Chapter

Deltamethrin Alters Thyroid Hormones and Delays Pubertal Development in Male and Female Rats

Shui-Yuan Lu, Pinpin Lin, Wei-Ren Tsai and Chen-Yi Weng

Abstract

Pyrethroid insecticides are suspected endocrine-disrupting chemicals. Deltamethrin has been reported to antagonize thyroid hormone receptor activity in a reporter assay. We hypothesized that deltamethrin alters thyroid function. Male and female rats were administered daily oral gavages with 0, 0.3, 1, or 3 mg/kg/day deltamethrin on postnatal days 23–53 and 22–42, respectively. Results showed that deltamethrin decreased the relative thyroid weight in 0.3 and 1 mg/kg/day in female but not in male rats. Although the histology and several parameters of thyroid were not affected, the decreased relative weight exhibited underlying meaning. Deltamethrin delayed the age of vaginal opening (VO) and increased body weight upon VO in 3 mg/kg/day. Deltamethrin failed to delay the age of preputial separation in male rats. In the respective of serum hormone concentration, deltamethrin increased 17β-estradiol (E_2) with dose-dependent manner in female rats. The novel finding is that deltamethrin decreased thyroxine (T_4), triiodothyronine (T_3), and thyroid-stimulating hormone (TSH) in the female rats. In contrast, deltamethrin increased T_3 and TSH but not in T_4 in male rats. We inferred that deltamethrin disrupts thyroid hormone and might be related to estrogen receptor agonist. The future work is to investigate if deltamethrin disrupts the hypothalamus-pituitary-thyroid axis.

Keywords: thyroid hormone, deltamethrin, vaginal opening, preputial separation, pubertal rats

1. Introduction

Environmental endocrine disruptors (EDs) have been the subject of public attention since the publication of Rachel Carson's Silent Spring [1]. Problems associated with EDs have been studied, and EDs have been suggested not only to produce reproductive and developmental toxicity in experimental animals but also to increase the incidence of cancers, such as those of the mammary gland, testis, and prostate, and to decrease sperm numbers and induce developmental abnormalities in wildlife and humans by disrupting the endocrine system [2, 3]. Faced with an emerging health threat, the U.S. Environmental Protection Agency (U.S. EPA) identified research requirements for future risk assessments regarding endocrine-disrupting chemicals (EDCs) [4, 5].

The Endocrine Disruptor Screening and Testing Advisory Committee of the U.S. EPA has recommended a screening strategy to investigate endocrine-disrupting compounds that are agonists/antagonists to estrogen/androgen receptors, steroid biosynthesis inhibitors, or altering thyroid hormone function [6]. Pubertal female and male rat models were designed to investigate alterations of female and male pubertal development, thyroid function, and hypothalamic-pituitary-thyroid (HPT) function, respectively. The intact 22- or 23-day-old weanling female and male rats were exposed to the test substance for 20 or 30 days during the pubertal development period [7, 8].

HPT function has been used extensively to review vertebrate species, teleosts, amphibians [9, 10], and mammals [11, 12]. In normal thyroid gland functioning, thyrotropin-releasing hormone (TRH) is released from the hypothalamus. TRH travels to the anterior pituitary and triggers the release of thyroid-stimulating hormone (TSH), or thyrotropin, from the thyrotrophic cells in the pars distalis of the adenohypophysis. An increase in TSH production leads to an increase in thyroid hormone (TH) synthesis. TSH binds to receptors on the membrane of the thyroid follicle cells, thereby stimulating the biosynthesis of the iodine-containing THs, thyroxine (T4), and triiodothyronine (T3). T4 is the principle TH secreted from the thyroid gland; however, it is quickly metabolized into T3, the more potent TH. T3 and T4 can exert negative feedback control over anterior pituitary to inhibit further release of TSH [13, 14].

Synthetic pyrethroids are a class of insecticides listed by the U.S. EPA as potential EDCs [15]. Pyrethroids are widely used in agriculture and public health. Deltamethrin ((S)-α-cyano-3-phenoxybenzyl-(1R)-cis-(2, 2-dibromovinyl)-2, 2-dimethylcyclo-propane carboxylate) is the membrane of the type-II synthetic pyrethroid pesticide, which is commonly used worldwide. The major site of action of deltamethrin seems to be the voltage-dependent sodium channel. However, most recent studies on deltamethrin-related toxicity have focused on testicular and epididymal toxicity with decreasing weights, damage to the diameter of seminiferous tubules [16], abnormal morphology of spermatozoa, and sloughing and vacuolization [17–23] in male rats and mice. The second related form of toxicity caused by deltamethrin is neurotoxicity. Previous studies have shown that deltamethrin-induced neurobehavioral toxicity [24] in parent rats and increased cytochrome P450 expression are involved in the neuroendocrine functions in their offspring [25, 26]. The most significant effects of neurotoxicity in rats include decreased neuronal sodium channel expression [27, 28], motor coordination deficit [29], decreased motor activity and activity of the striatal dopaminergic system [30], cell death in the hippocampus and deficits in hippocampal precursor proliferation [31], and decreased ambulatory motor activity [32]. A previous study showed that deltamethrin decreases messenger RNA expression of specific genes that may potentially disrupt normal adipogenesis and lipid and glucose metabolism [33]. Recently, an epidemiologic finding showed that deltamethrin might be a risk factor for attention deficit hyperactivity disorder [34].

Although deltamethrin induces male reproductive toxicity and neurotoxicity, it remains unclear whether deltamethrin causes endocrine disruption. According to structural similarities and in vitro research, pyrethroid insecticides (including deltamethrin and its metabolite, 3-phenoxybenzoic acid) can affect the TH system by interfering with the nuclear TH receptors [35]. Furthermore, daily administration of 6.25–25 mg/kg/day deltamethrin for 15 days was shown to decrease the serum and cerebral cortex levels of T4 and T3 in the mitochondria of the cortex and hippocampi of rats [36]. However, in another study, an oral gavage with a single 15 mg/kg dose of deltamethrin or 3 mg/kg/day of deltamethrin for 30 days failed to change TH levels [37].

However, the exposure time for the high dosage (15 mg/kg) may have been too short, and when a lower dosage (3 mg/kg/day for 30 days) was administered, exposure may have been too low to disturb TH levels. Little is known about the molecular and cellular mechanisms that mediate TH action in the developing brain or developmental events [38].

The Toxicology in the 21st Century (Tox21) program is a federal collaboration between U.S. government agencies. The program utilizes high-throughput screening (HTS) to quickly and efficiently test chemicals for activity across a battery of assays targeting cellular processes such as the agonistic and antagonistic activities of androgen receptors, estrogen receptors, and thyroid receptors (TRs) [39]. The Tox21 databank showed that deltamethrin exhibited TR antagonistic activity. Furthermore, Du et al. [35] showed that deltamethrin exhibited TR and androgen receptor antagonistic activities in reporter assays. Few studies have investigated endocrine-disrupting activity in nuclear receptors (especially in androgen receptors, estrogen receptors, and TRs) or deltamethrin-induced dysfunction of the hypothalamus-pituitary-gonad (HPG) and HPT axes; many studies, however, have investigated male reproductive toxicity and neurotoxicity. Based on previous reports regarding testicular and epididymal toxicity, neurotoxicity, and TR receptor antagonists from the Tox21 databank, we hypothesized that deltamethrin might disturb thyroid-related function. We studied pubertal development and thyroid function in male and female Wistar rats to investigate the possible effects of deltamethrin on endocrine activity [35].

2. Materials and methods

2.1 Chemicals

The following materials were obtained: testosterone propionate (TP, purity ≥ 97%), flutamide (purity ≥ 97%), corn oil (0.9 g/ml), 17α-ethinylestradiol (purity ≥ 98%) (Sigma-Aldrich Co., St. Louis, MO, USA), and deltamethrin (purity ≥ 97%; Sinon Co., Taichung, Taiwan, ROC).

