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Risk assessment of a formamidine pesticide, Amitraz, focusing on thyroid hormone receptors (*TR*s) in rainbow trout, *Oncorhynchus mykiss*

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Abstract: Amitraz, a formamidine pesticide, and their metabolites have the potential to disrupt endocrine homeostasis in a variety of organisms, nevertheless there is a lack of information concerning such effects and underlying mechanisms in any fish species.To evaluate the potential impacts of Trasil (EC; active constituent 200 g amitraz/L), a commercial product of amitraz, on thyroid hormone (TH) homeostasis of rainbow trout (*Oncorhynchus mykiss*); mRNA levels of thyroid hormone receptors (*TR*s), *TRα* and *TRβ*, were determined by RT-PCR soon after sub-lethal administration in a static bio-assay system. The sub-lethal exposure of 0.84 mg/L amitraz resulted in upregulation of both *TRα*and *TRβ*genes for muscle and liver, respectively in a tissue-manner, though the differences were found statistically insignificant ($P>0.05$). The present results emerged an endocrine interaction between amitraz based formulation and TH homeostasis, but still needs further detail studies to a better understanding of TH mechanism in teleosts in response to environmental compounds.

Key words: Formamidine pesticide; Amitraz; *Oncorhynchus mykiss*; Gene expression; mRNA

Introduction

The extensive pesticide applications in agriculture and urban areas possesses the risk for aquatic environments, due to the contamination and persistency potencial of themselves or their metabolites. A great deal of these compounds revealed endocrine disrupting effects on organisms by interfering the endocrine signaling via blocking, mimicking or synergizing endogenous hormones through binding their respective receptors (genomic pathway) or by a rapid non-genomic signaling pathway (1).

In respect to the new reported data substantiated by US Environmental Protection Agency (USEPA) (2)and a few researches that compiled in a comprehensive report by Pino et al. (3), regard amitraz as a Endocrine Disrupting Compound (EDC).

Amitraz,*N'*-(2,4-dimethylphenyl)-*N*-[[(2,4-dimethylphenyl)imino]methyl]-*N*-methylmethanimidamide(Figure 1)is a formamidine insecticide and acaricide, which is commonly used in control of ectoparasites (mites, ticks and protozoans) as a veterinary medicine (4) and pest management for fruits and cotton (5).It is metabolized into 2,4-dimethyphenylformamide and *N*-2,4-dimethylphenyl-*N*-methylformamidine and they were further both degradated to 2,4-dimethylaniline (6,7) and in some occurents, their influence on organisms can be more drastic comparison to amitraz, itself (3,8,9).It is highly liposoluble, thus rapidly absorbed through the skin and mucous membranes, distributed, metabolized and eliminated by the urine in the case of exposure (3,5).

Depending on the recent commercialization and comparatively the increased production and use of amitraz, it is not puzzling that the number of studies and the incidences concerning amitraz poisoning escalated in by a majority of human (10-12) and animals (13,14). Notwithstanding, few reports of critical toxic effects of amitraz on mammalian physiology which can be elaborated as neurotoxicity (15,16) reproductive toxicity (17) and genotoxicity were presented (5). As reported by Pino *et al*. (3) and according to USEPA (2), the toxicological database is insufficient for a detailed understanding of molecular mechanisms of amitraz toxicity, particularly emphasizing on aquatic organisms and needs further exhaustive studies to cover the lack of information. To the best of our knowledge, this paper is the first report on the molecular mechanism of amitraz toxicity in a fish species.

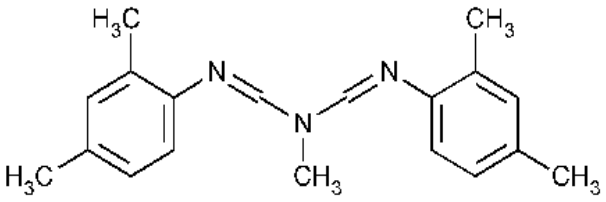

Figure 1. The chemical structure of amitraz.

