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Tri-m-cresyl phosphate and PPAR/LXR interactions in seabream hepatocytes: revealed by computational modeling (docking) and transcriptional regulation of signaling pathways

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ABSTRACT

The interactions between Tri-m-cresyl phosphate (TMCP: an organophosphate flame retardant), and peroxisome proliferator activated receptors (PPARs) **or** liver X receptor α (LXRα) were investigated in seabream hepatocytes. The study was designed to characterize the binding of TMCP to PPARα, PPARγ and LXRα by computational modeling (docking) and transcriptional regulation of signaling pathways. TMCP mainly established a non-polar interaction with each receptor. These findings reflect the hydrophobic nature of this binding site, with fish LXRα showing the highest binding efficiency. Further, we have investigated the ability of TMCP to activate PPARs and LXRs controlled transcriptional processes involved in lipid/cholesterol metabolism. TMCP induced the expression of all target genes measured. All target genes were up-regulated at all exposure doses, except fatty acid binding protein 7 (FABP7) and carnitine palmitoyltransferase 1B. Collectively, our data indicate that TMCP can affect fatty acid synthesis/uptake and cholesterol metabolism through LXRα and PPARs, together with interactions between these transcriptions factors in seabream liver.

Keywords: Tri-m-cresyl phosphate; PPARs; LXRα; molecular docking; seabream; gene transcription.

1. Introduction

Tri-m-cresyl phosphate (TMCP) belongs to the group of triaryl phosphate esters that are widely used as flame retardant.[1] These compounds are frequently used as stabilizers in several products ranging from polishing to lubricants and hydraulic fluids.[2] TMCP is also one of the major isomer of commercial tricresyl phosphate (TCP) that is used in jet turbine engine oil and is known for neurotoxic potential.[3] As TCP isomer, TMCP was found in engine oils from motor bikes and cars, showing levels of 1.5-6.8 μg TMCP/g oil.[4] Interestingly, TMCP was measured in exhaust gases from vehicles.[5] Based on available data for other TCP isomers, the half-life for basic hydrolysis in experiments with sodium carbonate (Na_2CO_3) was found to be 280 min for tri-o-cresylphosphate (TOCP) and 670 minutes for tri-p-cresyl phosphate (TPCP). In addition, available information indicates that TMCP undergoes hydrolysis in soil-water slurries, showing a 90 % degradation in 26 h.[6] Muir et al.[7] estimated bioconcentration factor (BCF) values for TMCP at 310 ± 52 l/kg and 462 ± 3 l/kg for rainbow trout and fathead minnows, respectively.

Previous studies have demonstrated that the potential leakage of engine oil that contains TCP isomers, to air conditioning systems of aircraft may affect human health through the aerotoxic syndrome.[8, 9] On the other hand, toxicological studies using mammalian models have demonstrated that exposure to TCP mixture (with 21% TMCP) produced high survival rate at all dose levels.[10] However, the presence of ovary, adrenal gland, spinal cord and sciatic nerve lesions were detected in all dose groups.[10] Recently, there are reports suggesting that pre- and postnatal exposure to a commercial mixture of flame retardants, containing 10-20% triphenyl phosphate (TPP), resulted in a variety of effects including anxiety, early puberty and obesity.[11] Long-term toxicity tests using rainbow trout showed the presence of plasma biochemical changes associated with enlarged livers in TMCP-treated fish.[12] TPP was also shown to bind to peroxisome proliferator activated receptor γ (PPARγ), inducing PPARγ-dependent transcription and potential obesogenic responses.[13] In this regard, the binding of environmental obesogens to nuclear receptors (NRs) that act as metabolic sensors can induce dysregulation of lipid homeostasis.[14] Besides PPARs, TPP has been shown to

interact with other NRs such as constitutive androstane receptor (CAR), pregnane X receptor (PXR), and estrogen receptor (ER).[15, 16] Recently, we reported that C10 phthalates such as the diisodecyl phthalate (DiDP) can bind efficiently to and activate PPARs and their obligate heterodimeric partner retinoid-X-receptor-α (RXRα), altering lipid metabolism in fish hepatocytes.[17] In addition, TMCP was reported to target the oxysterols-binding site of human liver X receptor α (LXRα), another NR involved in both lipid and glucose metabolism,[18, 19] displaying affinity values comparable to those of well-known LXRα agonists.[20] Interestingly, the down-regulation of lipid/cholesterol metabolism related genes was observed after exposure to diphenyl phosphate (DPP), which is considered as a primary metabolite of TPP.[21] Information regarding the potential binding efficiency of TMCP to fish lipid-sensing NRs, particularly PPARs and LXR, is currently not available. In addition, little is known about the effects of TMCP on PPAR and LXR signaling pathways in fish *in vitro* models. Accordingly, we have studied the ability of TMCP to bind to fish PPARα, PPARγ, RXRα and LXRα, activation of their controlled transcriptional processes that are involved in lipid/cholesterol metabolism using primary seabream hepatocytes. Our hypothesis is that TMCP will differentially bind to PPARs, RXR and LXR, and successfully activating their regulation of downstream molecular responses.

2. Material and methods

2.1. Chemicals

TMCP (CAS No. 563-04-2) and 3-aminobenzoic acid ethyl ester (MS-222) were purchased from Sigma (St. Louis, MO). Cell culture medium and serum [Leibovitz L-15 phenol red-free medium and fetal bovine serum (FBS)] were purchased from Life Technologies (Carlsbad, CA, USA).

2.2. Molecular docking

To evaluate the affinity of TMCP to fish nuclear receptor complex and corresponding geometry, we performed a homology modelling and molecular docking analysis as previously reported by Cocci

et al.[17] The three dimensional structures and homology of the four fish nuclear receptors (PPARα and γ from *Sparus aurata*, LXRα from *Salmo salar* and RXRα from *Danio rerio*) were modelled, starting with the corresponding receptors from human (1I7G[22], 1I7I[22], 3FC6[23] and 3DZY[24] and respective sequence identity of 66.67%, 65.64%, 81.67% and 87.96%), using Swiss PDB viewer (version 4.1) and Swiss-Model server.[25] The molecular docking procedure between the four receptors and the TMCP were completed using the Autodock Vina software (version 1.1.2)[26] on an Intel Core i7/Mac OS X 10.9 – based platform. The docking zone was set around the oxysterols-binding site with a dimension of 26×27×25Å. The predicted equilibrium dissociation constants were calculated from the free binding energies using the formula:

$$K_d = e^{\Delta G_{bind}\frac{1000}{RT}}$$

2.3. Experimental animals and hepatocytes isolation

Juveniles of Gilthead seabream (*S. aurata)* were provided by the hatchery of a local fish farm and kept in 1500 L tanks at Unità di Ricerca e Didattica of San Benedetto del Tronto (URDIS), University of Camerino in San Benedetto del Tronto (AP, Italy). Fish were fed a commercial diet once a day during the acclimation period (Tetra Werke, Germany). After acclimation, fish were randomly anaesthetized using MS-222 (0.1 g L^{-1}) and sacrificed by decapitation. The liver tissue was aseptically harvested to obtain hepatocytes under a laminar flow hood, according to Centoducati et al [27], with slight modifications. The detailed procedure for the isolation of sea bream hepatocytes was described in our previous publication.[17] After the isolation phases, purified hepatocytes were suspended in Leibovitz L-15 phenol red-free medium supplemented with 10% FBS, antibiotic-antimycotic solution (100 U/ml) and 10 mM HEPES. The cell density was estimated in a counting Burker Chamber and viability of hepatocytes used for experiments was over 90%, as assessed with trypan blue exclusion assay.[28]

Animal manipulation was performed according to the recommendations of the University Ethical

Committee, to the European Union directive (2010/63/EU) for animal experiments and under the supervision of the authorized investigators.