2.2 Animals

The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the Taiwan Agricultural Chemicals and Toxic Substances Research Institute. Five-week-old male and female Wistar rats were purchased from BioLASCO (Taipei, Taiwan, ROC). The rats were acclimated to the laboratory environment and reared under a controlled temperature (21 ± 2°C), humidity (40–70%), frequency of ventilation (at least 10/h), and alternating 12 h cycles of light and darkness. The rats were administered a pellet rodent diet and water ad libitum until they were sacrificed. At 12 weeks of age, the 18 male and 18 female rats within each treatment group were allowed to mate over a 14-day period. Gestation day (GD) 0 was defined as the day that sperm was observed in the vagina of the female following mating. Dams were allowed to deliver their pups naturally. Any litters with fewer than eight pups (i.e., including both male and female pups) or not delivered by GD 23 were excluded from the study. Sufficient litter numbers needed to be available to assure that 7–10 female and 10 male pups were available per treatment group and to avoid the need for placing littermates in the same experimental groups. A population of female and male pups that was as homogeneous as possible was selected by eliminating an equal number

of pups from the heavy and the light ends of the distribution, leaving the number of animals required in the middle of the distribution. The pups were assigned to treatment groups such that the mean body weights and variances for all groups were similar. Animal allocation to treatment groups was conducted on the basis of body weight randomization to ensure unbiased weight distribution across all groups.

2.3 Treatment

Each male offspring was weighed and clinically observed, and measurements were recorded daily prior to treatment. Treatments (control; TP 0.4 mg/kg/day; flutamide 3 mg/kg/day; deltamethrin 0.3, 1, or 3 mg/kg/day) were administered daily by oral gavage on postnatal day (PND) 23–53. Beginning on PND 30, males were examined daily for preputial separation (PPS). Males were dosed and sacrificed from 2 h after dosing on PND 53. Each female offspring was weighed and clinically observed, and its measurements were recorded daily prior to treatment. Treatments (control; 17α-ethinyl estradiol [EE] 5 mg/kg/day; deltamethrin 0.3, 1, or 3 mg/kg/day) were administered daily by oral gavage from PND 22 to 42. Females were examined daily for vaginal opening (VO). Females were dosed and sacrificed from 2 hours after dosing on PND 42.

2.4 Clinical signs and body weights

Throughout the study period, each female and male animal was observed at least once daily for clinical signs of toxicity related to chemical treatment. On working days, all cages were checked in the mornings and afternoons for dead or moribund animals. The body weight of each rat was recorded to the nearest 0.1 g daily prior to treatment.

2.5 Measurement of organ weights

Twenty-four hours after the final treatment, each rat was treated with the control (females and males); EE 5 mg/kg/day (females); TP 0.4 mg/kg/day (males); flutamide 3 mg/kg/day (males); and deltamethrin 0.3 or 1 mg/kg/day (females and males) and anesthetized with Zoletil 3 mg/kg/day (females and males) in the same sequence that the test substance was administered. Uteri and ovaries were dissected and carefully trimmed of fat to avoid loss of luminal content. The body of each uterus was cut just above its junction with the cervix and at the junction of the uterine horns with the ovaries. Each uterus was weighed with and without the luminal content. Thyroids, livers, kidneys, pituitary glands, adrenal glands, ovaries, seminal vesicles, and coagulating glands with and without fluid, prostates, levator ani plus bulbocavernosus muscles (LABC), epididymides, testes, and penes were carefully dissected and weighed.

2.6 VO

Each female animal was examined daily for VO from PND 21. On the day that VO was first detected, the age and body weight were recorded. Vaginal lavage was collected daily from the day following VO until the end of the study by repeated pipetting of 0.9% saline into the vagina. The lavage fluid was applied to a clean glass slide, and the smear was viewed immediately under low magnification (×100) with a microscope. Cytology was evaluated and the stage of the estrous cycle was determined using the method described by Everett [40]. The appearance of a small

"pinhole," vaginal thread, and complete VO were all recorded on the days they were observed. The day of complete VO was the endpoint in the analysis for the age upon VO; a pinhole or thread did not represent complete VO, even though it had to be recorded when observed. However, if any animal within any treatment group showed incomplete opening (such as persistent threads or a pinhole) for more than 3 days, a separate analysis was conducted using the ages at which an incomplete opening was first observed. Even if VO otherwise appeared complete, documentation of a vaginal thread was crucial. It was also critical that the "initiation" of VO be recorded and preferred that VO observations be taken daily after dosing. Whether collected before or after dosing, VO observations had to be collected at approximately the same time each day.

2.7 Estrous cyclicity

From the day of VO up to and including the day of necropsy, daily vaginal smears were obtained and evaluated under a low-power light microscope for the presence of leukocytes, nucleated epithelial cells, or cornified epithelial cells. The vaginal smears were classified as diestrus (predominance of leukocytes mixed with some cornified epithelial cells), proestrus (predominance of clumps of round, nucleated epithelial cells), or estrus (predominance of cornified epithelial cells), and the stages were recorded daily. Metestrus was classified as an early part of diestrus rather than a late part of estrus. Age upon first vaginal estrus was noted, and it was preferred (although not critical) that estrous cycle observations be made after daily dosing. Whether collected before or after dosing, estrous cycle observations had to be collected at approximately the same time each day.

At the end of the study, the overall pattern of each female was characterized as regularly cycling (having recurring 4- to 5-day cycles), irregularly cycling (having cycles with periods of diestrus longer than 3 days or periods of cornification longer than 2 days), or not cycling (having prolonged periods of either vaginal cornification or leukocytic smears). In cases where there were too few days between VO and the end of the study for more than one cycle to be observed, classification was based on the available data with the default assumption that the animals cycled regularly if the partial data fit the definition and irregularly if the study ended without being able to distinguish between irregular cycling and not cycling.

2.8 PPS

PPS, the separation of the foreskin of the penis from the glans, is an early reliable marker of the pubertal progression that normally occurs between 40 and 50 days of age, with an average of 43 days, depending on the rat species [41]. In the present study, PPS was monitored from PND 22 to 53. All males were monitored at approximately the same time each day. A partial separation with a thread of cartilage remaining was recorded as partial, but only the day of complete separation was used in the data analysis. The appearance of partial and complete PPS or a persistent thread of tissue between the glans and prepuce were all recorded on the days they were observed. The day of complete PPS was the endpoint used in the analysis for the age upon PPS. However, if any animal in any treatment group showed incomplete separation (including persistent threads) for more than 3 days, a separate analysis was conducted using the ages at which partial separation was first observed. Even if PPS otherwise appeared complete, documentation of a thread was crucial. It was also critical that "initiation" of PPS be recorded and preferred that PPS observations be taken after daily dosing. Whether collected before or after

dosing, the PPS observations had to be collected at approximately the same time each day.

2.9 Hematochemistry

Blood samples treated with control (females and males), EE 5 mg/kg/day (females), TP 0.4 mg/kg/day (males), flutamide 3 mg/kg/day (males), and deltamethrin 0.3, 1, and 3 mg/kg/day (female and male) were collected and coagulated for 30 min in an SST II tube (#367953, BD Co., Plymouth, UK). The blood samples were put on ice bath prior to centrifugation. After coagulation, the blood was centrifuged at 3000 × *g* for 15 min. The serum was transferred into siliconized microcentrifuge tubes and stored at −80°C until used. Serum creatinine and blood urea nitrogen levels were detected with an automated clinical chemistry analyzer (DRI-CHEM 4000i, Fujifilm Co., Tokyo, Japan).

2.10 Hormonal measurements

Luteinizing hormone (LH); follicle-stimulating hormone (FSH); T4, T3, and TSH levels in serum treated with control (females and males); EE 5 mg/kg/day (females); TP 0.4 mg/kg/day (males); flutamide 3 mg/kg/day; and deltamethrin 0.3, 1, and 3 mg/kg/day (females and males) were determined using a magnetic bead panel (#RPTMAG-86K, #PTHYMAG-30K, Millipore Co., St. Charles, MI, USA).

