Triphenyltin alters lipid homeostasis in females of the ramshorn snail *Marisa cornuarietis*

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Abstract

Molluscs are sensitive species to the toxic effects of organotin compounds, particularly to masculinization, and both, tributyltin (TBT) and triphenyltin (TPT) have been recently shown to bind to molluscs RXR. Being RXR is involved in lipid homeostasis, exposure to TPT would have an immediate effect on lipid homeostasis. To test this hypothesis, the ramshorn snail *Marisa cornuarietis* was exposed to environmentally relevant concentrations of TPT (30, 125, 500 ng/L as Sn) in a semi-static water regime for 7-days. Percentage of lipids and total fatty acid content decreased significantly in TPT-exposed females while the activity of peroxisomal acyl-CoA oxidase, involved in fatty acid catabolism, increased. In addition, fatty acid profiles (carbon chain length and unsaturation degree) were significantly altered in exposed females but not in males. This work highlights the ability of TPT to disrupt lipid metabolism in *M. cornuarietis* at environmentally realistic concentrations and the higher susceptibility of females in comparison to males.

Keywords: Fatty acids; lipids; molluscs; RXR; triphenyltin

Capsule: Short-term exposure to the fungicide TPT disrupts lipid metabolism in *M. cornuarietis* at environmentally realistic concentrations

1. Introduction

Since the late 1960s, organotin compounds, such as tributyltin (TBT) and triphenyltin (TPT), have been extensively used across the world as biocides in antifouling paints, applied on ship hulls and fishing nets, and as fungicides in agricultural corps. Despite their gradual removal from the market and their prohibition from use, their release into the environment combined with their low solubility in water and high octanol-water partition coefficient has resulted in worldwide contamination of the aquatic environment (Fent, 1996). Both TBT and TPT are potent endocrine disruptors; abnormalities in the endocrine system related to TBT and TPT exposure have been observed in vertebrates (Iguchi et al., 2007; Kanayama et al., 2005; McAllister and Kime, 2003) and invertebrates (Alzieu, 2000; Oehlmann et al., 2007), with gastropods and oysters being among the most susceptible organisms. A concentration of 1 ng/L TBT is enough for the induction of imposex (superimposition of male secondary sexual characteristics, including a penis and vas deferens) in females of the gastropod *Nucella lapillus* (Bryan et al., 1986; Spooner, 1991).

Imposex has been reported in over 150 species of gastropods worldwide (Horiguchi et al., 1997), and although the link between imposex in female gastropods and exposure to TBT or TPT has been established, the exact mechanism through which this phenomenon occurs remains unclear. Scientific data demonstrate that imposex induced females experience elevated levels of free testosterone and this has been attributed to non-genomic action of TBT and TPT such as inhibition of aromatase activity (Bettin et al., 1996), inhibition of the esterification of testosterone (LeBlanc et al., 2005) or alterations in the excretion of neurohormones that contribute to sexual differentiation in gastropods (Oberdörster and McClellan-Green, 2002).

However, recent scientific evidence suggests that TBT and TPT may act through interaction with nuclear receptors (Nakanishi, 2007). Different ligand binding assays show that both TBT and TPT bind to the human Retinoid X receptor (hRXR) with high affinity, similar to that of 9-*cis* retinoic acid (9-*cis* RA), the proposed natural ligand of RXR (Nishikawa et al., 2004). RXR homologues have been cloned from the gastropods *Thais clavigera* (Nishikawa et al., 2004) and *Nucella lapillus* (Castro et al., 2007), as well as from the freshwater snail *Biomphalaria glabrata* (Bouton et al., 2005); all of them showing high similarity with vertebrate RXR. In all three species, 9-*cis* RA was a high affinity ligand, suggesting that retinoid signalling pathways may exist in these species. Moreover, injections of *T. clavigera* and *N. lapillus* with 1 μg/g 9-*cis* RA resulted in induction of imposex, including an increase in penis length and vas deferens similar to the one produced by TBT and/or TPT in these species (Castro et al., 2007; Nishikawa et al., 2004). Therefore, imposex induction may be mediated through modulation of RXR signalling pathways. However, a retinoid synthesis pathway has not been described yet in invertebrates and the role of invertebrate RXR remains unclear. Thus, the significance of the activation of RXR by TBT and TPT can only be speculated.

In mammals, RXR forms heterodimers with orphan nuclear receptors (whose endogenous ligand is unknown: peroxisome proliferator-activated receptor, liver X receptor, farnesoid X receptor, and pregnane X receptor) as well as with retinoic acid receptor, thyroid hormone receptor and vitamin D receptor (Szanto et al., 2004). These orphan receptors are lipid sensors as they get activated by lipid molecules and therefore play an important role in lipid homeostasis, whereas the later regulate the endocrine system and resemble more closely the action of steroid hormone receptors (Chawla et al., 2001). The RXR heterodimer is activated by ligands of either receptors and subsequently binds to the corresponding response elements in the promoter region

of the target genes to modulate their transcription (Michalik et al., 2006). Knocking out RXR in mice disturbed lipid metabolism functions controlled by PPARα, PPARγ, LXRα, PXR and FXR (Szanto et al., 2004) showing the importance of this receptor in lipid homeostasis. Interestingly, TBT and TPT activate both RXR and PPARγ human receptors (Kanayama et al., 2005) and exposure of mice and the amphibian *Xenopus laevis* to TBT and RXR/PPARγ ligands stimulated lipid accumulation and ectopic adipocyte formation, respectively (Grün et al., 2006).

