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Expression of Estrogenic Response Genes in Black Mollies (*Poecilia sphenops*) Exposed to Pyrogenic Hydrocarbon and Petroleum from Campeche Sound

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ABSTRACT

The estrogenic effects of endocrine disrupting compounds (EDC´s) in animals are not reversible and can reduce populations. Sensitive methods such Q-PCR have been used to determine changes in gene expression and thus predict the effects before they become irreversible. The present study was designed to detect the expression on the estrogen receptors and vitellogenin genes in the Black Mollies fish (*Poecilia sphenops*) exposed to pyrogenic hydrocarbon and petroleum from Campeche Sound. The results indicate that the expression of transcript of the estrogen receptor and vitellogenin indicates are potentially useful as molecular biomarker for detecting the presence of endocrine-disruption compounds in environment.

Key words: Black Mollies fish, Endocrine Disrupting Compounds (EDC´s), Expression genes, Petroleum hydrocarbon, Pyrogenic hydrocarbon.

INTRODUCTION

The endocrine disruptors are knowing to mainly alter the hormonal and homeostatic of living systems. These systems are of primary importance since they are involved in the regulation of several significant process such as metabolism, sexual development, insulin production and utilization, growth, stress response, gender behaviour, reproduction and even in fetus development (Kabir *et al*., 2015).

Different studies demonstrate that endocrine disruptors are present in the air, water and even in the soil (Kabir *et al*., 2015). The evidences indicate that endocrine disruptors are responsible for different wildlife crisis. However, wildlife is not exposed to individual contaminants, but to a complex mixture of EDC´s. The exposure route of wildlife to EDC´s is also very critical, because many of those compounds do not persist in the environment and organisms. On the other hand, many chemicals persist in the food and habitat of wildlife. Although it largely depends on the properties and persistent nature of EDC´s, wildlife is normally exposed to them via air, water, food, soil and sediment and even via skin absorption (Kidd *et al*., 2012).

The EDC´s such as organochlorines and pesticides, as well as plasticizers, pharmaceutics and natural hormones can interact with various receptors, such as the estrogen receptor (ER), vitellogenin (VTG), androgen receptor, and aryl hydrocarbon receptor (Iguchi *et al.*, 2002). Clarifying the molecular basis of the EDC´s and the endogenous estrogens on developing organisms is essential, if we are to understand the linkages between exposure levels, timing of exposure, genes responsive to these chemicals and adverse effect (Iguchi *et al.*, 2007). Various modes of action of chemicals and non-traditional targets of the EDC´s have been summarized (Tarrant, 2005). Understanding the effect of the EDC´s on various species at the level of molecular biology is greatly needed to help explain observations at the cellular and organism levels that typically are obtained with traditional toxicological approaches.

Profiling of transcripts, proteins, and metabolites can help discriminate classes of the EDC´s and toxicants and clarify modes of action. Through systematic effort to generate mechanistic information, diagnostic and predictive assessments of the risk of the EDC´s and other toxic chemicals will be established in model species for ecological risk assessment. DNA microarray methodology has been applied to obtain genome-wide analysis of gene expression stimulated by hormones and/or chemicals (Watanabe and Iguchi, 2003). Understanding of the patterns of expression of estrogen-responsive genes is essential to comprehend the mechanisms of action of estrogenic chemicals on/non-target organs (Iguchi *et al*., 2007).

The Campeche Sound in the Southeast of Gulf of Mexico hosts a largest oil field in Mexico. It belongs to the world´s

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largest offshore oil provinces, producing more than 2 million barrels of crude oil per day and it´s important identify sentinel´s organisms to understand the effect of these activity in the enviroment. In this study, we evaluated the expression of genes involved in the VTG production by analyzing RNAm in male Black Mollies (*Poecilia sphenops*) exposed to pyrogenic hydrocarbon and petroleum from Campeche Sound, Mexico.

MATERIALS AND METHODS

The Black Mollies (*P. sphenops*) were acquired from Aquapolis Acuario (San Francisco de Campeche, Campeche, Mexico) and maintained for three weeks in 20 L glass aquarium containg 18 L of aerated and dechlorinated water according to Rendon von Osten *et al*. (2005). The organisms were fed *ad libitum*, three times a day with a commercial fish food (Treta Min, by Tetra Holding, USA). The stocking rate for the *in vivo* study was 3 fish/aquarium with four replicates per treatment. Fish were not fed during the experiment.

Table 1. Sequences of primer pairs used in Q-PCR study (Ishibashi *et al*., 2008).