The thyroid hormones (THs; T_4, T_3) regulate a wide range of cellular functions, including growth, development, differentation, metabolism and maintenance of homeostasis, in almost every tissue of teleost (18-20). Their pleiotropic effects are mediated via throid hormone receptors (TRs) (21) which are ligand-activated transcription factors termed nuclear receptors-NRs (22). NRs are phylogenetically related proteins clustered into a large superfamily, along with steroids, retinoids and

fatty acids (23). Two principal isoforms namely *TRα* and *TRβ* are encoded for TRs and they can enhance or inhibit gene expression subjecting to the nature of thyroid hormone response elements, the hormonal status and the cellular environment (24).The functioning of the thyroid system is intervened by EDCs through exposure, accumulation or food chain and lead to alterations in TSH expression (25). Therefore, the present paper aimed to unveil the endocrine disrupting potency of a commercial product of amitraz (Trasil) in a well-known teleost, *Oncorhynchus mykiss*, with respect to TH mechanism.

Materials and Methods

Pesticide

The commercial formulation tested, Trasil (EC), is manufactured by Bio Tarım & Agro Ankara, Turkey. It is an emulsifiable concentrate formulation containing 200 g amitraz/L.

Experimental setup

Rainbow trout, *Oncorhynchus mykiss*, (mixed sex) with a mean body weight of 77.27±15.24 g (Mean±S.D., *n*=252) were obtained from Cifteler Sakaryabasi Aquaculture and Research Station of Ankara University in Eskişehir, Turkey where the experiments were conducted. Prior to experiments for acclimatization, fish were kept in 200 L tank, aerated consistently, for two weeks and fed daily with commercial trout feed (45% crude protein) to satiation. Fish deprived of feed for 48 h before and during the experiment. To eliminate the fish faeces and excess feed, the water in the tanks were changed (10%) by siphoning and replaced with fresh water in the acclimatization period. All tanks were shielded with netting material to prevent fish from escape and minimize stress during the trials. The maintenance of the animals and the experiments were performed under approved protocols in accordance with the principles of Ankara University Animal Ethics Committee (Date/No: 16.02.2011/2011-105-384). In exposure system, providing triplicates per each concentration (fish per tank; preliminary, main and control: 4 / sub-lethal and control: 10), dosing solutions of amitraz were prepared from Trasil (200g amitraz/L) and the calculation of dosing solutions was done according to the active ingredient of pesticide. The dosing volumes never exceeded 0.2 ml.

Water quality parameters

The water quality was evaluated (26) and the measurements of physico-chemical parameters of water during the preliminary (range finding), main (LC_{50}) and sub-lethal toxicity tests were presented in Table 1. The values were the means of 3 measurements which were performed at the beginning, through and end of the trials.

LC_{50}-Median lethal concentration

LC_{50} tests were carried out in compliance with the standardized methods (27) at two stages in unrenewable static experimental conditions for 96 h with a total number of 192 fish. In stage I, a range finding (preliminary) test was performed to determine the concentrations of the main acute test by using wide ranges (28) as follows, 1, 2, 4, 5, 6, 8, 10 mg/L of dosing concentrations. In stage II, with regard to the preliminary test results, six concentrations (1, 2, 3, 4, 6 and 8 mg/L) that comprised 0% and 100% death were chosen as the main acute toxicity concentrations. Meanwhile, control groups (non-exposure) were maintained for both preliminary and main exposure tests in triplicates. In the course of exposure tests, dead individuals were removed immediately from the tanks and behavioral changes and mortalities were monitored closely and noted down every 24 h. The Probit regression, a dose-response, analysis was used to compute the median lethal concentration (LC_{50}) of Trasil (200 g amitraz/L), with confidence limit of 95%, for 96 h exposure (29).

Sub-lethal concentration

For the assesment of sub-lethal effects of Trasil, ¼ of 96 h LC_{50} value as computed in acute toxicity test, was considered and exposed as a sub-lethal concentration (the concentration which has no death to the experimental fish, however effects on its biochemical and physiological processes). The sub-lethal exposure tests were conducted in triplicates for both treatment and control groups with total number of 60 fish in static system likewise in LC_{50} tests.