Additionally, activation of RXR and/or PPARs has been linked to alterations in the steroidogenic pathway: modulation of STAR protein expression (Seto-Young et al., 2007) and P450aromatase activity (Mu et al., 2000; Saitoh et al., 2001) in human granulosa cells. Thus, alterations in steroid hormone levels observed after exposure to TBT and TPT may also be a consequence of the interaction of the compounds with RXR rather than a direct interaction at the enzyme level (Nishikawa, 2006). Although receptors such as PPARs appear to have emerged later in the evolution of the nuclear receptor family (Thornton, 2003) and up to date they have not been identified in invertebrates, a lipid regulation mechanism possibly mediated by RXR in gastropods cannot be excluded. Indeed, females of the freshwater snail *Marisa cornuarietis* exposed to different concentrations of TBT for 100 days showed increased percentage of lipids and total fatty acid content as well as significant alterations in the fatty acid profile (Janer et al., 2007).

Following the above observations, this study hypothesizes that being RXR involved in lipid homeostasis in gastropods as it is in mammals and vertebrates, exposure to TPT -a RXR agonist- will have an immediate effect on lipid homeostasis. More specifically, this study aimed at investigating changes on lipid content and fatty acid profiles in the ramshorn snail *Marisa cornuarietis* after short term exposure to environmentally realistic concentrations of TPT.

Additionally, the effect of TPT on the activity of acyl-CoA oxidase (AOX), the first and rate limiting enzyme of β-oxidation was examined. Peroxisomal AOX catalyzes the β-oxidation of very long (C>20) and long chain (C14-C18) fatty acids and its gene transcription is regulated by PPARα in mammals and vertebrates (Reddy and Hashimoto, 2001).

2. Materials and Methods

2.1. Chemicals

Triphenyltin chloride (TPT) was purchased from Merck (Darmstadt, Germany). Palmitoyl-CoA and NADPH were obtained from Sigma (Steinheim, Germany); 2,7-dichlorodihydrofluorescein (H_2DCF) diacetate was from Molecular Probes (Paisley, UK). All solvents and reagents were of analytical grade.

2.2. Animals

Ramshorn snails, *Marisa cornuarietis* (Mollusca: Prosobranchia: Ampullariidae), came from a laboratory breeding stock which was derived from a stock at Aquazoo Düsseldorf (Germany) in 1991 with regular cross-breeding of wild-caught animals from Florida (USA) to avoid inbreeding. The breeding stock was kept in a flow-through system with fully reconstituted water under constant conditions regarding temperature and light dark cycle (12:12 h). Water parameters (pH, temperature, conductivity, nitrite, oxygen concentration and saturation) were

measured twice a week per tank. Parameters of the fully reconstituted influx water were pH 7.5, 850 μS/cm, <1 mg NO_2/L and >95% O_2 saturation.

2.3. Exposure experiment

For the exposure experiments, two replicate groups of 10 sexually mature snails each were exposed to three nominal concentrations of TPT (30, 125, and 500 ng as Sn/L) for 7-days (June 2005) in fully reconstituted water at 24±1 ºC. TPT was added in absolute ethanol, the concentration of ethanol in water being 0.001% in all experimental groups, including solvent control (SC). Test concentrations were selected based on results from earlier studies in *M. cornuarietis* (Janer et al., 2006) and on reported values of TPT in the aquatic environment. The exposure experiment was performed in 60 L glass aquaria fitted with an Eheim filter system and additional aeration under 12-h light/12-h dark cycles. The exposure system was designed as semi-static renewal with addition of the test substance every 24 hours (weekend 48 hours) and 50% exchange of the water twice a week. Water parameters (pH, conductivity, temperature, nitrite, O2 concentration and saturation) were measured twice a week before the water was changed. Animals were fed daily with TetraMin® (Tetra, Melle, Germany) ad libitum. Exposed organisms were cooled in ice and the digestive gland/gonad complex was dissected, deep-frozen in liquid nitrogen, and stored at −80 ºC for determination of steroid levels and enzymatic activities.

2.4. Peroxisomal fatty acyl-CoA oxidase activity

Acyl-CoA (palmitoyl-CoA) oxidase was assayed by the determination of H_2O_2 production, coupled to the oxidation of leuco-DCF in a reaction catalysed by exogenous peroxidase. The method was modified after Small et al. (1985). Digestive gland/gonad complex (0.3-0.7 g) were homogenised in TVBE buffer pH 7.6 (4 ml buffer/g of tissue), containing 1 mM sodium bicarbonate, 0.1 M EDTA, 0.1% ethanol and 0.01% Triton X-100. After homogenisation, samples were centrifuged at 500 x *g* for 15 min and the supernatant containing the peroxisomes was assayed for acyl-CoA oxidase activity. The reaction was carried out at 25^{o}C in a final volume of 1 ml. The reaction mixture contained 0.05 mM leuco DCF (prepared weekly by hydrolysing 2.66 mM H_2DCF diacetate in 1:9 v/v, dimethylformamide: NaOH (0.01 M) and stored at -20^{o}C), 0.07 mg horseradish peroxidase, 40 mM sodium azide, 0.01% Triton X-100, 10 mM potassium phosphate buffer pH 7.4 and sample. This mixture was pre-incubated in the dark for 3 min, as some impurities in the peroxidase cause a small amount of oxidation of the leuco-DCF (Köchli and von Wartburg, 1978). After this time, the slow rate of auto-oxidation of the dye was determined by measuring spectrophotometrically the absorption at λ = 502 nm for 2 min. The reaction was then started by the addition of 30 μM of palmitoyl-CoA, and after 15 sec incubation in the dark, the enzymatic reaction rate was determined for 2 min. Rates were corrected by subtracting the blank and calculated by using a DCF molar extinction coefficient of 91,000 M^{-1} as obtained by Köchli and von Wartburg (1978) from the peroxidase-catalysed oxidation of leuco-DCF. Protein concentrations were determined by the method of Lowry et al. (1951) by using bovine serum albumin as a standard.