Testosterone (T) and estradiol (E2) levels in serum treated with control (female and male), 5 mg/kg/day 17α-ethinyl estradiol (EE 5) (female), 0.4 mg/kg/day testosterone propionate (TP 0.4) (male), 3 mg/kg/day flutamide, 0.3, 1, and 3 mg/kg/day deltamethrin (female and male) were assayed using EIA kit (#582701, #582251, Cayman Co.). Luteinizing hormone (LH), follicle-stimulating hormone (FSH), total tetraiodothyronine (T4), triiodothyronine (T3), and thyroid-stimulating hormone (TSH) levels in serum treated with control (female and male), 5 mg/kg/day 17α-ethinyl estradiol (EE 5) (female), 0.4 mg/kg/day testosterone propionate (TP 0.4) (male), 3 mg/kg/day flutamide, 0.3, 1, and 3 mg/kg/day deltamethrin (female and male) were determined using magnetic bead panel (#RPTMAG-86K, #PTHYMAG-30K, Millipore Co.). A cytochrome P450 19A1 ELISA kit (CSB-EL006394RA, Cusabio Co.) was used for determined aromatase level in serum treated with control (female and male), 5 mg/kg/day 17α-ethinyl estradiol (EE 5) (female), 0.4 mg/kg/day testosterone propionate (TP 0.4) (male), 3 mg/kg/day flutamide, 0.3, 1, and 3 mg/kg/day deltamethrin (female and male).

2.11 Histology

The tissues of the thyroid glands (females and males), pituitary glands (females and males); uteri, ovaries, testes, epididymides, and prostates of rats treated with control (females and males); EE 5 mg/kg/day (females); TP 0.4 mg/kg/day (males); flutamide 3 mg/kg/day; and deltamethrin 0.3, 1, and 3 mg/kg/day (females and males) were stored in 10% formaldehyde solution for at least 7 days. After routine processing, 3-μm-thick paraffin sections were cut and stained with hematoxylin and eosin. Thyroid sections were subjectively evaluated for several parameters of thyroid activity including total area analyzed (μm^2), total colloid area (μm^2), colloid/total area (%), number of colloid, average colloid area (μm^2), average colloid diameter (μm), total epithelial area (μm^2), and epithelial/total area (%) [42] by Tissue Phenomics (Definiens AG, Munich, Germany).

3. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Data for the mean initial or necropsy body weight, mean age, body weight upon VO or PPS, organ weight, and hormone levels were analyzed for homogeneity of variance by using Bartlett's test. When samples were proven to be homogeneous, nonparametric analysis of variance was applied. Absolute organ weight was analyzed using analysis of covariance with body weight upon necropsy as a covariate. When a significant treatment effect was observed, Dunnett's test (control vs. treatment groups) was used to compare the treatment groups. The level of statistical significance was set a priori at $\alpha = 0.05$.

4. Results

4.1 Effects on body and organ weights

During the study period, no clinical signs of toxicity were detected in any treatment group of male or female rats. EE significantly decreased the body weights of the female rats from PND 25 to 42 (**Figure 1A**). However, in contrast to the control group, deltamethrin did not change the body weight of the female or male rats (**Figure 1B**).

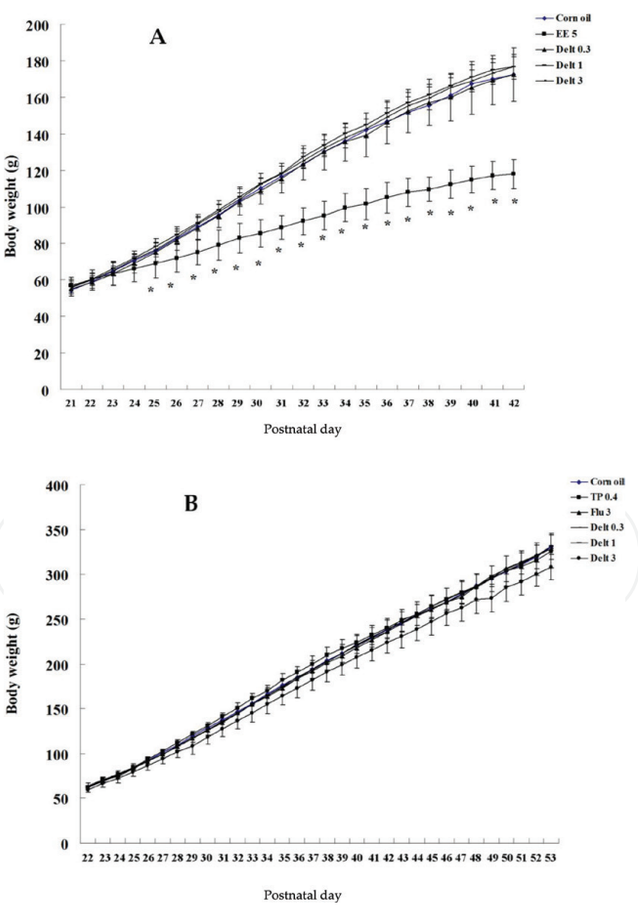

Figure 1.
*Body weight changes in Wistar female rats treated with 17α-ethinyl estradiol 5 mg/kg/day (EE5), testosterone propionate 0.4 mg/kg/day (TP 0.4), flutamide 3 mg/kg/day (Flu 3), and deltamethrin 0.3, 1, and 3 mg/kg/day (Delta 0.3, Delta 1, and Delta 3). Female rats were dosed daily starting at 21 days of age and this was continued until necropsy on day 42 (A), and male rats were dosed daily starting at 22 days of age and this was continued until necropsy on day 53 (B). EE significantly decreased the body weight during the treatment period. Data are expressed as mean ± SD of 10 animals per treatment group. *Significantly different from the vehicle control, $P < 0.05$.*

Treatments	Vehicle control	EE[1] (mg/kg/day)	TP[2]	Flutamide	Deltamethrin (mg/kg/day)		
		5	0.4	3	0.3	1	3
Female							
Sample size (n)	10	10			10	10	10
Initial body weight (g)	59 ± 3	60 ± 4			59 ± 4	61 ± 4	60 ± 2
Final body weight (g)	173 ± 10	119 ± 7***			173 ± 15	177 ± 6	177 ± 7
Final body weight (%)	100	69			100	102	102
Body weight gain (g)	114 ± 9	59 ± 6***			114 ± 14	116 ± 7	117 ± 7
Male							
Sample size (n)	10		10	10	10	10	10
Initial body weight (g)	69.6 ± 3.5		70 ± 3	69 ± 5	70 ± 3	71 ± 3	66 ± 4
Final body weight (g)	331 ± 18		328 ± 25	328 ± 25	328 ± 23	331 ± 14	308 ± 14**
Final body weight (%)	100		99	99	99	100	93
Body weight gain (g)	261 ± 16		260 ± 21	257 ± 21	258 ± 22	260 ± 12	242 ± 12

*[1]EE: 17α-ethinyl estradiol; [2]TP: testosterone propionate, p-value = *≤0.05, **≤0.01, ***≤0.005.*

Table 1.
General growth in the female and male rats.

Treatments	Vehicle control	EE[1] (mg/kg/day)	Deltamethrin (mg/kg/day)		
		5	0.3	1	3
Sample size (n)	10	10	10	10	10
Liver (g)	8.2 ± 0.6	6.1 ± 0.4***	8.0 ± 1.2	8.1 ± 0.7	8.1 ± 0.6
Kidney (g)	1.6 ± 0.2	1.2 ± 0.1***	1.6 ± 1.2	1.6 ± 0.1	1.6 ± 0.1
Pituitary (mg)	10.2 ± 1.4	15.7 ± 6.4*	10.0 ± 1.3	10.7 ± 1.8	12.1 ± 3.3
Adrenals (mg)	55.8 ± 7.0	36.8 ± 4.8***	50.9 ± 5.9	53.1 ± 5.2	52.3 ± 7.8
Ovaries (mg)	94.3 ± 14.5	59.4 ± 13.7***	90.9 ± 17.1	92.7 ± 14.5	98.5 ± 23.7
Uterus, wet (mg)	427.5 ± 191.8	583.3 ± 328.8	383.6 ± 237.8	508.6 ± 2773.6	431.7 ± 221.5
Uterus, blotted (mg)	344.5 ± 71.8	364.6 ± 58.8	303.9 ± 93.5	357.4 ± 100.2	340.2 ± 86.6
Thyroid w/o trachea (mg)	138.3 ± 10.0	113.0 ± 12.1***	130.5 ± 10.2	135.8 ± 8.7	140.5 ± 11.9
Thyroid w/o trachea (mg)	43.4 ± 7.8	38.3 ± 5.3	36.3 ± 6.4*	36.4 ± 6.6	42.5 ± 5.1

*[1]EE: 17α-ethinyl estradiol; propionate, p-value = *≤0.05, **≤0.01, ***≤0.005.*

Table 2.
Organ weight at necropsy in the female rats.