Tissue dissection and RNA extraction

At the end of the sub-lethal exposure of amitraz, the tissue samples (liver and white muscle) of rainbow trout were collected from all individiuals of both exposure and control groups. The dissection of liver and muscle

Table 1. Somewater quality parameters throughout the toxicity tests (Mean values±S.D.) (*n*=3).

Parameters	P[1]	M[2]	S[3]
Dissolved oxygen (mg/L)	6.47±0.02	6.36±0.00	7.15±0.20
pH	7.24±0.04	7.00±0.11	7.01±0.01
Temperature (°C)	20.06±0.06	19,95±0.49	20.23±0.15
Electrical conductivity (µS/cm)	416.00±0.42	362.20±7.40	311.35±29.50
NH_3-N (mg/L)	1.00±0.14	0.76±0.29	0.38±0.08
NO_2-N (mg/L)	0.01±0.00	0.01±0.00	0.01±0.00
NO_3-N (mg/L)	1.39±0.08	1.68±0.44	1.38±0.13
Alkalinity (mg/L $CaCO_3$)	40.00±0.00	40.00±0.00	40.00±0.00
Hardness (mg/L $CaCO_3$)	39.50±1.70	46.50±3.30	51.90±6.80

[1](P) Preliminary; [2](M) Main-LC_{50}; [3](S) Sub-lethal.

tissues was performed on crushed ice and the samples were flash-frozen in liquid nitrogen and preserved at -80°C for further procedures. Total RNA was isolated from the frozen tissues using High Pure RNA Tissue Kit (Roche Diagnostics) according to the manufacturer's instructions. The purity of isolated RNA was quantified by spectrophotometric absorbance (A_{260}/A_{280} ratio) using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Schwerte, Germany) and the integrity of RNA samples was verified by agarose gel electrophoresis on agarose gel that comprised ethidium bromide (EB).

Real time PCR(RT-PCR) procedure

Real time PCR analyses were performedto examine the tissue-specific and gene-specific expression of *TR*s in rainbow trout associated with the sub-lethal exposure of amitraz based pesticide.

The complementary DNAs (cDNA) used for expression analyses were synthesized by reverse transcribing from total RNA using the transcriptor first strand cDNA Synthesis Kit (Roche Diagnostics) following the protocol of the manufacturer. Random hexamer primer was chosen in reverse transcribing of cDNAs due to the use of *18S* rRNA as a housekeeping gene. The cDNAs were stored at -20°C until used for real-time PCR (RT-PCR). Gene-specific primer pairs and probes were provided from Universal ProbeLibrary (UPL) system (Roche Diagnostics) which constituted a web-based software (ProbeFinder software). UPL probes are hydrolysis probes substituted with Locked Nucleic Acids (LNA) and they are labeled at the 5′ end with FAM (Fluorescein amidite) and at the 3′ end with a dark quencher dye. The sequences of primer pairs and probes were presented in Table 2.

The amplification was performed with a LightCycler 480 RT-PCR system (Roche, Switzerland) using LightCycler 480 Probes Master Kit (Roche Diagnostics). The thermal cycling conditions consisted of an initial pre-incubation for 10 min at 95°C, followed by 45 cycles of denaturation of the target DNA at 95°C for amplification of the target DNA with for 10 s, primer annealing at 60°C for 30 s, extension at 72°C for 1 s and 1 cycle of cooling for 30 s at 40°C. A standart curve was constituted for target and housekeeping gene seperately and efficiency of each reaction (1.8-2.0) was determined. PCR samples were run in duplicates for standarts, samples and negative controls. Negative control samples (nuclease-free water instead of cDNA) were used in each run to test the target specifity of the cDNA amplification.

Data normalization and statistics

The housekeeping gene *18S* was used as an endogenous standart and expressions of target genes of *TRα* and *TRβ* were normalized to the corresponding level of *18S* mRNA relatively to the untreated control by using comparative Ct ($2^{-\Delta\Delta CT}$) method (30).

The all data were expressed here as means±standart error of the mean (SEM) unless indicated otherwise. A total of ten fish were used from exposure and control groups and two technical replicates per fish and treatment were made. Paired Samples T-test, a confidence level of 95%, was applied using statistical software IBM SPSS Statistics 23.0 to determine the presence of significant differences among the groups.