2.5. Fatty acid analysis

The digestive gland/gonad complex of *M. cornuarietis* individuals (0.4-0.7 g) were lyophilised and processed for lipid and fatty acid analysis. Lipids were extracted from the lyophilised samples by homogenisation in 2 ml ice-cold chloroform/methanol (2:1 v/v) plus 0.01% (w/v) butylated hydroxytoluene (BHT) as an antioxidant, following a modification of the method of Folch et al. (1957). After homogenisation, 0.25 ml of 0.88% KCl was added to the homogenates and the solution was mixed. After phase separation, the chloroform layer containing the lipids, was removed, filtered and the solvent evaporated by flushing with nitrogen. The solid residue was then weighted to determine the total lipid levels, and afterwards redissolved in chloroform/methanol (2:1, v/v) with 0.01 % BHT, flushed with nitrogen and stored at -20^{o}C in a screw cap vial. Lipid aliquots were transmethylated overnight (Christie, 1982) after addition of a known amount of nonadecanoic acid (19:0) as internal standard (Sigma). Fatty acid methyl esters (FAME) were extracted with hexane/diethyl ether (1:1, v/v), and purified by thin layer chromatography (silica gel G60, Merck) using hexane/diethyl ether/acetic acid (85:15:1.5, v/v/v) as solvent system. FAME were analysed with a Fissons 8000 gas chromatograph equipped with a fused silica 30 x 0.25mm open tubular column (Tracer, TR-WAX, film thickness: 0.25 μm), and a cold on-column injection system, using helium as carrier, and a 50-220^{o}C thermal gradient. Peaks were recorder and integrated in a personal computer using Azur software (Datalys, France), and identified by comparison with a well characterised sardine oil named Marinol (Fishing Industry Research Institute, Rosebank South Africa).

2.6. Statistical analysis

Results are mean values ± SEM. Differences between control groups (absolute control and solvent control) was assessed with Student's t-test and they were not statistically significant (p>0.05). Thereafter, exposure groups were compared with control groups by using one way ANOVA (Dunnett's test).

3. Results

3.1. Peroxisomal acyl-CoA oxidase activity

AOX activity was determined in peroxisomal enriched fractions obtained from the digestive gland/gonad complex of *M. cornuarietis.* After one week exposure to TPT, the activity AOX was significantly increased in females exposed 30 and 500 ng TPT/L, resulting in 1.3- and 1.4-fold increase, respectively (Figure 1). AOX activity was also significantly increased (1.4-fold) in males exposed to 30 ng TPT/L, but no further differences were observed at higher TPT concentrations (Figure 1).

3.2. Fatty acid profile and lipid content

A detailed description of the fatty acid composition of control and exposed males and females of *M. cornuarietis* is given in Table 1. At least 33 fatty acids with carbon atoms from 14 to 24 were detected in the digestive gland/gonad complex of both males and females.

Unsaturated fatty acids were the major group, accounting for 54% and 61% of total fatty acids in control males and females, respectively. Within this group, monounsaturated fatty acids constituted 28-30% of the total fatty acids and polyunsaturated 26 to 31%. The most abundant unsaturated fatty acids were linoleic (18:2n-6) and oleic (18:1n-9) acids. Highly unsaturated fatty acids (3 or more saturations) of 20 or more atoms of carbon (HUFA) represented only about 10% of total fatty acids. The major forms were in decreasing order, arachidonic (20:4n-6), docosahexaenoic (DHA; 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3). Saturated fatty acids represented 33% of the total fatty acids in males and 30% in females, and among them palmitic (16:0) and stearic (18:0) were the most abundant.

One week exposure to TPT caused a shift in the fatty acid profile in the digestive gland/gonad complex of *M. cornuarietis* with alterations being more evident in females (Table 1). Thus, a ~10% decrease of MUFA (% FAME) and a ~20% increase of HUFA was observed in females exposed to 125 and 500 ng/L TPT; the increase in HUFA was associated to a relative increase of the n-6 HUFA group. To further understand the effect of TPT exposure on individual fatty acids, those were expressed as mg/g dry tissue. Interestingly, one week exposure to TPT resulted in a decrease in fatty acids, both in terms of chain length and saturation degree, and this decrease was mainly detected in females (Tables 2 & 3). Almost all exposed females revealed a decrease in fatty acid content in terms of chain length (Table 2). C18 fatty acids, that accounted for 50% of total fatty acids (control groups), showed a 40% decrease in females exposed to 30 and 500 ng/L TPT whereas no significant alteration was observed in exposed males. In terms of unsaturation degree, saturated (ΣC:0), mono-unsaturated (ΣC:1) and di-unsaturated (ΣC:2) fatty acids, which account for 32, 34 and 23% of total fatty acids in control groups, decreased in TPT-exposed females (30-40%) (Table 3). Only the tetra-unsaturated fatty acids (ΣC:4) were not

significantly altered by TPT-exposure in females; within this group, arachidonic acid (20:4n-6) was the most abundant (97%).