2.4. Hepatocytes culture and exposure

Isolated hepatocytes were seeded on 24-well Falcon Primaria™ culture plates (1 x 10^6 cells per well) in Leibovitz L-15 phenol red-free medium supplemented with 10% FBS, antibiotic-antimycotic solution (100 U/ml) and 10 mM HEPES. Cells were cultured in an incubator (3% CO_2) at 23 °C to allow for attachment, before chemical exposure.[17] After a 24 h incubation period, the L-15 phenol red-free medium culture was removed and hepatocytes were exposed to medium containing the vehicle (ethanol, final concentration 0.01%) and 0.1, 1.0 or 10 μM of TMCP or bezafibrate (BZF; an established ligand for **PPAR** receptors[29]). Concentrations of TMCP were chosen on the basis of binding affinities obtained **through** molecular docking analysis and taking into account the concentration range used in previous *in vitro* studies.[13, 21, 30] Hepatocytes were incubated with 3% CO_2 at 23 °C for 48 h. After 24 h of culture, 90 % of the medium was removed and replaced with fresh appropriate medium. Exposure of primary seabream hepatocytes was performed using 24-well plates and six independent wells were set-up for both control and each TMCP concentration. **The experiments were repeated with three independently prepared pools of hepatocytes.** At the end of exposure, all cell layers remained attached to the bottom of the plates. At this point, cell viability was again assessed by microscopic examination of the cell morphology and the trypan blue exclusion test.

2.5. Quantitative (real-time) PCR (q-PCR)

After exposure, medium was carefully removed and cells were lysed with the Trizol reagent (Invitrogen Life Technologies, Milan, Italy). Total RNA was isolated according to the manufacturer's specifications. DNase digestion (2 U, 30 min, 37 °C; Ambion, Austin, TX) was performed to eliminate genomic DNA contamination. RNA concentration and purity were assessed

spectrophotometrically at absorbance of 260/280 nm, and the integrity was confirmed by electrophoresis through 1% agarose gels stained with ethidium bromide. The complementary DNA (cDNA) was synthesized from 1 μg of total RNA using random hexamers (50 ng μL^{-1}) and 200 U of SuperScript™ III RT according to manufacturer's instruction (Invitrogen Life Technologies, Milan, Italy). SYBR green-based real-time PCR was used to evaluate expression profiles of PPARα, PPARβ, PPARγ, RXRα, LXRα, carnitine palmitoyltransferase 1A-1B (CPT1A, CPT1B), hepatic lipase (HL), lipoprotein lipase (LPL), fatty acid desaturase 2 (FADS2), sterol regulatory element-binding protein 1c (SREBP-1c), fatty acid binding protein 7 (FABP7; Figure S1 and Table S1), stearoyl-CoA desaturase 1A-1B (SCD1A, SCD1B), apolipoprotein A1 (APO-A1) target genes (**Table 1**). Analysis of the 18S rRNA gene expression confirmed that its expression was unaffected by exposure to flame retardants (data not shown), and thus it was selected as reference gene for the qPCR analysis.[31] Quantitative-PCR was performed according to previously described methods.[17] Results were calculated using the relative $2^{-\Delta\Delta Ct}$ method[32] and expressed as normalized fold expression corrected for 18S rRNA and with respect to control levels. Values are given as mean ± standard deviation (SD) of three independent observations.

2.6. Pathway mapping analysis

Public domain database tools were used to annotate changes in gene expression within the pathway. First, we used the functional annotation tool of the database for annotation, visualization and integrated discovery (DAVID) bioinformatics resource (http://david.abcc.ncifcrf.gov/)[33, 34] to obtain a global overview of the biological processes regulated by the studied genes. Using the Kyoto encyclopedia for genes and genomes (KEGG) database (http://www.genome.jp/kegg/pathway.html)[35] we conducted a pathway analysis of selected genes. The graphical representation of the pathway was performed with Pathvisio 3.2.0 (http://www.pathvisio.org/)[36] to understand known biological processes regulated by TMCP.

2.7. Western blotting analysis

Total proteins were extracted following the modified Trizol protocol described in Simões et al.[37] Total protein concentration in samples were determined according to the Bradford method[38] using bovine serum albumin (BSA) as standard. Western blotting was performed according to standard protocol[39] before blotting as previously described.[40] A detailed procedure for western blotting analysis was reported in Cocci et al.[17]

2.8. Statistical analysis

All statistical analyses were performed using R.[41] Data were first examined for their fit to a normal distribution and homogeneity of variance using Shapiro-Wilks and Levene median tests. A one-way analysis of variance (ANOVA) was used to compare results among exposure groups, followed by the Tukey *post-hoc* test. Differences between means were considered significant when $P<0.05$. For protein analysis, western blotting technique was mainly used for qualitative purposes to obtain a visual evaluation of protein expression after exposure to test compound. Principal components analysis (PCA)[42] was conducted on the data matrix containing all gene expression values for evaluating response similarities between genes. Volcano plots of log2-transformed fold changes (induction ratios) versus $\log_{10}$-transformed p-values of individual treatment effects were used to highlight the upregulation of these genes by TMCP.