In the female rats, EE significantly decreased final body weight and weight gain in female rats, while deltamethrin did not. In the male rats, TP, flutamide, and deltamethrin did not affect the body weight and weight gain except 3 mg/kg/day deltamethrin (**Table 1**). EE significantly changed the weights of the livers, kidneys, pituitary glands, adrenal glands, ovaries, and thyroid with trachea. For absolute organ weight treatment with 0.3 mg/kg/day, deltamethrin significantly reduced 16% of thyroid weight in the female rats, and the reduction was not dose-dependent (**Table 2**). For relative organ weight, EE significantly increased relative thyroid weight, while 0.3 and 1 mg/kg/day deltamethrin significantly decreased it (**Figure 2A**) in female rats but not in male rats (**Figure 2B**).

In the male rats, TP significantly increased the absolute organ weights of the adrenals, seminal vesicles plus coagulating glands with and without fluid, left and right epididymides, and left and right testes. Flutamide significantly decreased the absolute organ weights of the seminal vesicles plus coagulating glands, LABC, epididymides, and penes. Treatment with 3 mg/kg/day deltamethrin significantly decreased the weights of the thyroids, livers, and penes in male rats. Although treatment with 3 mg/kg/day deltamethrin significantly reduced 13% of penis weight in the male rats, the reduction was not dose-dependent (**Table 3**).

4.2 Effects on BUN, creatinine in serum

In order to investigate the effect of deltamethrin on blood chemistry, the blood urea nitrogen (BUN) and creatinine were selected as indicators. EE significantly increased BUN, while deltamethrin did not affect BUN and creatinine in female rats. TP and flutamide did affect the BUN and creatinine, while deltamethrin decreased marginally serum BUN in male rats (**Table 4**).

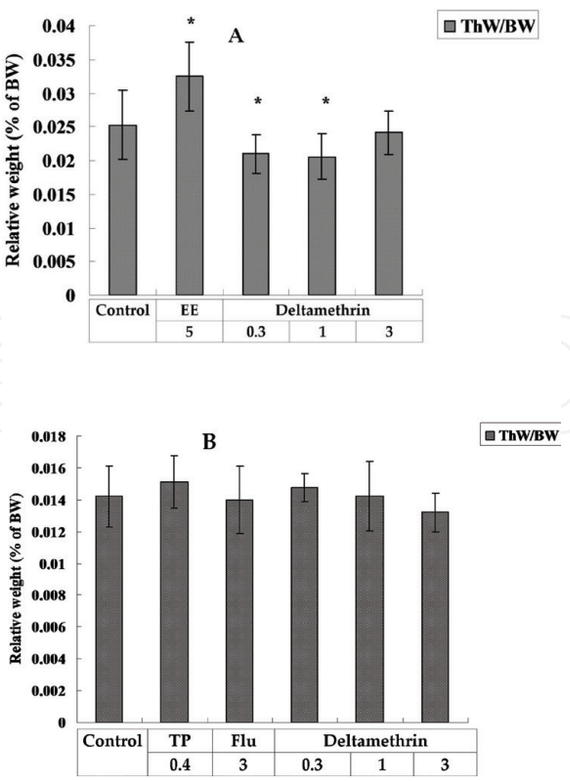

Figure 2.
*Relative thyroid weight in Wistar female (A) and male (B) rats treated with 17α-ethinyl estradiol 5 mg/kg/day (EE5), testosterone propionate 0.4 mg/kg/day (TP 0.4), flutamide 3 mg/kg/day (Flu 3), and deltamethrin 0.3, 1, and 3 mg/kg/day (Delta 0.3, Delta 1, and Delta 3). EE significantly increased relative thyroid weight, while deltamethrin in low and middle dose significantly decreased it. There is no significant difference of relative thyroid weight between control and positive, negative and deltamethrin treatments. Data are expressed as mean ± SD of 10 animals per treatment group. *Significantly different from the vehicle control, P < 0.05.*

Treatments	Vehicle control	TP[1]	Flutamide		Deltamethrin	
		0.4	3	0.3	1	3
Sample size (n)	10	10	10	10	10	10
Liver (g)	15.7 ± 1.1	14.8 ± 1.4	15.2 ± 1.0	14.8 ± 1.4	15.2 ± 0.8	13.6 ± 0.8***
Kidney (g)	2.7 ± 0.2	2.6 ± 0.3	2.5 ± 0.2	2.6 ± 0.2	2.6 ± 0.2	2.5 ± 0.2
Pituitary (mg)	13.3 ± 1.1	13.4 ± 1.6	13.4 ± 2.3	12.9 ± 2.2	13.1 ± 1.1	12.2 ± 1.7
Adrenals (mg)	57.5 ± 4.8	67.8 ± 5.8***	58.2 ± 7.0	56.9 ± 6.5	58.4 ± 6.0	55.5 ± 5.5
Seminal vesicle + coagulating gland, with fluid (mg)	859 ± 100	1050 ± 111***	627 ± 120***	956 ± 151	954 ± 124	852 ± 103
Seminal vesicle + coagulating gland, without fluid (mg)	540 ± 46	675 ± 109**	447 ± 67**	574 ± 68	599 ± 85	571 ± 77
Prostate (mg)	233 ± 47	255 ± 53	210 ± 57	246 ± 26	233 ± 37	218 ± 45
LABC[2] (mg)	732 ± 109	779 ± 99	585 ± 97**	733 ± 94	716 ± 82	648 ± 75
Left epididymis (mg)	229 ± 14	199 ± 10***	196 ± 24**	241 ± 20	236 ± 28	224 ± 16
Right epididymis (mg)	235 ± 21	197 ± 10***	196 ± 24**	247 ± 21	234 ± 28	217 ± 18
Left testis (mg)	1409 ± 75	1013 ± 209***	1415 ± 118	1410 ± 115	1380 ± 105	1394 ± 116
Right testis (mg)	1407 ± 59	1018 ± 218***	1432 ± 139	1388 ± 110	1409 ± 99	1390 ± 134
Thyroid with trachea (mg)	183.4 ± 23.8	188.8 ± 18.2	182.1 ± 12.4	190.3 ± 15.7	191.7 ± 12.4	175.1 ± 11.4
Thyroid without trachea (mg)	47.0 ± 7.7	49.1 ± 5.6	45.7 ± 5.8	48.5 ± 5.3	46.9 ± 6.3	40.6 ± 3.7*
Penis (mg)	253 ± 24	268 ± 19	228 ± 26*	256 ± 24	252 ± 17	234 ± 16*

*[1]TP: testosterone propionate, [2]LABC: levator ani plus bulbocarvernosus muscles, p-value = *≤0.05, **≤0.01, ***≤0.005.*

Table 3.
Organ weight at necropsy in the male rats.

Treatments	Vehicle control	EE[1] (mg/kg/day)	TP[2] (mg/kg/day)	Flutamide (mg/kg/day)	Deltamethrin (mg/kg/day)		
		5	0.4	3	0.3	1	3
Female							
Sample size (n)	10	10			10	10	10
BUN[3] (mg/dL)	19.1 ± 4.0	24.2 ± 3.4**			18.0 ± 2.0	17.0 ± 4.7	16.8 ± 4.4
Creatinine (mg/dL)	0.27 ± 0.05	0.24 ± 0.05			0.26 ± 0.05	0.23 ± 0.05	0.25 ± 0.05
Male							
Sample size (n)	10		10	10	10	10	10
BUN (mg/dL)	19.1 ± 2.2		19.2 ± 2.2	18.1 ± 1.9	17.5 ± 2.0	16.8 ± 2.2*	16.2 ± 2.2*
Creatinine (mg/dL)	0.29 ± 0.03		0.27 ± 0.08	0.28 ± 0.04	0.28 ± 0.04	0.3 ± 0.00	0.26 ± 0.05

*[1]EE: 17α-ethinyl estradiol; [2]TP: testosterone propionate; [3]BUN: blood urea nitrogen; p-value = *≤0.05 , **≤0.01.*

Table 4.
BUN, creatine in the female and male rats.

4.3 Effects on VO and estrous cyclicity in female rats

The mean age and body weight upon VO were 27.9 days and 95.5 g, respectively, in the control female rats. Treatment with EE significantly advanced VO to 25.4 days of age and substantially reduced the mean body weight at the time of VO to 70.7 g. VO in EE-treated rats was first detected at 24 days of age in most rats. Treatment with 3 mg/kg/day deltamethrin significantly delayed the mean time of VO (32.3 days) and increased the mean body weight to 127.3 g at the time of VO (**Figure 3A**).