Overall, one week exposure to 30, 125 and 500 ng TPT/L resulted in a drop in the total fatty acid levels (FAME) in females equal to 33, 20 and 35% (Figure 2). TPT caused a significant decrease (20%) in total fatty acid levels in males as well but only at the highest TPT concentration (Figure 2). Furthermore, TPT exposure resulted in a significant decrease (20%) in the percentage of lipids in the digestive gland/gonad complex of females at the highest TPT concentration (500 ng/L) but had no significant effect in exposed males (Figure 2).

4. Discussion

One week exposure to TPT had a significant effect on the percentage of lipids, fatty acid content and fatty acid metabolism in the digestive gland/gonad complex of females of *M. cornuarietis*, whereas males demonstrated very few significant alterations. Percentage of lipids, total fatty acid content as well as carbon chain length and unsaturation degree, all decreased significantly in TPT-exposed females. In parallel, the activity of peroxisomal AOX, the enzyme responsible for the break down of C14-C18 and C>20 fatty acids was significantly induced, which supports the observed decrease in fatty acid content.

In vertebrates, the peroxisomal AOX gene is transcriptionally activated by PPARα (Reddy and Hashimoto, 2001). Activation of the enzymes involved in the peroxisomal β-oxidation pathway, including AOX, with a parallel increase in volume and density of peroxisomes is a phenomenon known as peroxisome proliferation that has been related to hepatocarcinogenesis in rats and mice (Yu et al., 2003). Although PPARs have not been

identified in invertebrates (Thornton, 2003), existing data demonstrate that peroxisome proliferation in response to organic contaminant exposure occurs. Thus, induction of AOX activity with a parallel increase in peroxisomal volume density have been observed in mussels *Mytilus edulis* exposed to specific peroxisome proliferators (fibrates and phthalates) and various organic pollutants (PAHs and PCBs) (Cajaraville and Ortiz-Zarragoitia, 2006; Ortiz-Zarragoitia and Cajaraville, 2006), in slugs *Arion ater* exposed to a Cd-kerosene mixture (Zaldibar et al., 2007) and in the land snail *Helix aspera* exposed to air-born urban pollutants (Regoli et al., 2006). Therefore, a mechanism of peroxisome-proliferation analogous to the one promoted by PPARα activation in vertebrates also exists in invertebrates.

The fact that total lipids and almost all fatty acid groups decreased in such a short exposure period in exposed females is of special concern, taking into account the multi-functional role of fatty acids in cell structure and function, energy metabolism and storage, bioactive signaling and synthesis of various compounds involved in physiological regulation (e.g. steroids, eicosanoids, etc.) (Benatti et al., 2004). Toxicity of organotin compounds has been related to their interference with cell's membrane permeability, fluidity and signaling (Ortiz et al., 2005). Thus, exposure of ovaries of *Ciona intestinalis* to TBT for 5 hours caused a reduction in total lipids and triglycerides but an increase in phospholipids and PUFA, including HUFA and arachidonic acid (Puccia et al., 2005); phospholipids and PUFA are involved in maintaining membrane fluidity and the authors suggest that this increase is an adaptive mechanism to TBT toxicity. Enrichment of yeast *Saccharomyces cerevisiae* with linoleic acid (18:2n-6) caused resistance of the membranes to the toxic action of TBT (Masia et al., 1998), suggesting that although membrane fluidity was enhanced, toxicity of TBT was blocked probably by an increase of the lipophilicity of the membrane that would prevent the passive diffusion of TBT. In the

present work, a relative increase of arachidonic acid was observed in 7-days exposed females (Table 1). Arachidonic acid is required in cell signaling and specifically as a substrate for eicosanoids synthesis (Nakamura and Nara, 2004). Eicosanoids, which include prostaglandinds, thromboxanes, leukotrienes, hydroxyl FA and lipoxines are also critical in a very wide range of physiological processes in invertebrates; these include regulating egg-production, egg-laying, spawning and hatching, mediating immunological responses to infections, and regulating neurophysiology among other processes (Stanley-Samuelson 1994). Thus, the relative increase of arachidonic acid (30%) may be a short-term response in exposed females in order to maintain endogenous levels stable (2.0-2.4 mg/g) and minimize the effect of TPT on physiological functions.

Additionally, the proportion of arachidonic acid was markedly increased in females exposed to 125 and 500 ng/L TPT (Table 1), but not in males. These changes paralleled the alterations observed in total lipid and fatty acid levels, suggesting a link between the relative increase of this potential regulator of lipogenesis (Yoshikawa et al., 2002) and the observed decrease in total lipid and fatty acid levels in exposed individuals.