3. Results and discussion

3.1. Computational analysis of TMCP binding to fish NRs

Molecular docking between TMCP and four fish nuclear receptors was performed in order to predict their feasible geometric poses and affinities on the base of equilibrium dissociation constant. TMCP was docked onto each fish receptor oxysterols-binding site producing four best complexes with affinities reported in **Table 2**, in the range from 4.15×10^{-07} to 1.87×10^{-09} M, similar to binding affinities of specific drugs.[43] For BZF, these affinities show values at least one order of

magnitude lower for both PPARγ and RXRα, as reported by Cocci et al.[17], and as calculated for the LXR (Kd = 9.08 × 10^{-08} M) or similar for PPARα[17]. TMCP mainly established a non-polar interaction with each receptor, reflecting the hydrophobic nature of this binding site (Figure 1 and 2). The values of free binding energy were strictly related to these hydrophobic contributions (**Table 2**), which are critical in determining the affinity between TMCP and individual fish receptor. The theoretical H-bonds between TMCP and PPARs appear to be irrelevant in the stabilization of the complexes. In comparison with the results obtained for DiDP[17], TMCP has about 10-fold lower predicted equilibrium dissociation constant for interaction with fish PPARα and RXRα showing greater potential to modulate receptor-mediated signalling pathways. Interestingly, this value reaches a 100-fold level, lower than that of DiDP for PPARγ.[17] It has been established that PPARγ is the most important regulator of adipocyte differentiation, mediating the effects of thiazolidinediones and obesogenic pollutants.[14, 44] In this regard, Pillai et al.[13] demonstrated that TPP binds with PPARγ ligand-binding domain (LBD) in a similar manner, compared to partial selective agonists, resulting to the induction of adipocyte differentiation. In the present study, we observed that TMCP showed the highest binding efficiency with fish LXRα (K_{δ}=1.87·10^{-09} M). This finding confirms our previous results on the ability of TMCP to target the oxysterols-binding site of human LXRα with affinities in the nano molar range.[20] The stability of the resulting TMCP/LXRα complex was found to be due to a slower dissociation phase. Overall, our findings suggest that TMCP is a pan-agonist for all PPAR isotypes, but with higher potency and affinity for LXRα that produces the regulation of multiple LXR target genes in fish hepatocytes with similar efficacy, compared to established LXR ligands.

3.2. Modulation of mRNA expression in seabream hepatocytes exposed to TMCP

In order to examine the ability of TMCP to bind to fish NRs, resulting in potential regulation of lipid/cholesterol metabolism, we investigated the effect of TMCP on selected PPAR/RXRα and LXRα target genes in primary seabream hepatocytes. In addition, the present study also focused on

possible cross-talk between PPARs and LXRα in mediating transcription of their related genes. Using the KEGG database, three pathways - PPAR signaling pathway (dre03320), biosynthesis of unsaturated fatty acids (dre01040) and adipocytokine signaling pathway (dre04920) were obtained. To understand the molecular signaling associated with genomic changes, we mapped our gene list on a custom PPAR signaling pathway showing the expression profiles of target genes affected by TMCP exposure (Figure 3). We demonstrated that expression of each PPAR and their heterodimeric partner RXRα was increased by TMCP at ~2−3-fold (Figure 4a-d). Figure 4 shows similar findings from analysis of BZF-induced PPAR and RXR expression, suggesting more pronounced effects on either PPARβ or PPARγ. Likewise, an up-regulation of PPARβ was observed in fish exposed to high concentrations of BZF for 21 days.[45] On the contrary, no changes in the levels of PPARα mRNA were observed in both testis and liver of fish exposed to waterborne BZF.[45, 46] In accordance with the present study, increased expression of PPARγ was observed in fish hepatocytes exposed to BZF or clofibrate.[47, 48] In addition, elevated levels of PPAR subtype mRNAs were reported in fish following exposure to a broad range of environmental contaminants including 4-nonylphenol and clofibric acid.[49, 50]. Taken together, our results suggest that TMCP is not dependent on PPARα in exerting its effect on fish hepatocytes, since it may produce biological effects through the PPARβ and RXRα. This hypothesis is supported with the observed lower TMCP Kd-value for RXRα and TMCP-induced expression level of PPARβ. The up-regulation of PPAR and RXRα mRNA is also in accordance with previous data from our sea bream *in vitro* hepatocyte model after exposure to DiDP[17]. In addition, it further supports the potential auto-regulation of the expression of these genes following exposure to TMCP.

In mammals, liver FABPs (L-FABPs) may function as carriers and selectively enhancing the distribution of long-chain fatty acyl CoAs (LCFA-CoAs) and long-chain fatty acids (LCFAs) to the nucleus for potential interaction with nuclear receptors. Findings from the present study may indicate that L-FABP has the potential to regulate PPARα transcriptional activity in hepatocytes through direct interaction with PPARα.[51] In this regard, we observed that expression of FABP7 (a

lipid transporter gene) was significantly induced (7-fold) after exposure to TMCP at 10 μM concentration (Figure 5a). Similar increases were observed after exposure to the highest BZF concentration (Figure 5a). Recently, it was reported that exposure of primary bone marrow cells to a commercial mixture of organophosphate flame retardants up-regulated FABP4 expression.[13] FABP4 is a major PPARγ target in mature adipocytes.[52] Kamstra et al.[53] reported that the brominated flame retardant (BDE-47) induced adipocyte differentiation by activating adipogenic gene programmes including, the elevation of FABP4 mRNA expression levels. In fish, 0.5% clofibrate activated PPARs, which directly induced the peroxisome proliferator response element (PPRE)-mediated transcription of FABP7 in the liver.[54] There are no data known to us that links TMCP with PPAR/RXRα or regulation of downstream target genes in fish. However, we have previously shown that phthalates (*i.e.* DiDP) are able to increase FABP7 mRNA levels in seabream primary hepatocytes, suggesting a direct involvement of this protein in the transport of lipophilic xenobiotic to liver PPARα.[17] Interestingly, the dose-dependent effects of DiDP on FABP7 expression were opposite in comparison to that obtained after TMCP exposure. In fact, TMCP-related effects were found exclusively at the highest concentration. In the context of lipid transport, APO-AI is another target of PPARα.[55] Expression of APO-AI was significantly induced in hepatocytes exposed to all TMCP or BZF concentrations (Figure 5b). It has been demonstrated that PPARα activation resulted to increased levels of both plasma protein and hepatic mRNA of APO-AI in human[56], but not in rodents.[57] Fibrates (*e.g.* fenofibrate), that is considered a weak PPARα agonist increased human APO-AI at 10-fold higher concentration than classical PPARα agonists.[58] In fish hepatocytes, APO-AI expression was increased after exposure to DiDP at 0.1 - 1 μM concentration range.[17]

In fish, peroxisome proliferators such as hypolipidemic drugs, plasticizers and some herbicides, produced increases in the activity of enzymes of peroxisomal *β*-oxidation.[47] Herein, we observed that transcript levels of CPT1A, but not CPT1B, were increased after exposure of seabream hepatocytes to TMCP or BZF, at all concentrations (Figure 5c, d). It has been shown that fibrates