The estrous cycles of individual animals were evaluated from the day after VO until the end of the study. Most control rats exhibited regular cycling, although three

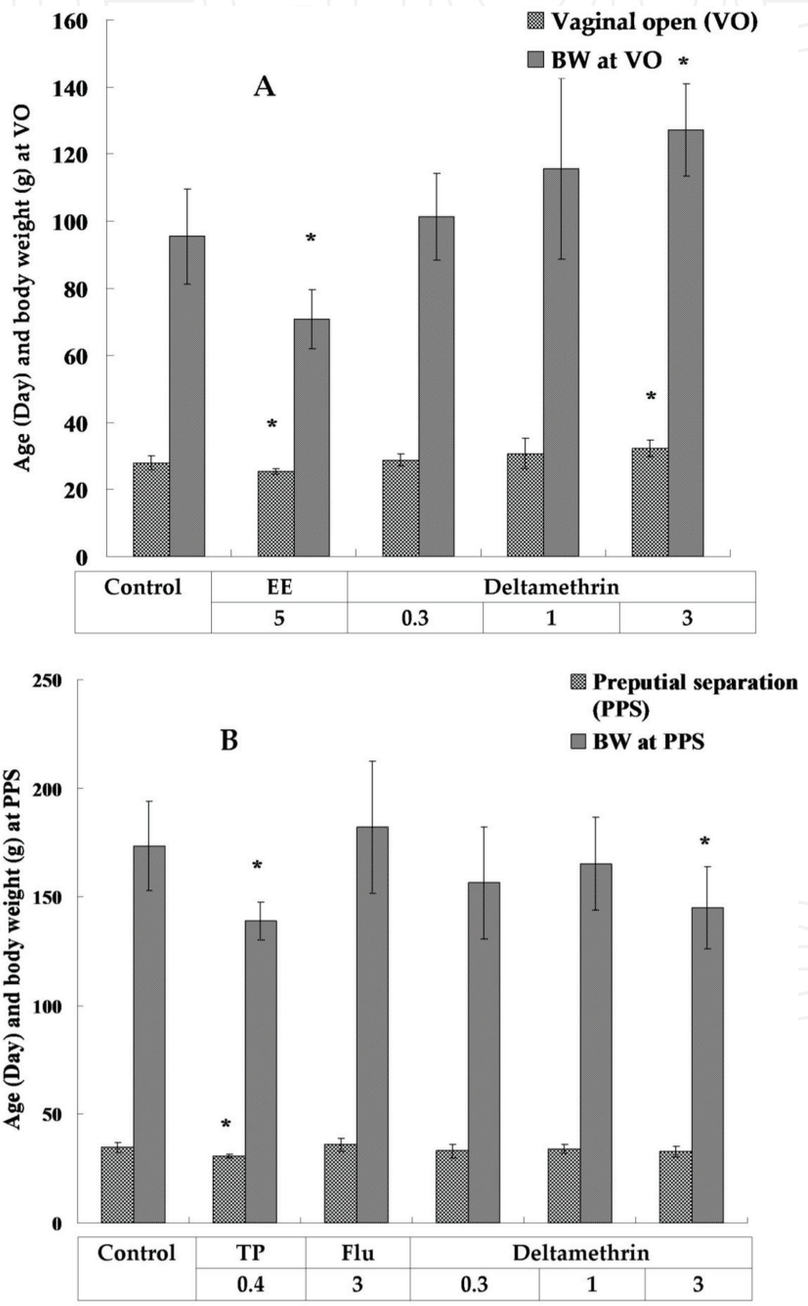

Figure 3.
*Mean age and body weight at vaginal opening (VO) in Wistar female (A) and male (B) rats treated with 17α-ethinyl estradiol 5 mg/kg/day (EE5) testosterone propionate 0.4 mg/kg/day (TP 0.4), flutamide 3 mg/kg/day (Flu 3), and deltamethrin 0.3, 1, and 3 mg/kg/day (Delta 0.3, Delta 1, and Delta 3). Animals were dosed daily starting at 21 days of age and examined from the time and body weight of vaginal opening for 21 days for female rats. Animals were dosed daily starting at 22 days of age and examined from the time and body weight of preputial separation (PPS) for 31 days for male rats. *Significantly different from the vehicle control, $P < 0.05$.*

Treatments	Vehicle control	EE[1] (mg/kg/day)	TP[2] (mg/kg/day)	Flutmaide (mg/kg/day)	Deltamethrin (mg/kg/day)		
		5	0.4	3	0.3	1	3
Female							
Sample size (n)	10	10			10	10	10
Total area analyzed (μm^2) ($\times 10^6$)	2.4 ± 1.1	3.6 ± 0.9			2.2 ± 0.2	2.7 ± 1.7	3.2 ± 0.6
Total colloid area (μm^2) ($\times 10^6$)	1.1 ± 0.5	1.9 ± 0.5			1.2 ± 0.2	1.2 ± 0.6	1.6 ± 0.3
Colloid/total area (%)	0.46 ± 0.04	0.51 ± 0.01			0.53 ± 0.05	0.48 ± 0.10	0.51 ± 0.06
Number of colloid	437 ± 206	869 ± 265			409 ± 16	479 ± 291	656 ± 157
Average colloid area (μm^2) ($\times 10^3$)	2.6 ± 0.2	2.2 ± 0.1			2.8 ± 0.6	2.7 ± 0.6	2.6 ± 0.9
Average colloid diameter (μm)	57.2 ± 2.3	52.5 ± 1.3			59.8 ± 6.4	58.1 ± 6.3	56.9 ± 10.3
Total epithelial area (μm^2) ($\times 10^6$)	1.2 ± 0.5	1.7 ± 0.3			0.9 ± 0.2	1.3 ± 0.9	1.5 ± 0.3
Epithelial/total area (%)	0.48 ± 0.06	0.46 ± 0.01			0.43 ± 0.07	0.47 ± 0.09	0.48 ± 0.06
Male							
Sample size (n)	10		10	10	10	10	10
Total area analyzed (μm^2) ($\times 10^6$)	4.0 ± 1.2		3.0 ± 0.7	2.8 ± 1.2	2.9 ± 0.9	3.4 ± 0.3	3.6 ± 0.4
Total colloid area (μm^2) ($\times 10^6$)	1.7 ± 0.4		1.6 ± 0.4	1.2 ± 0.5	1.4 ± 0.3	1.6 ± 0.03	1.7 ± 0.4
Colloid/total area (%)	0.44 ± 0.08		0.51 ± 0.05	0.46 ± 0.14	0.56 ± 0.07	0.47 ± 0.03	0.46 ± 0.07
Number of colloid	619 ± 304		347 ± 133	435 ± 261	515 ± 304	669 ± 80	711 ± 152
Average colloid area (μm^2) ($\times 10^3$)	2.8 ± 0.8		4.7 ± 0.8*	3.7 ± 2.6	3.2 ± 1.1	2.4 ± 0.3	2.4 ± 0.5
Average colloid diameter (μm)	59.9 ± 9.3		76.9 ± 6.8*	66.3 ± 23.2	62.7 ± 11.7	54.8 ± 3.0	55.0 ± 5.1
Total epithelial area (μm^2) ($\times 10^6$)	2.1 ± 1.0		1.4 ± 0.4	1.5 ± 0.8	1.4 ± 0.7	1.8 ± 0.3	1.8 ± 0.1
Epithelial/total area (%)	0.53 ± 0.07		0.46 ± 0.04	0.56 ± 0.13	0.48 ± 0.06	0.52 ± 0.05	0.51 ± 0.04

*[1]EE: 17α-ethinyl estradiol; [2]TP: testosterone propionate, p-value = *≤0.05.*

Table 5.
Measurements of several parameters of thyroid activity in treatment with deltamethrin.

rats exhibited diestrus for ≥4 days. VO occurred from PND 26 to 31. EE induced a lasting estrus in most rats and VO occurred from PND 24 to 26. Most rats treated with a low dosage (0.3 mg/kg/day) of deltamethrin had regular estrus and experienced VO from PND 26 to 32. One rat exhibited no VO. Treatment with a medium-strength dosage (1 mg/kg/day) of deltamethrin caused irregular cycling in four rats and delays in VO (PND 42) in one rat. Treatment with a high dosage (3 mg/kg/day) of deltamethrin prevented VO in two rats, irregular cycling in one rat, and a delay in VO between PND 30 and 36 in eight rats (data not shown).