Results and discussion

Endocrine disrupting chemicals (EDCs) are endocrine active compounds causing specific effects on endocrine systems at several levels without relevant toxic actions (25). The mode of actions (MOAs) of EDCs comprise mimicking and antagonizing the effects of endogenous hormones, altering the pattern of synthesis and metabolism of normal hormones but also modifying hormone receptor levels (31,32).Since, it has been declared as an endocrine disrupter and due to the wide commercialization, amitraz and its metabolites take great attention and the specification of risk assessment, particulary endpoints and molecular mechanisms regarding to toxicity is a dictate of environmental sustainability.

Here, in the present study, the 96-h acute LC_{50} value of commercial formulation of amitraz, Trasil (200 g amitraz/L), in a static bio-assay system for rainbow trout was found 3.361 mg/L (χ^2=0.796) with 95% confidence limits of 2.952-3.850 using Probit regression analysis (Table 3). The sub-lethal concentration was 0.84 mg/L and determined as ¼ of 96-h LC_{50} value. With corroborating our data, the previous LC_{50} studies in rainbow trout, bluegill sunfish and harlequin fish as reported by EXTOXNET(33) for 48 and 96-h exposure resulted with the values of ranging between 2.7-4.0 mg/L, 1.3 mg/L and 3.2-4.2 mg/L, respectively and the report also characterised the toxicity as moderate for fish species. Moreover, Quantitative Structure Activity

Table 2. Primer and probe sequences of reference (housekeeping) and target genes of *18S*, *TRα*, *TRβ* respectively, in rainbow trout, *Oncorhynchus mykiss*.

Gene	Primer/Probe	Sequence	Gene Bank Accession No.
18S	Forward primer	ATGGTCTAACATCTTATAAGCGGCTT	AF308735.1
	Reverse primer	GCGCAGAGAAGTTGACTGG	
	UPL*(#118, cat.no. 04693523001)	CACTGGGA	
TRα	Forward primer	GAGAAGAGGAAGAAGGAGGAGAT	AF302245.1
	Reverse primer	GAACTTGCGTTTCTGTTTCCA	
	UPL (#24, cat.no. 04686985001)	CAGCTCCC	
TRβ	Forward primer	GAGGCCCACATGTCAACTAAC	AF302246.1
	Reverse primer	TGGTTTCCTTCACCCCCACT	
	UPL (#37, cat.no. 04687957001)	TGCCCTGG	

*UPL; Universal ProbeLibrary Probe.

Table 3. The 96-h acute LC_{50} value of commercial formulation of amitraz in rainbow trout*.

Probability	95% Confidence Limits		
	Estimate (mg/L)	Lower Bound	Upper Bound
LC_{10}	2.832	1.894	3.148
LC_{20}	3.004	2.248	3.310
LC_{30}	3.134	2.523	3.461
LC_{40}	3.249	2.755	3.633
LC_{50}*	3.361	2.952	3.850
LC_{60}	3.477	3.121	4.138
LC_{70}	3.605	3.268	4.529
LC_{80}	3.762	3.412	5.089
LC_{90}	3.989	3.584	6.047

Relationships-QSAR modelling, an alternative method for the prediction of LC_{50} values, have been premediated this characterization too (34).

The mechanism of amitraz toxicity have been studied for mammals in detail comparison to other vertebrates and the existing results were compiled by Pino *et al*. (3) comprehensively, nevertheless, the researchs concerning fish is scarcely any. The endocrine disrupting potency of amitraz and its metabolites have been evaluated for rats and they were concluded that the detrimental effects on hormones mainly depends on the activation of α_2-adrenergic receptor (35). It was stated that amitraz (1.85 mg/kg) escalated plasma glucose and concussed the insulin release in dogs (36), as in rats that exposured to its metabolite, 2,4-dimethyphenylformamide (37) through the activation of α_2 receptors.Chou *et al*. (17) reported that amitraz induces hepatic estradiol (E2) and testosterone metabolism in rats for both gender, controversially Ueng *et al*. (18) who notified that amitraz is a week antiestrogen. As mentioned above, depending on the lack of researches, further studies are needed to understand the dose-response relationship between amitraz treatment and endocrine disruption centering upon non-target organisms as fish.