and fatty acids induce peroxisomal β-oxidation enzyme activities in fish[47, 59] suggesting species-related differences in their sensitivity to peroxisome proliferators. The increase in the expression of CPT1A (the predominant isoform in the liver) may be explained by the activation of PPARs. In addition, our results suggest that the expression of CPT1A resembled the expression pattern of either PPARβ or PPARγ. In contrast, the lack of CPT1B mRNA changes after exposure to TMCP or BZF exposure, may be attributed to the low abundance of this isoform in the liver, compared to skeletal muscle.[60] Both PPARα and PPARγ are involved in the regulation of target gene such as SCD1A, SCD1B and FADS2 that are associated with lipogenesis. In the present study, the expression of these genes was significantly increased after exposure to TMCP at all test concentrations (Figure 5e, f, g). On the contrary, transcript levels of these hepatic lipogenic genes were significantly elevated after exposure to 10 μM BZF, compared with respective control, with the exception of FADS2. FADS2 mRNA levels were significantly elevated at all BZF concentrations, compared to control group. Previous studies have shown that phthalates and polychlorinated biphenyls (PCBs), induced the over-expression of SCD1 in mammals[61, 62] and fish.[17] Elevated expression of CPT1 and SCD1 may lead to increased hepatic lipid accumulation resulting to metabolic perturbations. In mice, it has been shown that combined exposure to di (2-ethylhexyl) phthalate (DEHP) and Aroclor 1254 increased CPT1 and SCD1 mRNA levels and altered the liver to body weight ratio.[62] In a previous study, bisphenol A (BPA) was found to affect the expression of lipogenic enzyme, including SCD1, showing a non-monotonic dose-response curve, with most pronounced effects at lower doses.[63] In contrast, in the present study, the effect of TMCP on SCD1A, SCD1B and FADS2 mRNA levels was more pronounced at higher concentrations.

In addition to lipogenic genes, all three PPARs are involved in controlling the expression of transcription factors such as LXRα, SREBP-1c, HL and LPL, that in turn, are downstream effectors responsible for cholesterol synthesis and fatty acid transport. Expression of these genes was significantly induced at all TMCP concentrations (Figure 5h, i, l). Interestingly, both HL and

LPL were significantly increased (up to 3-fold) at the highest TMCP concentration. Otherwise, no transcription changes of both HL and LPL were observed after 48h exposure to BZF (Figure 5i, l). It has been demonstrated that most PPARα activation is linked to multiple aspect of lipoprotein uptake and metabolism.[64] Expression of both HL and LPL was slightly up-regulated by PPARα agonists in liver.[65, 66] Previously, LPL activity was induced in adipose tissue of rat exposed to dietary DEHP.[67] Thus, Quistad et al.[68] have suggested the potential sensitivity to organophosphorus (OP) inhibitors that is based on differences in lipase classes. In addition, our previous findings showed that HL and LPL mRNA levels were significantly increased in seabream hepatocyte *in vitro* model.[17] LPL is a known SREBP-1c target gene, but also contains DR4 LXR response elements (LXRE)[69], suggesting a possible transcriptional activity in response to LXR activation. These findings indicate a possible and combined LXRα- and PPARα- or PPARγ-mediated regulation of LPL expression by TMCP.

Accordingly, our results showed parallel increases in SREBP-1c and LXRα mRNA levels after 48 h exposure to both TMCP and BZF (Figure 5h, 6). This is interesting, because LXR is involved in the regulation of gene transcripts that are responsible for controlling multiple pathways, such as cholesterol homeostasis, fatty acid synthesis, carbohydrate metabolism and anti-inflammatory mechanisms. Nevertheless, there is limited information about the specific contribution of LXR mediated lipid metabolism in fish species. In this regard, fish LXR was activated by LXR ligand binding in similar way as in mammals, thus resulting in the induction of a battery of genes involved in lipid metabolism.[70] Indeed, exposure to LXR agonists, significantly up-regulated LPL expression in trout myocytes.[71] Similarly, activation of LXR increased SREBP-1c gene expression and promoting fatty acid synthesis and triglycerides accumulation in mammals.[69, 72] These reports are in accordance with our previous findings showing LXRα-mediated activation of SREBP-1c after exposure of human liver hepatocellular carcinoma cells (HepG2) to TMCP.[20] Moreover, our results indicated the auto-regulation of LXRα after TMCP exposure in seabream hepatocytes. Several studies have demonstrated positive auto-regulation of LXR in various human cells[69, 73] and in fish

myocytes.[71] This mechanism has been suggested to facilitate the induction of target genes in a tissue-specific manner.[74]

It is interesting to note that human LXRα is considered a common target gene for both PPARγ and LXRs.[73] A functional PPRE has been identified in the promoter of the murine LXRα gene suggesting its potential role as target for PPARα.[40] Transcriptional cross-talk between LXR and PPARs was demonstrated in fish and PPAR mRNA expression was modulated by LXR ligands in trout myocytes.[71] In particular, the authors showed that PPARα mRNA levels were up-regulated by T091317 in the range of 0.01 to 1 μM demonstrating transcriptional regulation through LXR activation. Thus, it is possible that the increase in PPARs expression observed in the study is due to TMCP-mediated activation of LXRα. Previously, it was shown that different PPAR agonists increased the expression of LXRα and PPAR isoforms in an LXRα-dependent manner.[75] Interestingly, the authors also observed an increase in LXRE-luciferase activity by PPAR agonists in mouse fibroblast, demonstrating that this activation was LXRα-dependent due to most of the tested compounds.

The relationship between the expression levels of all genes was further analyzed using PCA. The model showed that the first two principal components (PC1 and PC2) accounted for 80.4% of the total variance in the dataset (Figure **7**a). PC1 explained most (69.3%) of the total variability observed with almost all variables lying in this dimension. Moreover, PC2 described a little part of the total variation (11.1%) with FABP7, HL, FADS2 and PPARα, which are located closer to PC2 in comparison to the other genes. In particular, FABP7 is the only gene located in the left upper corner of the PCA plot indicating differential response to TMCP concentrations, than all other genes. In fact, the majority of differentially expressed genes of interest (based on fold changes of 1.5 or greater) were active at more than one TMCP concentration (Figure **7**b). On the contrary, only CPT1B was found to be non-responsive to the treatments. It is also noted that no gene showed decreased expression level at any treatment concentrations, indicating consistency for the regulated genes at different treatment concentrations. This condition was also evident despite the nonlinear

concentration-response relationships observed after exposure to TMCP. SREBP-1c, APO-AI, PPARγ, CPT1A, PPARβ, LXRα, RXR and SCD1B grouped together in the bottom right corner of the PCA plot, demonstrating the absence of a concentration-dependent effect. On the contrary, LPL clustered with SCD1A, showing a U-shaped response over the three concentrations.

In summary, the present study showed that TMCP has the potential for binding and activating both PPARs and LXRα in seabream primary hepatocytes. In particular, we suggested the presence of a specific interaction between fish LXRα and TMCP, which results to increased mRNA expression of genes involved in lipid and cholesterol metabolism. Moreover, we observed auto-regulation of these transcription factors, as previously described in mammals. On the basis of our findings, we conclude that TMCP can affect FA synthesis/uptake and also cholesterol metabolism through LXRα and PPARs, and interaction between these transcriptions factors in seabream liver. These findings should be validated *in vivo* for better understanding on the hepatic lipid metabolism after exposure to TMCP and related chemicals.

Conflicts of interest

There are no conflicts of interest to declare.