In vertebrates, fatty acids are endogenous ligands of various nuclear steroid receptors and control transcript signaling. All three isoforms of PPAR are activated by fatty acids, specifically PUFAs, regulating an extensive network of genes involved in glucose and lipid metabolism (Benatti et al., 2004). Palmitic (16:0), stearic (18:0), palmitoleic (16:1n-7), oleic (18:1n-9), linoleic (18:2n-6), arachidonic acid (20:4n-6) and EPA (20:5n-3) are endogenous ligands of PPARα which is involved in fatty acid oxidation and catabolism, whereas linoleic, arachidonic acid and eicosanoids are endogenous ligands of PPARγ which plays a central role in adipocyte differentiation and storage of fatty acids (Willson and Wahli, 1997; Reddy and Hashimoto, 2001;

Kota et al., 2005; Mochizuki et al., 2006). Other transcriptional factors have been identified to be targets of fatty acid regulation such as the Liver X receptor and RXR, which are both involved in lipid regulation (Benatti et al., 2004). Since fatty acids appear to be PPAR ligands at a concentration range that is consistent with their physiological circulating levels (Braissant et al., 1996), alterations in the abundance of endogenous fatty acids may trigger different mechanisms of lipid regulation further down the cascade of events. Indeed, Janer et al. (2007) exposed *M. cornuarietis* to TBT for 100 days and found a significant increase in the percentage of total lipids and total fatty acid content in females exposed to 500 ng TBT/L. Furthermore, the percentage of PUFAs, including HUFAs, decreased and MUFAs increased. The discrepancies with the present study are probably a reflection of long- and short-term effects of organotin compounds on lipid homeostasis rather than a different effect of TBT and TPT. Both studies indicated higher susceptibility of females than males of *M. cornuarietis* to lipid alterations.

In vertebrates and some invertebrate species, steroids are conjugated with fatty acids to form apolar esters that are retained in the lipoidal matrices of the body from where they can be hydrolysed by esterases and liberate the active steroids upon demand (Borg et al., 1995). In *Marisa cornuariets* most of the estradiol and testosterone have been found to exist in the esterified form (Janer et al., 2006). Esterification of steroids occurs upon acyl-CoA moieties, which activation is depended on the concentration of the corresponding fatty acids (Hochberg, 1998). In the oyster *Crassostrea virginica*, estradiol esters formation was achieved using the fatty acid moieties C16:0, C16:1, C18:0, C18:1, C18:2 and C20:4 (Janer et al., 2004). Exposure of mussels *Mytilus edulis* to estradiol resulted in the formation of estradiol esters with C16:0, C16:1 and C16:2 fatty acid moieties (Labadie et al., 2007). Additionally, acyl-CoAs are substrates of AOX enzyme. Interestingly, in the present experiment, one week exposure to TPT

resulted in a significant increase of AOX activity in exposed females together with a significant increase in esterified testosterone levels (60-85%) and a concomitant decrease in esterified estradiol (50-84%) (Lyssimachou et al., 2008). The observed alterations in esterified steroids were not directly related to changes in P450 aromatase activity or to changes in 17β-HSD, 5α-reductase, involved in the metabolism of the androgen precursor androstenedione. Thus, the hypothesis that changes in fatty acid availability might trigger alterations in endogenous steroid levels is a challenging one. In molluscs, the esterification of steroids with fatty acids appears to be an important regulation mechanism of endogenous steroid levels (Gooding and LeBlanc, 2001; Janer et al., 2005).

Overall, short-term exposure of *Marisa cornuarietis* to environmentally relevant doses of TPT lead to a decrease of total lipids and fatty acid content and an increase in AOX activity, which is involved in fatty acid catabolism. Since fatty acids have a pivotal role in organisms (cell membrane composition, bioactive signalling, steroid and eicosanoid synthesis), the observed effects are of special concern. Further research should focus on the higher sensitivity of females in comparison to males, the potential link of these alterations with the development of the imposex phenomena and the role of fatty acid composition on the control of adipogenesis in different species. Finally, being TPT a high affinity ligand of RXR, the obtained data further support the hypothesis that RXR may also be implicated in lipid homeostasis in gastropods (alone or in combination with putative PPARs) as it is in vertebrates.

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Table 1. Fatty acid profile in the digestive gland/ gonad complex of *Marisa cornuarietis* exposed to different concentrations of TPT for 1 week.