4.4 Effects on PPS in male rats

The mean age and body weight of the male control rats upon PPS were 34.8 days and 173.5 g, respectively. TP substantially advanced the mean age of PPS to 30.8 days

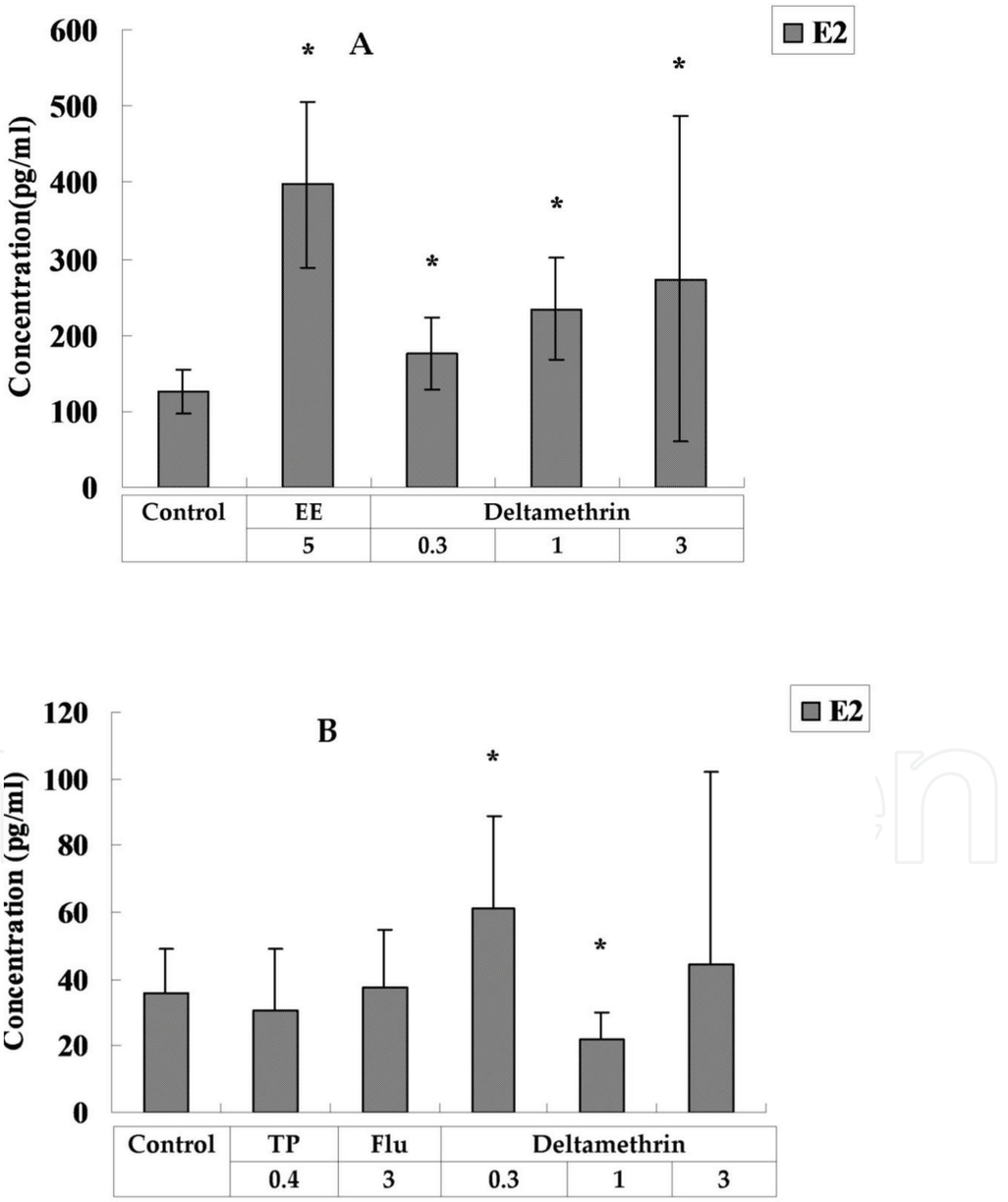

Figure 4.
Measurements of serum 17β-estradiol (E2) treated with control, 17α-ethinyl estradiol 5 mg/kg/day (EE5) testosterone propionate 0.4 mg/kg/day (TP 0.4), flutamide 3 mg/kg/day (Flu 3), and deltamethrin 0.3, 1, and 3 mg/kg/day (Delta 0.3, Delta 1, and Delta 3) in rats. EE significantly increased serum E2 concentration, while deltamethrin decreased it in female rats (A). Deltamethrin significantly increased E2 in low dose but decreased it in middle dose in male rats (B).

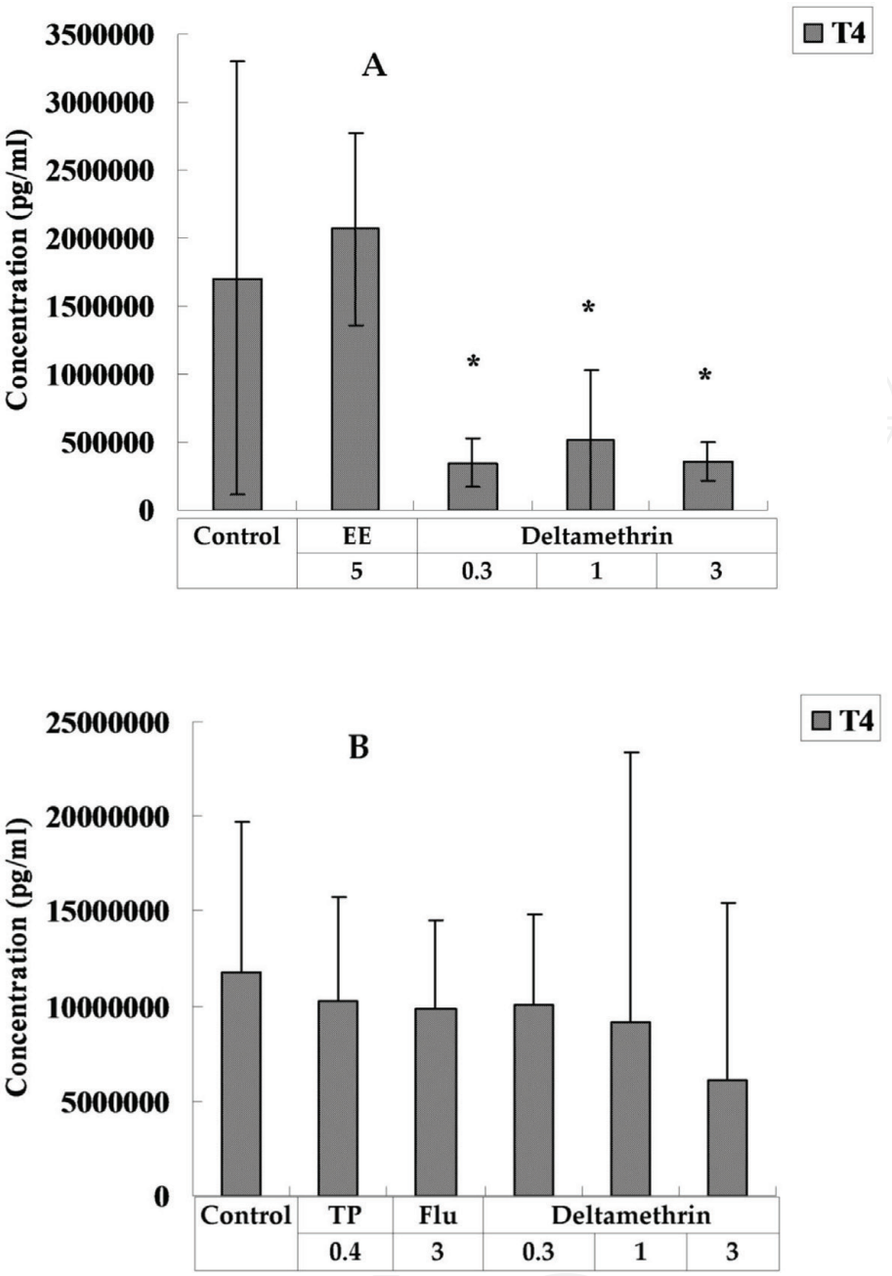

Figure 5.
Measurements of serum thyroxine (T4) treated with control, 17α-ethinyl estradiol 5 mg/kg/day (EE5) testosterone propionate 0.4 mg/kg/day (TP 0.4), flutamide 3 mg/kg/day (Flu 3), and deltamethrin 0.3, 1, and 3 mg/kg/day (Delta 0.3, Delta 1, and Delta 3) in rats. Deltamethrin significantly decreased serum T4 concentrations (A). No obvious effect was observed in male rats (B).

and reduced the mean body weight at the time of PPS to 138.9 g. However, neither deltamethrin nor flutamide affected the time or body weight at PPS, except for a reduction of body weight to 145 g induced by 3 mg/kg/day of deltamethrin (**Figure 3B**).