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Table 1
List of primers used in this study

Gene	Primer sequence (5′-3′)	Genebank	Reference
PPARα	GCAGCCTGTGAGTCTTGTGAGTGA CTCCATCAGGTCTCCACACAGC	AY590299	Fernández et al.[76]
PPARβ	CGTGTTCGGGATTCGGGACT CACCCTGTCGTGCTGCTCTGTA	AY590301	Fernández et al.[76]
PPARγ	CGGAGAGAGAAGCAAGAACAAGAA GAGGAGGAGGAGATGGAGGTGTA	AY590304	Fernández et al.[76]
RXRα	GGGCTTCTTCAAGAGGACAGT TGCACCGCTTCTCTCTTCAT	HS092100	Ribecco et al.[77]
LXRα	GCACTTCGCCTCCAGGACAAG CAGTCTTCACACAGCCACATCAGG	FJ502320	Benedito-Palos et al.[78]
CPT1A	GTGCCTTCGTTCGTTCCATGATC TGATGCTTATCTGCTGCCTGTTTG	JQ308822	Pérez-Sánchez et al.[79]
CPT1B	CAAGCCCCGACACAGACTCATACC CCCATTTCCCAGCTGCGTTATTTT	DQ866821	Boukouvala et al.[60]
LPL	CGTTGCCAAGTTTGTGACCTG AGGGTGTTCTGGTTGTCTGC	AY495672	Benedito-Palos et al.[78]
FADS2	GCAGGCGGAGAGCGACGGTCTGTTCC AGCAGGATGTGACCCAGGTGGAGGCA	AY055749	Benedito-Palos et al.[78]
SREBP-1c	AGGGCTGACCACAACGTCTCCTCTCC GCTGTACGTGGGATGTGATGGTTTGGG	JQ277709	Benedito-Palos et al.[78]
FABP7	AAATGGTTGAGGCTTTCTGTGCTAC ATCGCTACTGTCGGCTTGGTG	HQ228170	Varó et al.[80]
SCD1A	CGGAGGCGGAGGCGTTGGAGAAGAAG AGGGAGACGGCGTACAGGGCACCTAT	JQ277703	Benedito-Palos et al.[78]
SCD1B	GCTCAATCTCACCACCGCCTTCATAG GCTGCCGTCGCCCGTTCTCTG	JQ277704	Benedito-Palos et al.[78]

HL	TTGTAGAAGGTGAGGAAAACTG GCTCTCCATCAGACCATCC	EU254479	Pérez-Sánchez et al.[79]
APO-A1	GAATACAAGGAGCAGATGAAGCAGAT GTGGTGACGGAGGCAGCGATG	AF013120	Varó et al.[80]
18s	GCATTTATCAGACCCAAAACC AGTTGATAGGGCAGACATTCG	AY993930	Pérez-Sánchez et al.[81]

Table 2
Energy contributions, free energies of binding and predicted equilibrium dissociation constants of the TMCP/fish nuclear receptor complexes.

Receptor	PPARα	PPARγ	LXRα	RXRα
$K_{d,pred}$ (M)	4.15×10^{-07}	2.11×10^{-07}	1.87×10^{-09}	7.67×10^{-08}
Gauss1	85.43046	108.21243	105.13935	81.88045
Gauss2	1420.72652	1479.48434	1577.28502	1520.77443
Repulsion	1.25515	4.21133	1.11833	1.24524
Hydrophobic	53.32514	94.41652	146.22619	97.30421
Hydrogen	0.91187	1.63538	0.00000	0.00000
ΔG (kcal/mol)	-8.67125	-9.04382	-11.88683	-9.71512

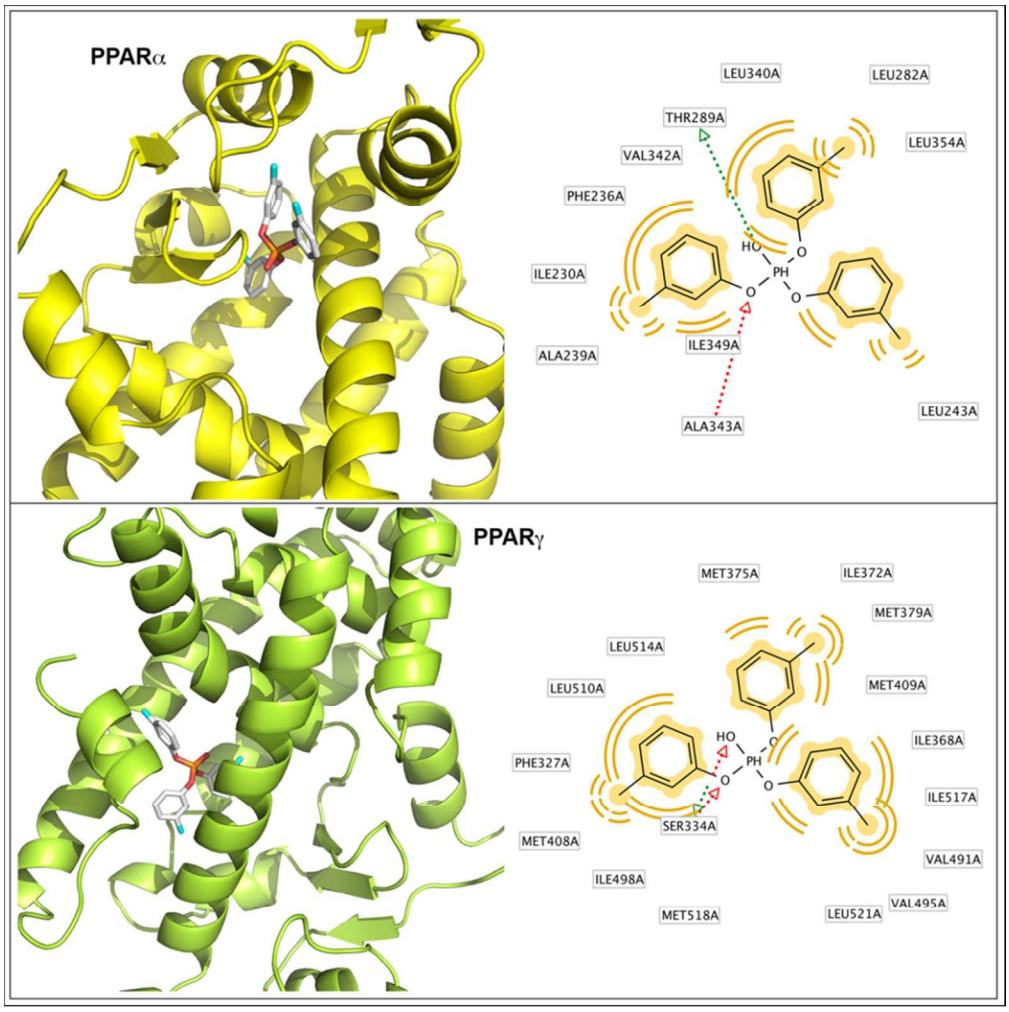


Fig. 1. 3D and 2D predicted models of the TMCP/fish PPARα (a)-PPARγ (b)complexes obtained by molecular docking. In the 3D representations, the receptor is shown in cartoon mode whereas the TMCP is shown as stick. Predicted hydrophobic interactions (yellow areas) and H-bonds (red and green dotted lines) are reported in the 2D schemes. These intermolecular interaction features were obtained using LigandScout software (version 3.12) whereas the 3D representations were rendered by MacPyMOL software (Python Molecular Graphics – version 1.3).
177x177mm (150 x 150 DPI)