	Males					Females				
Fatty acid	Control	Solvent	30 ng /L	125 ng /L	500 ng /L	Control	Solvent	30 ng /L	125 ng /L	500 ng /L
14:0	2.72±0.25	2.84±0.19	2.97±0.28	2.63±0.22	2.37±0.49	1.10±0.07	1.13±0.08	1.55±0.66	1.17±0.06	1.06±0.05
15:0	0.36±0.03	0.37±0.02	0.37±0.02	0.43±0.04	0.41±0.05	0.39±0.01	0.37±0.04	0.38±0.05	0.38±0.02	0.31±0.01*
16:0	18.88±0.92	19.59±0.78	18.86±0.67	20.02±1.08	19.58±1.80	17.06±0.19	16.09±0.24	17.10±1.65	16.27±0.20	15.75±0.33
16:1n-9	0.36±0.01	0.40±0.03	0.35±0.03	0.30±0.05	0.46±0.05	0.42±0.02	0.43±0.02	0.48±0.03	0.44±0.01	0.40±0.01
16:1n-7	1.14±0.05	1.24±0.19	1.17±0.04	1.33±0.08	1.32±0.18	1.24±0.04	1.30±0.10	1.00±0.17	1.22±0.12	1.12±0.09
16:2	0.21±0.01	0.19±0.03	0.20±0.01	0.21±0.01	0.21±0.01	0.23±0.02	0.19±0.01	0.20±0.01	0.19±0.01	0.20±0.01
17:0	1.04±0.04	1.03±0.06	0.94±0.05	1.05±0.06	1.23±0.08	1.15±0.02	1.03±0.03	1.23±0.03**	1.08±0.02	1.11±0.03
16:3	0.19±0.04	0.21±0.06	0.15±0.04	0.24±0.05	0.13±0.02	0.13±0.02	0.21±0.02	0.18±0.05	0.12±0.01*	0.11±0.00*
18:0	8.35±0.37	8.72±0.32	8.25±0.39	8.40±0.45	8.76±0.55	7.96±0.15	6.97±0.33	8.12±0.57	7.32±0.41	7.43±0.24
18:1n-11	0.18±0.01	0.14±0.01	0.16±0.02	0.20±0.05	0.19±0.04	0.21±0.03	0.11±0.01	0.15±0.03	0.17±0.04	0.13±0.01
18:1n-9	13.68±0.51	13.98±1.61	13.87±0.36	16.43±0.92	15.89±2.06	16.87±0.69	15.98±0.47	13.51±1.34	14.53±0.55*	14.43±0.32**
18:1n-7	1.98±0.10	1.93±0.11	2.01±0.15	1.81±0.09	1.90±0.20	1.53±0.09	1.47±0.09	1.36±0.23	1.37±0.06	1.34±0.05
18:2n-6	13.12±1.62	13.44±1.60	14.43±1.18	13.38±2.42	11.83±3.10	17.30±1.13	19.25±0.57	14.82±2.26	18.06±0.48	17.68±0.64
18:3n-6	0.13±0.01	0.12±0.003	0.16±0.01	0.10±0.01	0.11±0.02	0.09±0.00	0.10±0.00	0.10±0.00	0.09±0.00	0.09±0.00
18:3n-3	0.44±0.17	0.59±0.14	0.66±0.08	0.61±0.17	0.49±0.17	0.80±0.09	0.96±0.04	0.60±0.14	0.88±0.06	0.78±0.08
18:4n-3	0.08±0.01	0.08±0.01	0.06±0.01	0.09±0.02	0.08±0.02	0.11±0.02	0.12±0.01	0.11±0.00	0.12±0.01	0.11±0.01
20:0	1.14±0.07	1.04±0.06	1.08±0.07	1.25±0.13	1.17±0.19	1.19±0.06	0.87±0.05	0.74±0.08	0.88±0.13	0.85±0.03
20:1n-9	4.76±0.19	4.38±0.21	4.25±0.09	3.99±0.61	5.07±0.34	4.62±0.25	4.16±0.15	4.72±0.33	4.54±0.25	4.35±0.23
20:1n-7	2.79±0.11	2.68±0.25	2.73±0.08	2.73±0.35	3.15±0.36	3.21±0.17	3.03±0.10	2.58±0.25	2.82±0.05*	2.86±0.11
20:2n-6	1.04±0.12	0.96±0.04	0.97±0.05	0.95±0.16	0.93±0.18	1.18±0.09	1.29±0.09	1.29±0.20	1.19±0.10	1.30±0.02
20:3n-6	0.63±0.04	0.53±0.07	0.67±0.05	0.50±0.05	0.49±0.11	0.39±0.01	0.45±0.03	0.46±0.01	0.49±0.04	0.31±0.07
20:4n-6	3.61±0.2	3.59±0.55	3.35±0.16	2.96±0.35	3.65±0.82	3.52±0.24	4.00±0.31	5.66±1.33	4.97±0.34*	4.92±0.33*
20:4n-3	0.05±0.01	0.06±0.00	0.06±0.00	0.06±0.00	0.06±0.01	0.06±0.00	0.07±0.00	0.07±0.01	0.06±0.005	0.05±0.002
20:5n-3	1.30±0.13	1.27±0.24	1.28±0.18	1.26±0.27	1.22±0.40	1.75±0.25	1.96±0.04	1.92±0.40	1.99±0.07	2.21±0.05
22:0	0.75±0.05	0.73±0.04	0.69±0.06	0.83±0.08	0.74±0.12	0.81±0.03	0.65±0.03	0.54±0.04	0.64±0.07	0.64±0.03*
22:1n-11	2.91±0.09	2.65±0.36	2.94±0.13	2.98±0.32	2.72±0.22	2.85±0.27	2.81±0.09	2.23±0.41	2.44±0.18	2.74±0.22
22:1n-9	0.09±0.01	0.09±0.02	0.15±0.06	0.09±0.01	0.16±0.08	0.08±0.01	0.08±0.01	0.09±0.004	0.07±0.01	0.08±0.005
22:1n-7	0.05±0.00	0.06±0.01	0.06±0.00	0.06±0.00	0.05±0.01	0.06±0.004	0.05±0.0	0.07±0.007	0.05±0.003	0.05±0.0
22:2n-6	1.02±0.04	0.99±0.08	0.96±0.02	0.84±0.09	1.09±0.21	1.11±0.08	1.27±0.11	1.68±0.33	1.46±0.13	1.50±0.13
22:5n-6/22:3n-3	1.33±0.09	1.28±0.19	1.48±0.24	0.97±0.19	0.85±0.32	0.09±0.02	0.12±0.01	0.46±0.3	0.10±0.01	0.10±0.01
22:5n-3	0.34±0.06	0.32±0.06	0.35±0.06	0.33±0.08	0.64±0.28	0.43±0.04	0.48±0.01	0.41±0.08	0.46±0.01	0.49±0.01
22:6n-3	2.93±0.39	2.64±0.50	3.23±0.39	2.48±0.44	1.96±0.44	2.18±0.32	2.43±0.08	1.82±0.25	2.26±0.09	2.23±0.13
24:1n-9	0.24±0.02	0.25±0.03	0.22±0.02	0.28±0.01	0.25±0.02	0.24±0.02	0.23±0.01	0.18±0.03	0.22±0.01	0.23±0.01
SFA	33.25±1.57	34.31±1.27	33.15±1.31	34.60±1.87	33.96±3.18	29.67±0.45	27.10±0.57	29.65±3.00	27.74±0.66	27.14±0.53
MUFA	28.15±0.58	27.79±2.53	27.88±0.62	30.19±1.91	31.14±3.14	31.38±1.50	29.63±0.60	26.29±2.14	27.85±0.74*	27.73±0.49*
PUFA	26.38±2.27	26.20±2.86	27.96±1.78	24.93±3.81	23.52±5.46	29.29±1.98	32.89±0.29	29.56±3.81	32.41±0.62	32.09±0.43
n-3	5.11±0.69	4.90±0.96	5.60±0.69	4.81±0.96	4.25±1.19	5.29±0.71	6.02±0.10	4.84±0.81	5.76±0.20	5.87±0.26
n-6	20.87±1.68	20.90±1.99	22.01±1.08	19.68±2.87	18.93±4.31	23.64±1.31	26.47±0.24	24.35±3.10	26.35±0.48	25.90±0.22
HUFA	10.18±0.46	9.66±1.49	8.77±1.72	8.54±1.10	8.68±1.93	8.35±0.82	9.50±0.25	10.65±1.50	10.32±0.31*	10.31±0.28*
HUFA n-3	4.61±0.56	4.26±0.82	4.88±0.61	4.12±0.78	3.70±1.01	4.39±0.60	4.94±0.06	4.19±0.66	4.77±0.14	4.98±0.17
HUFA n-6	7.62±0.18	7.34±0.78	7.43±0.39	6.21±0.68	6.99±1.33	6.25±0.32	7.12±0.46	9.44±1.63	8.20±0.52*	8.13±0.55*
Total FAME (mg/g d.w.)	59.44±8.96	52.07±3.34	62.16±6.50	64.22±4.17	41.71±3.20*	56.44±1.63	61.51±5.68	41.22±4.53*	49.09±0.83*	40.12±2.15**
Total lpids (% of d.w.)	15.97±0.91	15.79±0.52	17.66±0.89	17.48±0.99	14.67±1.44	14.48±0.56	14.12±0.80	12.74±1.52	13.65±0.69	11.59±0.16**