THs presented their biological activity by binding to *TR*s (genomic pathway) or through a rapid non-genomic signaling pathway (39). *TR*s have a core role in the genomic regulation of THs (40), particularly they appear in the early development and metamorphosis in fish (20,39) and serve different functions and demonstrate tissue-specific and developmental state-specific expression (41,42), nonetheless the action mechanism for fish is not fully understood. In the present study, TRα was upregulated in white muscle tissue in comparison to liver with respect to toxic treatment (Table 4).

Unlikely from *TRα* expression pattern, the relative expression levels of liver for *TRβ* gene slightly upregulated, despite the differences were found nominal according to Paired Samples T-test (P>0.05).Similar observations of upregulated TR subtypes, mainly*TRα* soon after administration of two synthetic pyrethroidswere reported for zebrafish (42). In the case of exposure with pyrethroids, the disruption of motor activity associated with the alterations of TH levels can be responsible for upregulated expression levels (1). Considering the results for this study, it can be interpreted that, *TR*s elicited their activity both tissue- and gene-specific manner. Though the mechanism is not explicit, such expression levels may be related with *TR*s subtypes (*TRα* and *TRβ*) as mentioned by Filby *et al*. (20) and can be also elucidated by distinct tissue specifity of α and β subtypes in fish body (43). Moreover, it has been represented that triiodothyronine (T_3) can elevate *TR* expression levels and exposure to chemicals induced *TR* transcription related with increased levels of T_3as a feedback mechanism (42, 44) to respond regarding to the disturbance of hypothalamus-pituarity-thyroid (HPT) axis homeostasis(45).

Table 4. mRNA expression levels of *TRα* and *TRβ* genes in white muscle and liver tissue of rainbow trout that are expressed relative to control values and normalized to *18S* with their respective standard errors in response to 0.84 mg/L of Trasil (200 g amitraz/L).

Gene	Fish Tissue	
	White muscle	Liver
TRα	1.109±0.51$^{N.S.*}$	0.819±0.26
TRβ	0.916±0.45	1.404±0.42

*N.S. (Non-significant); Means (Means±S.E.M) (n=5) in the same row and column were found in-significant according to Paired Samples T-test (P>0.05).

In conclusion, the results of this study suggest that sub-lethal exposure of a commercial product of amitraz, entailed alterations in expression of *TR*s subtypes subjecting to tissue- and gene-manner, emanating wider potential impacts on the physiological function of fish.

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Interest conflict
The authors declared that there is no conflict of interest regarding to the publication of this manuscript.

Author's contribution
İ. Meriç Turgut conceived and designed the research, also performed the whole process of the manuscript. E.Keskin contributed the acute and sub-lethal toxicity part of the research.