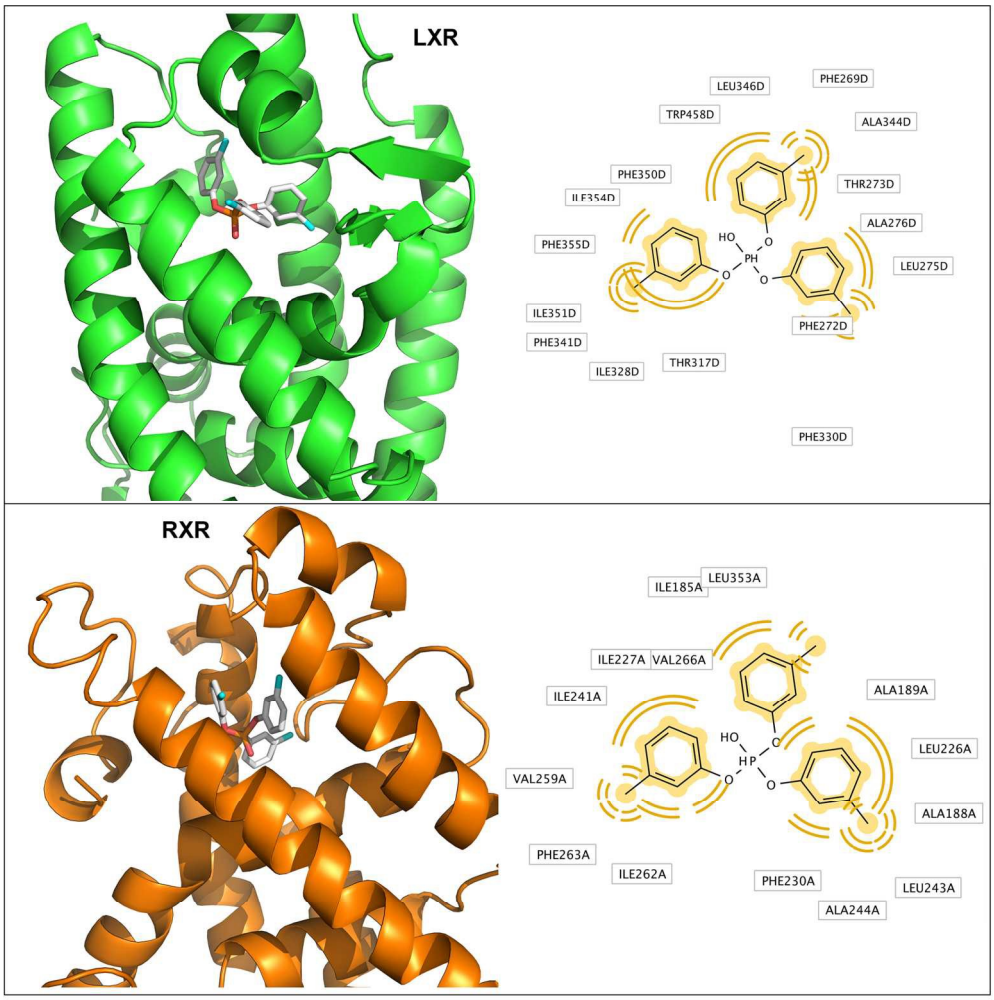


Fig. 2. 3D and 2D predicted models of the TMCP/fish LXRα (a)- RXRα (b) complexes obtained by molecular docking. In the 3D representations, the receptor is shown in cartoon mode whereas the TMCP is shown as stick (see caption of Figure 1 for further details).
177x177mm (300 x 300 DPI)

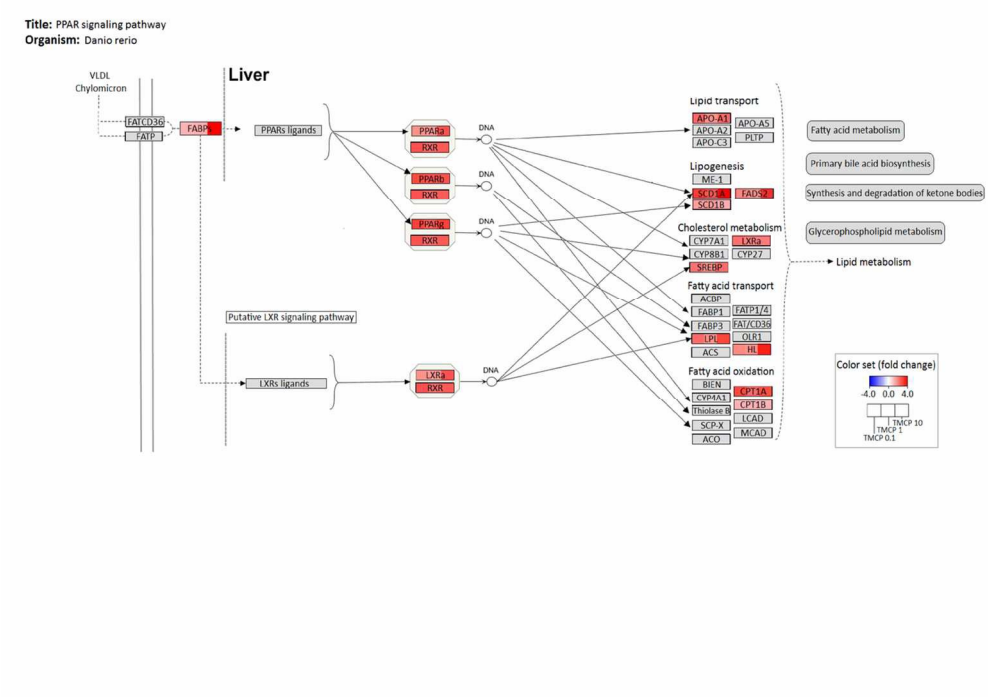


Fig. 3. Schematic representation of a selected part of PPAR signalling KEGG pathway using Pathvisio 3.2.0. The pathway created was comprehensively modified from KEGG pathway 03320, "PPAR signaling pathway" in *Danio rerio* to indicate the differentially expressed genes from the genomic analysis. Coloured squares next to the gene name represent the fold change in gene expression in TMCP-treated hepatocytes with respect to control. Marked up-regulation of genes is indicated by red coloured box.
119x83mm (300 x 300 DPI)