4.5 Effects on histology and several parameters of thyroid activity

The tissues of the thyroid glands in females and males, and the other tissues including pituitary glands (females and males), uteri, ovaries, testes, epididymides, and prostates (data not shown) of rats treated with control, EE, TP, flutamide, and deltamethrin were examined histologically. Histology of all tissues was comparable to that of the control group. No significant differences between the pituitary glands, uteri, ovaries, testes, epididymides, and prostates were detected between the controlled and treated male and female rats. Because 0.3 mg/kg/day deltamethrin

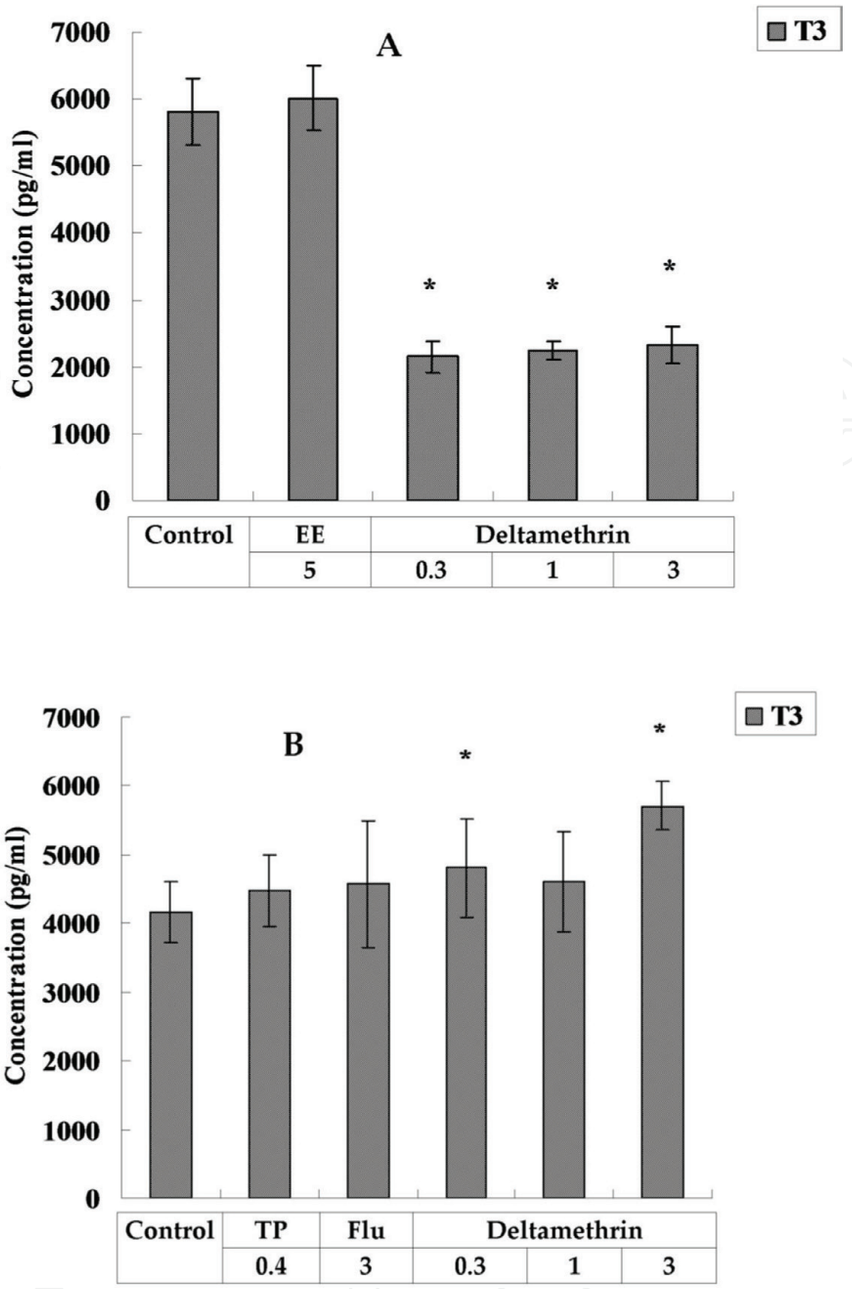

Figure 6.
Measurements of serum triiodothyronine (T3) treated with control, 17α-ethinyl estradiol 5 mg/kg/day (EE5) testosterone propionate 0.4 mg/kg/day (TP 0.4), flutamide 3 mg/kg/day (Flu 3), and deltamethrin 0.3, 1, and 3 mg/kg/day (Delta 0.3, Delta 1, and Delta 3) in rats. Deltamethrin significantly decreased T3 concentrations in female rats (A), while low and high dose significantly increased in male rats (B).

significantly reduced absolute thyroid weight (**Table 2**) and 0.3 and 1 mg/kg/day deltamethrin significantly decreased the relative thyroid weight (**Figure 2A**) in female rats, we measured the several parameters of thyroid activity. EE, TP, flutamide, and deltamethrin did not affect total area analyzed (μm^2), total colloid area (μm^2), colloid/total area (%), number of colloid, average colloid area (μm^2), average colloid diameter (μm), total epithelial area (μm^2), and epithelial/total area (%) except TP increasing average colloid area and average colloid diameter (**Table 5**).

4.6 Effects on serum hormone concentrations in female and male rats

For the female rats, EE significantly decreased serum luteinizing hormone (LH) and testosterone but increased 17ß-estradiol (E2) (**Figure 4A**) and TSH (**Figure 7A**) serum concentrations, and there is no effect on aromatase (data now