References

1. Brander SM, Gabler MK, Fowler NL, Connon RE, Schlenk D. Py-

rethroid pesticides as endocrine disruptors: Molecular mechanisms in vertebrates with focus on fishes. Environ Sci Technol2016; 50: 8977-8992.
2. USEPA (United States Environmental Protection Agency). Amitraz proposed tolerance actions. US Environmental Protection Agency, Office of Prevention, Pesticide and Toxic Substances, Washington DC, 2013.
3. Del Pino J, Moyano-Cires PV, Anadon MJ, Díaz MJ, Lobo M, Capo MA, Frejo MT. Molecular Mechanisms of amitraz mammalian toxicity: a comprehensive review of existing data. Chem Res Toxicol 2015; 28: 1073-1094.
4. Balta F, Kyis S, Altinok I. External protozoan parasites in three trout species in the Eastern Black Sea region of the Turkey: intensity, seasonality, and their treatments. Bull Eur Ass Fish Pathol 2008; 28: 157-162.
5. Ponzinibbio MV, Padula G, Picco S, Seoane A. Assessment of the adverse effects of the acaricide Amitraz: in vitro evaluation of genotoxicity. Toxicol Mech Method 2012; 22: 657-661.
6. Pierpoint AC, Hapeman CJ, Torrents A. Kinetics and mechanism of amitraz hydrolysis. J Agric Food Chem 1997; 45: 1937-1939.
7. Zaranyika MF, Mandizha NT. Adsorption of amitraz by a river sediment: apparent thermodynamic properties. J Environ Sci Heal B 1998; 33: 235-251.
8. Osano O, Oladimeji AA, Kraak MH, Admiraal W. Teratogenic effects of amitraz, 2,4-dimethylaniline, and paraquat on developing frog (Xenopus) embryos. Arch Environ Contam Toxicol 2002; 43: 42-49.
9. Le T, Li B, He H, Chen Y, Niu X. Development of a GC-MS method for the simultaneous determination of amitraz in fish muscle. International Conference on Biomedical Engineering and Biotechnology, IEEE 2012; 144-148.
10. Yaramis A, Soker M, Bilici M. Amitraz poisoning in children, Hum Exp Toxicol 2000; 19: 431-433.
11. Yilmaz HL, Yildizdas DR. Amitraz poisoning, an emerging problem: epidemiology, clinical features, management, and preventive strategies. Arch Dis Child 2003; 88: 130-134.
12. Veale DJ, Wium CA, Muller GJ. Amitraz poisoning in South Africa: a two year survey (2008-2009). Clin Toxicol 2011; 49: 40-44.
13. Andrade SF, Sanchez O, Tostes RA. Effects of experimental amitraz intoxication in cats. Arq Bras Med Vet Zootec 2004; 53: 38-42.
14. Marafon CM, Delfim CI, Valada CA, Menotti R, Andrade SF. Analysis of amitraz in cats by gas chromatography. J Vet Pharmacol Therap 2009; 33: 411-414.
15. Del Pino J, Martínez MA, Castellano VJ, Ramos E, Martínez-Larrañaga MR, Anadón A. Effects of prenatal and postnatal exposure to amitraz on norepinephrine, serotonin and dopamine levels in brain regions of male and female rats. Toxicology 2011; 287: 145-152.
16. Del Pino J, Martínez MA, Castellano VJ, Ramos E, Martínez-Larrañaga MR, Anadón A. Effects of exposure to amitraz on noradrenaline, serotonin and dopamine levels in brain regions of 30 and 60 days old male rats. Toxicology 2013; 308: 88-95.
17. Chou CP, Lu SY, Ueng TH. Modulation odf serum concentrations and hepatic metabolism of 17beta-estradiol and testosterone by amitraz in rats. Arch Toxicol 2008; 82: 729-737.
18. Brent GA. Thyroid hormones (T4, T3). In: Endocrinology: Basic and Clinical Principles. Melmed, S. (ed.), Totowa: Humana Press, 1996.
19. Marchand O, Safi R, Escriva H, Rompaey EV, Prunet P, Laudet V. Molecular cloning and characterization of thyroid hormone receptors in teleost fish. J Mol Endocrinol 2001; 26: 51-65.
20. Filby AL, Tyler CR. Cloning and characterization of cDNAs for hormones and/or receptors of growth hormone, insulin-like growth factor-I, thyroid hormone, and corticosteroid and the gender-, tissue-, and developmental-specific expression of their mRNA transcripts in fathead minnow (Pimephales promelas). Gen Comp Endocrinol 2007; 150: 151-163.
21. Kawakami Y, Adachi S, Yamauchi K, Ohta H. Thyroid hormone receptor β is widely expressed in the brain and pituitary of the Japanese eel, Anguilla japonica. Gen Comp Endocrinol 2007; 150: 386-394.
22. Jones I, Rogers SA, Kille P, Sweeney GE. Molecular cloning and expression of thyroid hormone receptor alpha during salmonid development. Gen Comp Endocrinol 2002; 125: 226-235.
23. Kawakami Y, Shin DH, Kitano T, Adachi S, Yamauchi K, Ohta H. Transactivation activity of thyroid hormone receptors in fish (Conger myriaster) in response to thyroid hormones. Comp Biochem Physiol 2006; 144: 503-509.
24. Wu Y, Koenig RJ. Gene regulation by thyroid hormone. Trends Endocrinol Metab 2000; 11: 207-211.
25. Kloas W, LutzI. Amphibians as model to study endocrine disrupters. J Chrom A 2006; 1130: 16-27.
26. APHA. Standart Methods for the Examination of Water and Wastewater. 19th. Edition, Washington, USA, 1995).
27. OECD. Guideline for the Testing of Chemicals: Fish, Acute Toxicity Test, No: 203, 1992.
28. Schäperclaus W. Fischkankheiten, Akademie Verlag, Berlin, 1979.
29. Unsal M. Kirlilik Deneyleri-Yöntemler ve Sonuçların Değerlendirilmesi, ODTÜ Deniz Bilimleri Enstitüsü, Mersin, Turkey, 1996.
30. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^(-ΔΔC_T)method. Methods 2001; 25: 402-408.
31. Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FT. The E-screen assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ Heal Perspec 1995; 103(Suppl. 7): 113-122.
32. Pait AS, Nelson JO. Endocrine Disruption in Fish: An Assessment of Recent Research and Results. NOAA Technical Memorandum NOS NCCOS CCMA 149, Silver Spring, MD: NOAA, NOS, Center for Coastal Monitoring and Assessment http://aquaticcommons.org/2177/1/NOS_TM149.pdf, 2002; 55pp.
33. EXTOXNET. Amitraz. The Extension Toxicology Network, http://extoxnet.orst.edu/, 1996.
34. Agrawal SP, Saxena VL. Prediction of LD50 of some common pesticidesthrough QSAR (an alternative method to save experimental animals). Int J Pharma Sci Res 2014; 5: 5356-5373.
35. Goldman JM, Cooper RL. Assessment of toxicant-induced alterations in the luteinizing hormone control of ovulation in the rat. In: female reproductive toxicology. Heindel J, Chapin R. (eds.), Academic Press, San Diego, 1993, pp. 79-91.
36. Hsu WH, Schaffer DD. Effects of topical application of amitraz on plasma glucose and insulin concentrations in dogs. Am J Vet Res 1988; 49: 130-131.
37. Chen TH, Hsu WH. Inhibiton of insulin release by a formamidine pesticde amitraz and its metabolites in a rat beta-cell line: an action mediated by alpha-2 afrenoceptors, a GTP-binding protein and a decrease in cyclic AMP. J Pharmacol Exp Ther 1994; 271: 1240-1245.
38.Ueng TH, Hung CC, Wang HW, Chan PK. Effects of amitraz on cytochrome P450-dependent mono-oxygenases and estrogenic activity in MCF-7 human breast cancer cells and immature female rats. Food Chem Toxicol 2004; 42:1785–1794.
39. Manchado M, Infante C, Rebordinos L, Cañavate JP. Molecular characterization, gene expression and transcriptional regulation of thyroid hormone receptors in Senegalese sole.Gen Comp Endocrinol