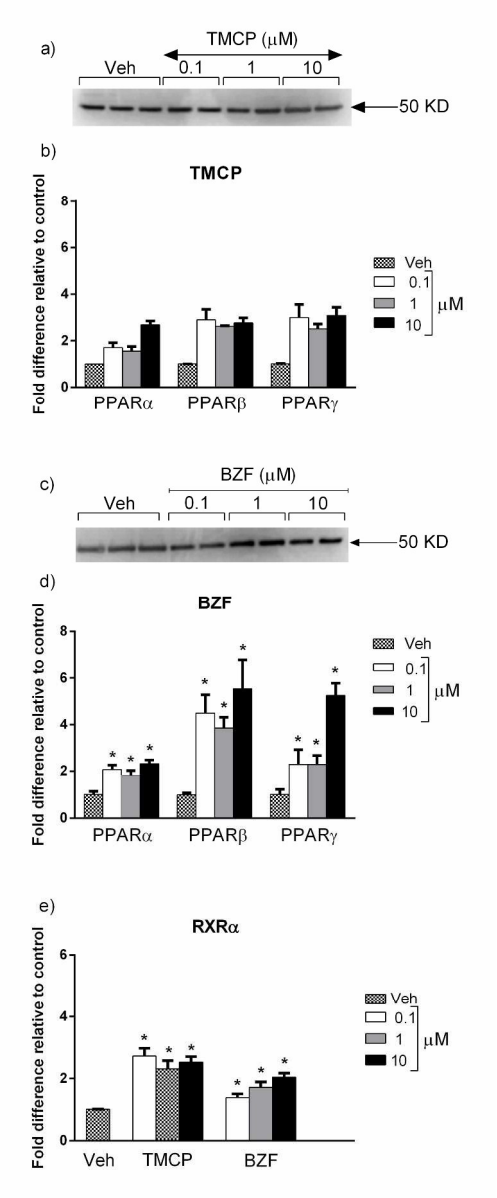


Fig. 4. Representative samples of peroxisome proliferator-activated receptor PPAR protein (a, c), PPARα, PPARβ, PPARγ (b, d), and retinoid X receptor α (RXRα) (e) mRNA levels in *Sparus aurata* hepatocytes exposed to different doses (μM) of TMCP or BZF for 48 h. q-PCR results are expressed as normalized fold expression corrected for 18s rRNA and with respect to control levels. Values are mean ± S.D. of three independent experiments. Asterisks indicate significant difference between control and treated samples (Tukey, $p<0.05$).
201x488mm (300 x 300 DPI)

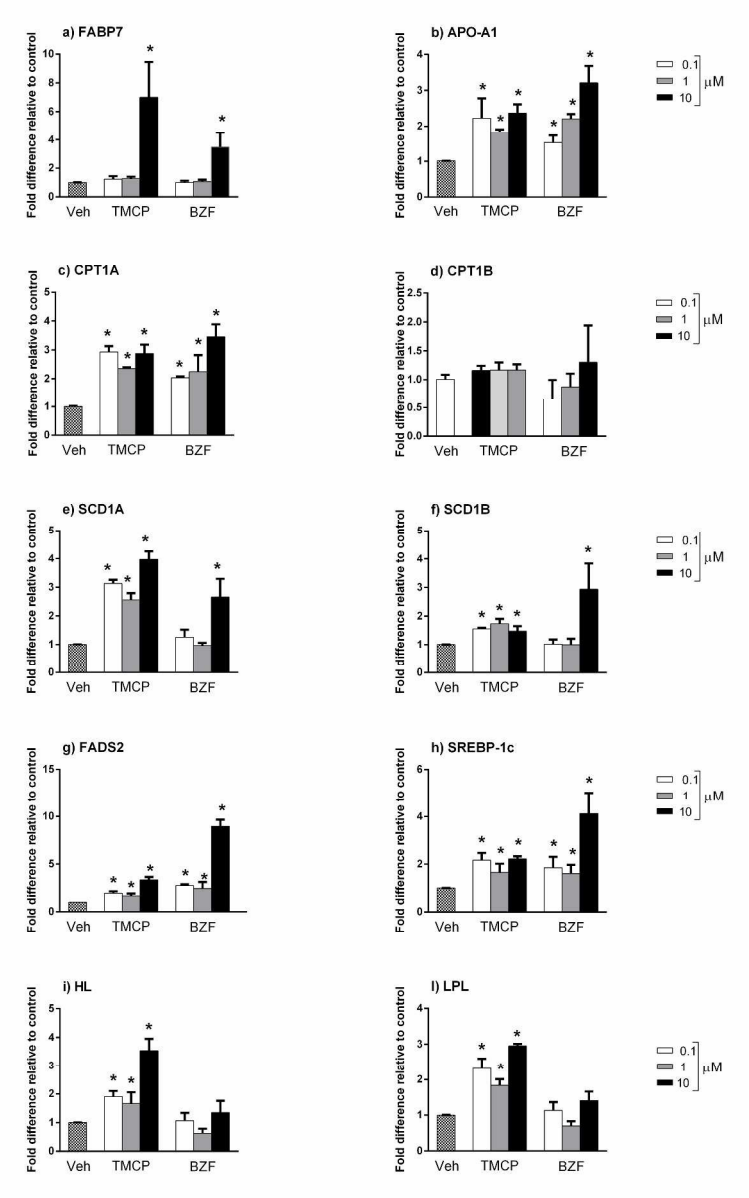


Fig. 5. Target gene expression relative to mean controls and corrected for 18s rRNA in *Sparus aurata* hepatocytes exposed to various concentrations (0.1, 1, 10 µM) of TMCP or BZF for 48 h. Target genes are FABP (a), APOA1 (b), CPT1A (c), CPT1B (d), SCD1A (e), SCD1B (f), FADS2 (g), SREBP-1c (h), HL (i), LPL (l). Values are mean ± S.D. of three independent experiments. Asterisks indicate significant difference between control and treated samples (Tukey, $p<0.05$).
274x439mm (300 x 300 DPI)