Data are expressed as % of total fatty acid methyl esters (mean±SEM; n=4). * and **significant differences respect to controls ($p<0.05$ and $p<0.01$ respectively).

Table 2. Levels of fatty acids grouped by chain length in the digestive gland/gonad complex of *Marisa cornuarietis* exposed to different concentrations of TPT for 7-days.

	Control	Solvent	30ng TPT/L	125ng TPT/L	500ng TPT/L
Females					
Σ C14	0.62±0.04	0.70±0.10	0.70±0.36	0.58±0.03	0.42±0.03**
Σ C15	0.22±0.01	0.65±0.08	0.28±0.07	0.19±0.01*	0.12±0.01*
Σ C16	10.77±0.39	11.20±1.02	8.01±1.55	8.95±0.10*	7.06±0.43**
Σ C17	0.65±0.03	0.63±0.05	0.50±0.05	0.53±0.02	0.45±0.03*
Σ C18	25.32±0.80	27.72±2.80	16.11±2.20**	20.85±0.37*	16.81±0.76**
Σ C20	8.96±0.24	9.73±0.94	6.94±0.24**	8.31±0.32	6.78±0.53**
Σ C22	4.03±0.21	4.83±0.45	2.96±0.43*	3.67±0.07*	3.13±0.09**
Σ C24	0.13±0.02	0.14±0.02	0.08±0.02*	0.11±0.01*	0.09±0.00**
Males					
Σ C14	1.56±0.11	1.46±0.03	1.80±0.04*	1.67±0.07	1.00±0.21
Σ C15	0.21±0.03	0.38±0.08	0.52±0.10	0.27±0.02	0.17±0.03
Σ C16	12.15±1.38	11.22±0.73	12.79±1.02	14.16±1.05	9.15±1.43
Σ C17	0.61±0.07	0.53±0.03	0.58±0.05	0.67±0.05	0.51±0.06
Σ C18	22.95±4.48	20.45±2.06	24.81±3.25	26.46±2.50	16.32±1.20
Σ C20	9.08±1.31	7.51±0.43	8.93±0.99	8.72±0.34	6.40±0.44
Σ C22	5.70±1.18	4.56±0.37	6.12±0.70	5.53±0.61	3.36±0.48
Σ C24	0.14±0.02	0.13±0.02	0.13±0.02	0.18±0.02	0.10±0.02

Values are expressed as mg/g of dry weight (mean ± SEM; n=4). Significant differences respect to controls indicated by *p<0.05 and **p<0.01.

Table 3. Levels of fatty acids grouped by unsaturation degree in the digestive gland/gonad complex of *Marisa cornuarietis* exposed to different concentrations of TPT for 7-days.

