has focused on estrogenicity (Harvey and Everett 2003), more recently it has been shown that there are many processes and toxicological targets in the endocrine system that could be subjected to chemical disruption and have equally important potential consequences for human health.

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