2009;160: 139-147.
40. Power DM, Llewellyn L, Faustino M, Nowell MA, Bjornsson BT, Einarsdottir IE, Canario AV, Sweeney GE. Thyroid hormones in growth and development of fish. Comp Biochem Physiol Part C,2001;130 (4): 447-459.
41. Chen H, Xiao J, Hu G, Zhou J, Xiao H, Wang X. Estrogenicity of organophosphourous and pyrethroid pesticides. J Toxicol EnvironHeal Part A 2002;65: 1419-1435.
42. Tu W, Xu C, Lu B, Lin C, Wu Y, Liu W. Acute exposure to synthetic pyrethroids causes bioconcentration and disruption of the hypothalamus-pituitary-thyroid axis in zebrafish embryos.Sci Tot Environ 2016;542: 876-885.
43. Yamano K, Miwa S. Differential Gene Expression of thyroid hormone receptor α and β in fish development. Gen Comp Endocrinol 1998; 109: 75-85.
44. Crump D, Jagla MM, Chiu S, Kennedy SW. Detection of PBDE effects on mRNA expression in chicken (Gallus domesticus) neuronal cells using real-time RT-PCR and a new differential display method. Toxicol in Vitro 2008; 22: 1337-1343.
45. Yu L, Chen M, Liu Y, Gui W, Zhu G. Thyroid endocrine disruption in zebrafish larvae following exposure tohexaconazole and tebuconazole. Aquat Toxicol 2013; 138-139: 35-42.