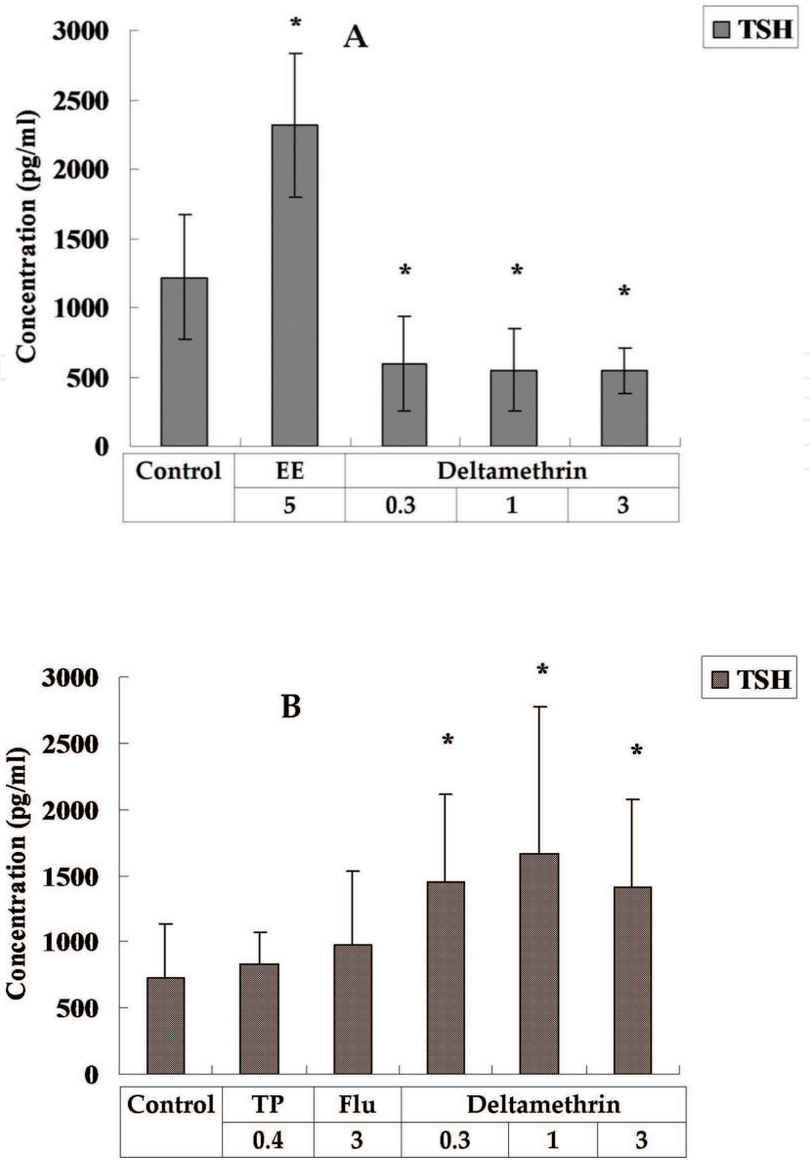

Figure 7.
Measurements of serum thyroid-stimulating hormone (TSH) treated with control, 17α-ethinyl estradiol 5 mg/kg/day (EE5) testosterone propionate 0.4 mg/kg/day (TP 0.4), flutamide 3 mg/kg/day (Flu 3), and deltamethrin 0.3, 1, and 3 mg/kg/day (Delta 0.3, Delta 1, and Delta 3) in rats. EE significantly increased TSH concentration, while deltamethrin significantly decreased it in female rats (A). In contrary to female rats, deltamethrin increased TSH concentrations in male rats (B).

shown), follicular-stimulating hormone (FSH) (data now shown), thyroxine (T4) (**Figure 5A**), and triiodothyronine (T3) (**Figure 6A**). Deltamethrin tended to decrease the serum aromatase concentration and did significantly in 1 mg/kg/day (data now shown). Deltamethrin showed no effect on serum concentrations of LH, FSH, and testosterone (data now shown). Deltamethrin significantly increased 17β-estradiol (E2) with dose-dependent manner (**Figure 4A**). Also, deltamethrin significantly decreased serum concentrations of T4 (**Figure 5A**), T3 (**Figure 6A**), and TSH (**Figure 7A**).

For the male rats, TP significantly decreased serum concentrations of LH and FSH, and there were no effect on aromatase, E2, T, T4, T3, and TSH. Flutamide significantly increased serum T, while there were no effect on aromatase (data now shown), LH (data now shown), FSH (data now shown), E2 (**Figure 4B**), T4 (**Figure 5B**), T3 (**Figure 6B**), and TSH (**Figure 7B**).

For the female rats, EE significantly increased 89% of TSH. However, deltamethrin significantly decreased 60–64% of T3, 70–80% of T4, and 51–55% of TSH concentrations. By contrast, for the male rats, 0.3 and 3 mg/kg/day of deltamethrin significantly

increased 14 and 36% of T3 concentrations, and 0.3, 1, and 3 mg/kg/day deltamethrin significantly increased 100, 130, and 96% of TSH concentrations (**Table 3**).

5. Discussion

This study investigated whether deltamethrin alters pubertal development and thyroid function on the basis of previous reports regarding testicular and epididymal toxicity, neurotoxicity in juvenile/peripubertal rats, and TR antagonism in Tox21 HTS assays. Furthermore, the study was aimed at verifying whether Tox21 reporter activity data can predict the endocrine-disrupting activity of deltamethrin. Deltamethrin was observed to delay age and increase body weight at VO, accompanied with decreased T3, T4, and TSH concentrations in female rats. Based on the reductions of T3, T4, and TSH concentrations, delay in VO age, and induction of an irregular estrous cycle, we propose that deltamethrin disrupts the HPT and hypothalamic-pituitary-ovarian (HPO) axes in female rats. By contrast, deltamethrin failed to delay the age of PPS and increased T3 and TSH concentrations in the male rats. Because deltamethrin reduced T3, T4, and TSH levels in a sex-dependent manner and correlated with disturbances in pubertal development, the delay in female pubertal development might be a consequence of thyroid disruption.

The HPO axis has been demonstrated by Ortega et al. [43, 44]. Disruption of thyroid function with 133-iodine in adult female rats resulted in irregular cycles, atrophied and underweight ovaries, and decreased serum T3 and T4. However, T3 replacement restored normal cycles and ovary weights [43]. Similarly, T4 replacement restored estrous cycles and pubertal patterns of gonadotropin secretion in hypothyroid female rats [44]. Jiang et al. [45] also reported that T4 treatment improved follicular development rather than gonadotropin secretion in infertile immature hypothyroid rdw rats. By contrast, Tamura [46] reported that hypothyroidism increases ovarian hormonal secretion and folliculogenesis during equine chorionic gonadotropin-induced follicle development in immature female rats. Thus, the involvement of the HPG ovary in hypothyroidism-induced disruption of female puberty development is still unclear. In the present study, the FSH and LH levels of deltamethrin did not change (data not shown), suggesting that the HPG ovary might not be involved in the mechanism for the delay of female puberty by deltamethrin.

The results of the present study show that deltamethrin decreased concentrations of T3, T4, and TSH in female rat serums. Because the morphology of the thyroid glands was normal after deltamethrin treatment, it is unlikely that deltamethrin disturbed the biosynthesis function of TH in the thyroid glands. Given that TSH positively regulates the biosynthesis of TH, the decreases in T3 and T4 levels might be consequences of the decrease in TSH by deltamethrin. TSH is released from the anterior pituitary, which also releases LH and FSH. Because LH and FSH levels were not affected by deltamethrin, it is likely that the anterior pituitary function remained normal after deltamethrin treatment. Therefore, it is plausible that deltamethrin disturbed the regulatory mechanism for TSH synthesis or release through the anterior pituitary.

TSH is produced in pituitary thyrotrophs. It activates thyroid follicular cells and promotes thyroid cell proliferation and TH synthesis [47, 48]. TSH synthesis is largely dependent on serum TH levels. Patients with primary hyperthyroidism or primary hypothyroidism consistently demonstrate suppressed or increased serum TSH levels, respectively [49]. On the basis of the results of the present study, we infer that deltamethrin might damage TSH production or release, subsequently resulting in reductions of T3 and T4 in female rats. Furthermore, the reduction in TSH level caused by deltamethrin was sex-dependent. A possible explanation for the difference might be responsiveness to the thyrotropin-releasing hormone. The presence of a

sex difference in HPT function was suggested by McClain et al. [50]. Phenobarbital increased TSH secretion as a compensatory response to the increased T4 metabolism and excretion. Higher TSH responsiveness to TRH has been observed in male rats but not in female rats, and this difference was attributed to testosterone [51]. Therefore, a possible explanation for the difference in responses to deltamethrin between male and female rats might be that male rats are more susceptible to deltamethrin than female rats are in terms of TSH response.

In contrast to the negative regulation of TSH production by THs, TSH production is positively regulated by TRH. Mice devoid of the TRH gene were shown to exhibit hypothyroidism accompanied by low circulating TSH levels and reduced numbers of TSH immunopositive cells in their pituitary glands [52]. In TRH and TR β-subunit double knockout mice, basal serum TSH levels were shown to be low and hypothyroidism failed to increase serum TSH concentrations [53]. These studies have demonstrated the pivotal role of TRH in the regulation of TSH production. The reduction of TSH in female rats might be regulated by TRH.

TSH secretion is increased by hypothyroidism [49]. In the present study, because deltamethrin increased the TSH levels in the male rats, it is possible that deltamethrin moderately reduced TH levels, which were overcome by the normal TSH regulatory system in the male rats. However, the reduction in TH levels could not activate the positive regulatory mechanism for TSH secretion in the female rats, and TH levels remained low. These results are strongly correlated with sex-dependent delay in pubertal development. A more detailed study must be conducted to elucidate the mechanisms.

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Conflicts of interest

The authors declare no conflicts of interest.

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