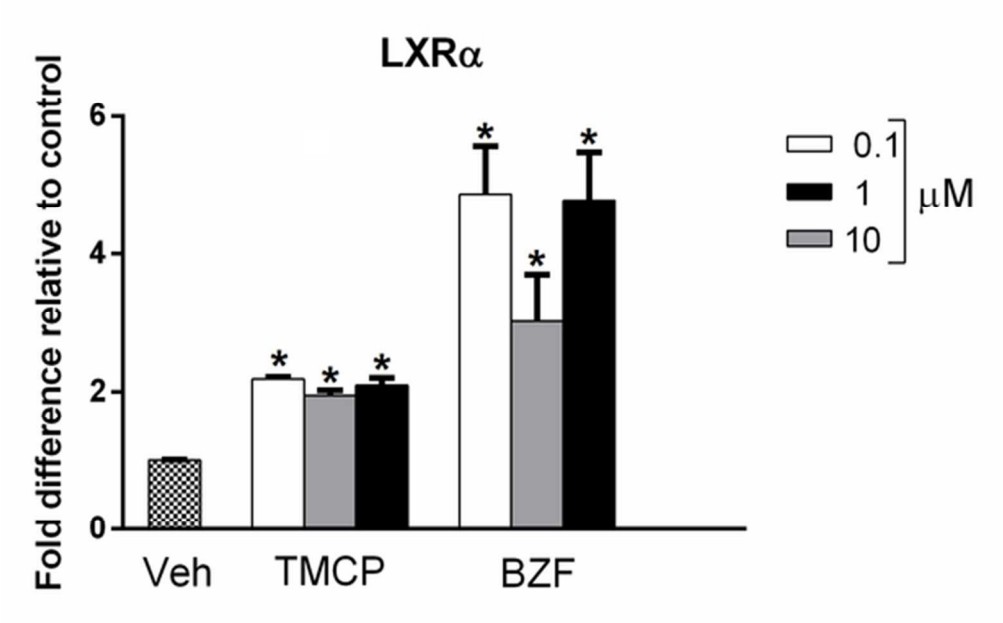


Fig. 6. Liver X receptor *α* (LXRα) mRNA levels in *Sparus aurata* hepatocytes exposed to different doses (μM) of TMCP or BZF for 48 h. q-PCR results are expressed as normalized fold expression corrected for 18s rRNA and with respect to control levels. Values are mean ± S.D. of three independent experiments. Asterisks indicate significant difference between control and treated samples (Tukey, $p<0.05$).
51x32mm (300 x 300 DPI)

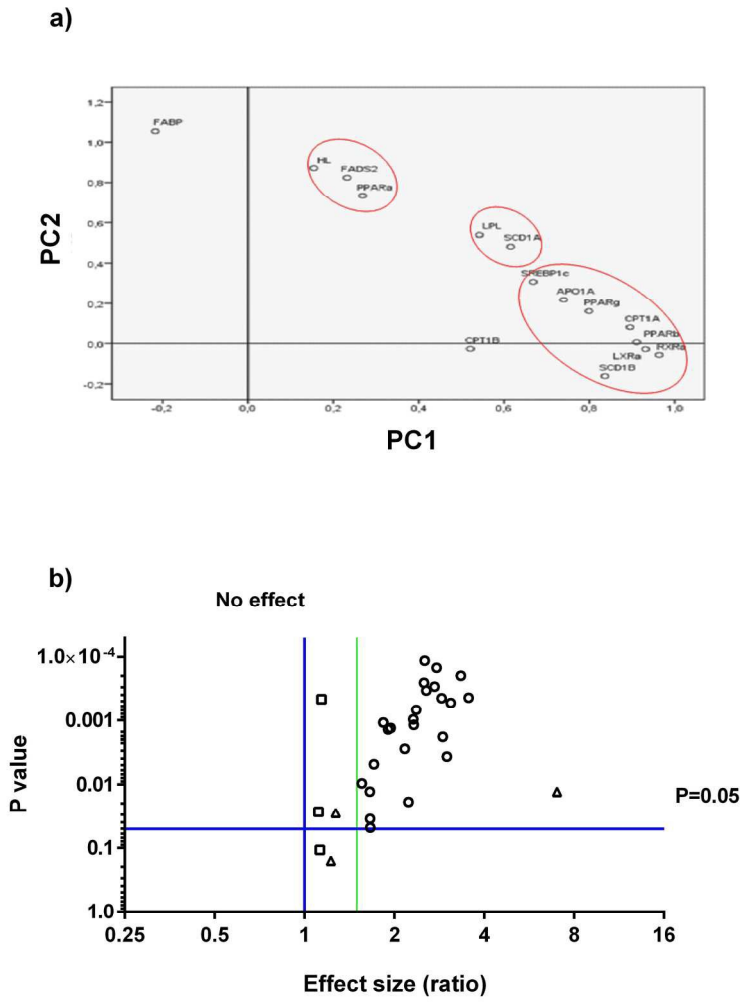
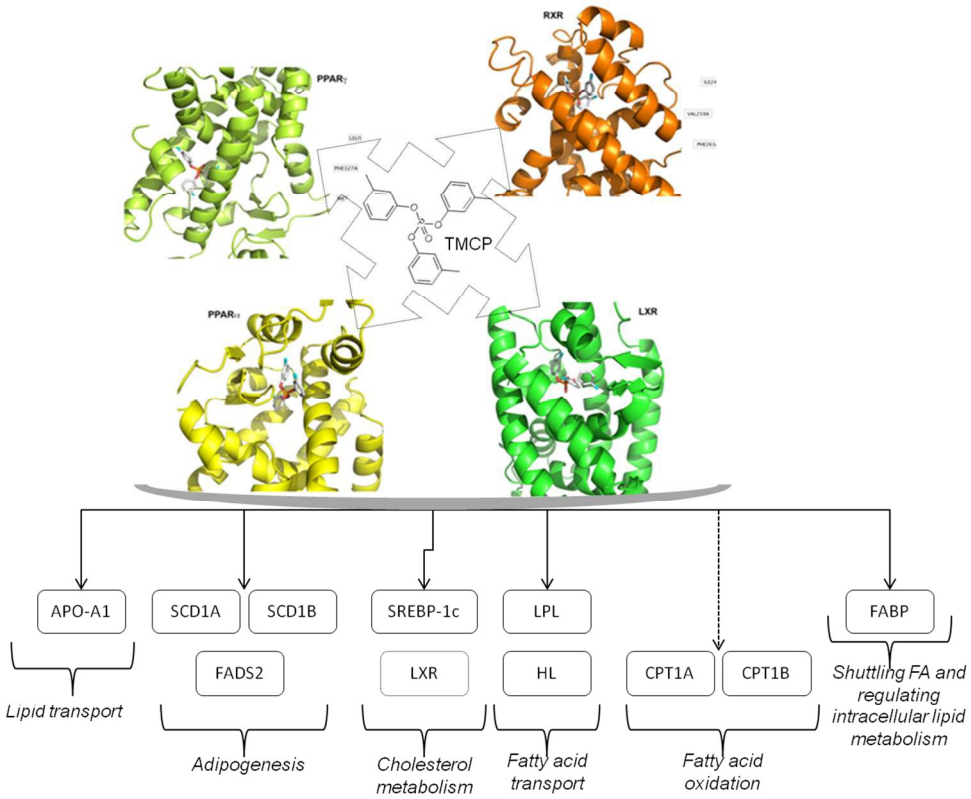


Fig. 7. Transcriptomic effects of TMCP on seabream hepatocythes. Principal Component Analysis (PCA) was conducted using expression data from all genes examined (a). Rotation method was Oblimin with Kaiser Normalization. Volcano plots of all genes of interest highlighting the upregulation of these genes by TMCP at all 3 treatment concentrations (b). CPT1B (square), FABP (triangle). The vertical green line shows where fold change = 1.5.
200x225mm (300 x 300 DPI)



Graphical Abstract
237x192mm (144 x 144 DPI)