	Control	Solvent	30ng TPT/L	125ng TPT/L	500ng TPT/L
Females					
Σ C:0	16.76±0.68	16.60±1.32	12.53±2.51	13.69±0.19**	10.89±0.63**
Σ C:1	17.72±1.34	18.31±1.97	11.12±1.96*	13.66±0.33*	11.10±0.40**
Σ C:2	10.92±0.58	13.59±1.43	7.25±1.12**	10.26±0.28	8.29±0.41**
Σ C:3	0.79±0.04	1.06±0.12	0.55±0.07**	0.77±0.04	0.52±0.04**
Σ C:4	2.06±0.09	2.56±0.25	2.19±0.23	2.53±0.20	2.05±0.22
Σ C:5	1.25±0.13	1.57±0.12	1.08±0.09*	1.25±0.04	1.12±0.07*
Σ C:6	1.22±0.16	1.50±0.17	0.75±0.13**	1.11±0.03	0.89±0.05**
Males					
Σ C:0	19.42±2.14	17.78±0.90	20.39±1.42	22.14±1.53	14.33±2.17
Σ C:1	16.83±2.83	14.56±1.89	17.38±2.06	19.42±1.87	13.18±2.23
Σ C:2	9.49±2.30	8.19±1.15	10.48±1.73	9.96±2.06	5.65±1.28
Σ C:3	0.86±0.22	0.76±0.08	1.03±0.14	0.94±0.17	0.50±0.09
Σ C:4	2.18±0.27	1.90±0.29	2.13±0.24	1.97±0.20	1.52±0.33
Σ C:5	1.79±0.34	1.49±0.27	1.93±0.25	1.63±0.23	1.05±0.29
Σ C:6	1.84±0.54	1.38±0.30	2.04±0.37	1.60±0.34	0.80±0.19*

Values are expressed as mg/g of dry weight (mean ± SEM; n=4). Significant differences respect to controls indicated by *p<0.05 and **p<0.01.

Figure 1. AOX activity in the digestive gland/gonad complex of *Marisa cornuarietis* exposed to TPT for 7-days. Values are the mean ± SEM (n=4). *Significant differences respect to control (p<0.05).

Figure 2. Fatty acid methyl esters (FAME, mg/g dry mass) and total lipids (% dry weight) in the digestive gland/gonad complex of *Marisa cornuarietis* exposed to TPT for 7-days. Values are mean ± SEM (n=4). *Significant differences respect to control (p<0.05).

Figure 1

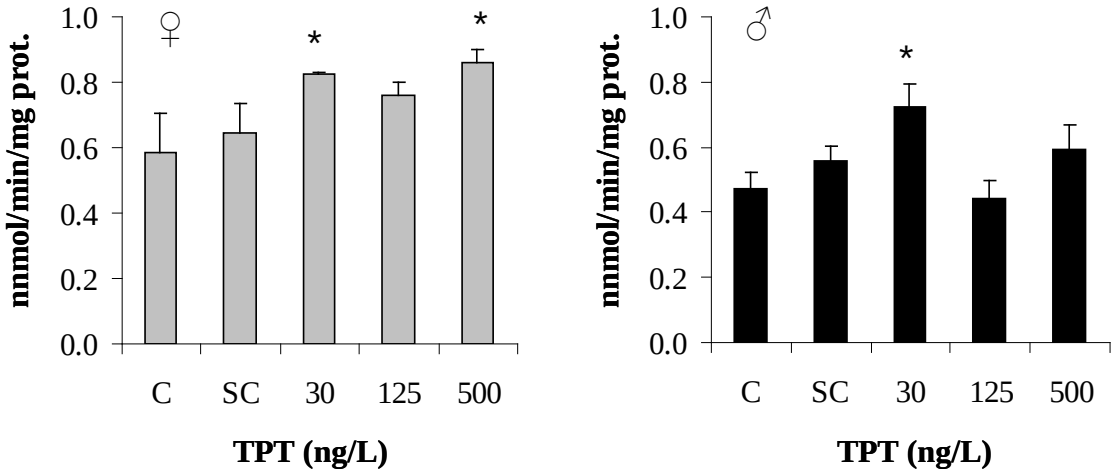
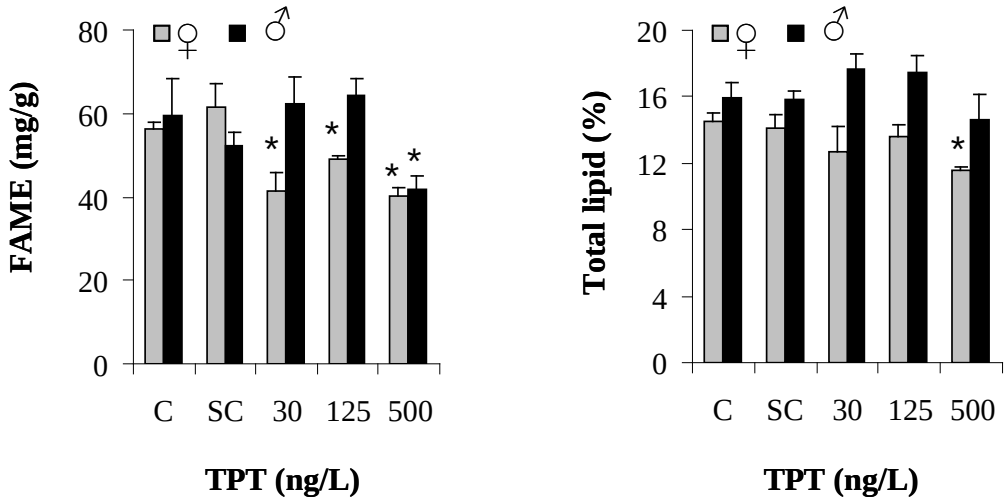


Figure 2.


FAME (mg/g)
80
60
40
20
0
♀
♂
*
*
* *
C
SC
30
125
500
TPT (ng/L)
Total lipid (%)
20
16
12
8
4
0
♀
♂
*
C
SC
30
125
500
TPT (ng/L)