GENE NAME	PRIMER SEQUENCES
Estrogen receptor	5′-GTCAGTCGGTTACTTGGCC-3′
α (AB033491)	5′-CATCACCTTGTCCCAACCTG-3′
Estrogen receptor	5′-GTGGACTCAACTTTCGGC-3′
ß (AB070901)	5′-CACGTCGCAGCAGGATCTT-3′
Vitellogenin I	5′-TGGAAAGGCTGATGGGGAAG-3′
(AB064320)	5′-AACTGCAGGCATGGTGAGCC-3′
Vitellogenin II	5′-GTCTTCAGGAGGTCTTCTTC-3′
(AB075891)	5′-GGTAGACAATGGTATCCGAC-3′
β-Actin	5′-AGACCACCTACAGCATC-3′
(S74868)	5′-TCTCCTTCTGCATTCTGTCT-3′

A total of 12 fishes were exposed to 1 mg L^{-1} of burned grass as a source of pyrogenic hydrocarbon, another group of 12 fishes was exposed to 1 mg L^{-1} of a sample form an oil spill occurred in 2007 in the Gulf of Mexico. The chemical analysis of the oil indicates the presence of aliphatic hydrocarbons of C14 to C39 and aromatic hydrocarbons acenaphthene, acenaphthylene, anthracene, benzo [k] fluoranthene, fluoranthene, fluorene, indeno [1,2,3-cd] pyrene and phenanthrene (Rendon von Osten, 2009) and 12 fishes were kept as controls. All the fishes were stocked in ASTM hard water. The bioassays were carried out for 36 h in static test design according to chronic exposure criteria by Orlando *et al*. (2002). Every 12 h, four fish from each treatment were sacrificed to remove the liver.

Total RNA was isolated from tissue of the liver of the fishes in accordance with the manufacturer´s instructions (RNA purification kits GeneJet, Thermo Fisher Scientific, Foster City, USA). RNA was quantified using NanoDrop ND-1000 Spectrophotometer (NanoDrop, Wilmington, DEE) and its quality was assessed by the presence of ribosomal bands in ethidium-bromide stained agarose gels. The RNA was diluted to approximately 1 mg mL^{-1} for RT-PCR. The RT-PCR was performed according to the manufacturer´s instructions (TaqMan Reverse Transcription Reagent, Thermo Fisher Scientific, Foster City, USA). For the relative quantification of gene expression, Q-PCR on StepONE Q-PCR equipment (Applied Biosystems, Foster City, USA) using Maximum SYBRGreen/ROX QPCR Master Mix (Thermo Fisher Scientific, Foster City, USA) was used. The sequences of the primers are shown in Table 1. PCR conditions were as follows: initial denaturation at 94°C for 30 sec, hybridization at 60°C for 30 sec and extension at

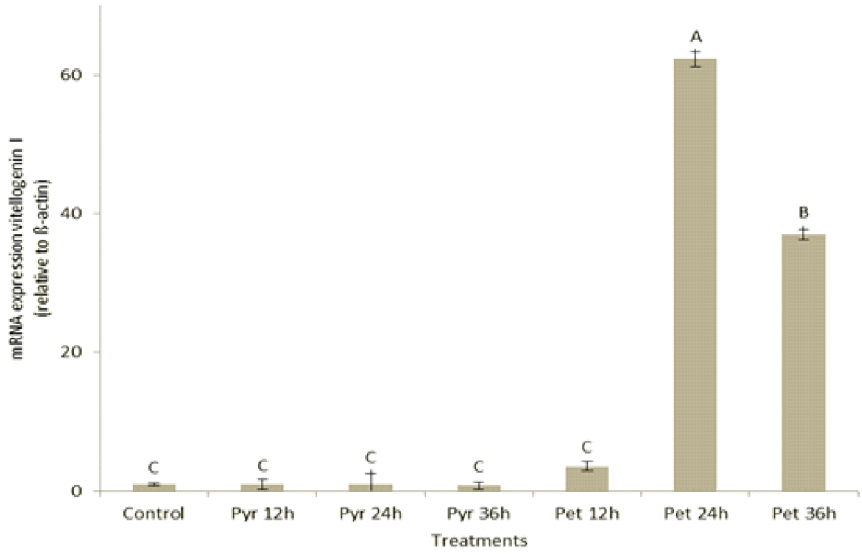

Fig 1: Mean and standard deviation of the relative intensity of Vitellogenin I receptor transcript in the gonads of Black Mollies fishes exposed to Petrogenic (Pet) and Pyrogenic (Pyr) hydrocarbon. Values with the same superscript are not statistically different (p>0.05).

72°C for 1 min. For quantification gene β-actin was used as reference and $2^{\Delta CT}$ method, to calculate the expression (Kenneth *et al*, 2001).

Values of parameters were used to detect interaction between petroleum and pyrogenic hydrocarbons and exposure time by two-way ANOVA. If a significant interaction was detected between the main effects, the variable was analysed using a one-factor ANOVA. If there was a significant difference, *F*-test were performed using the STATGRAPHICS Centurion X (The Plains, VA, USA).

RESULTS AND DISCUSSION

The expression of the VTG I gene in Black Molly in response to pyrogenic and petrogenic hydrocarbon is shown in Fig 1. At 24 h the VTG I expression was a maximum decreasing after 36 h of exposure to petrogenic hydrocarbon; in contrast, there no evidence of effect in the organisms when there exposed to pyrogenic hydrocarbon.

The maximum expression of the VTG II was observed at 36 h of exposure to the pyrogenic hydrocarbon; on the other hand, with petrogenic hydrocarbon the maximum expression was at 12 h with linear decrease over time (Fig 2).

For the Estrogen Receptor α gene, an increase over time with maximum values at 36 h in both hydrocarbon source (Fig 3).

Only after 24 h and increase was observed in the expression of Estrogen Receptor β gene in organisms exposed to petrogenic hydrocarbons, without expression in the other samples (Fig 4).

The hepatic VTG is a sensitive biomarker for estrogenic

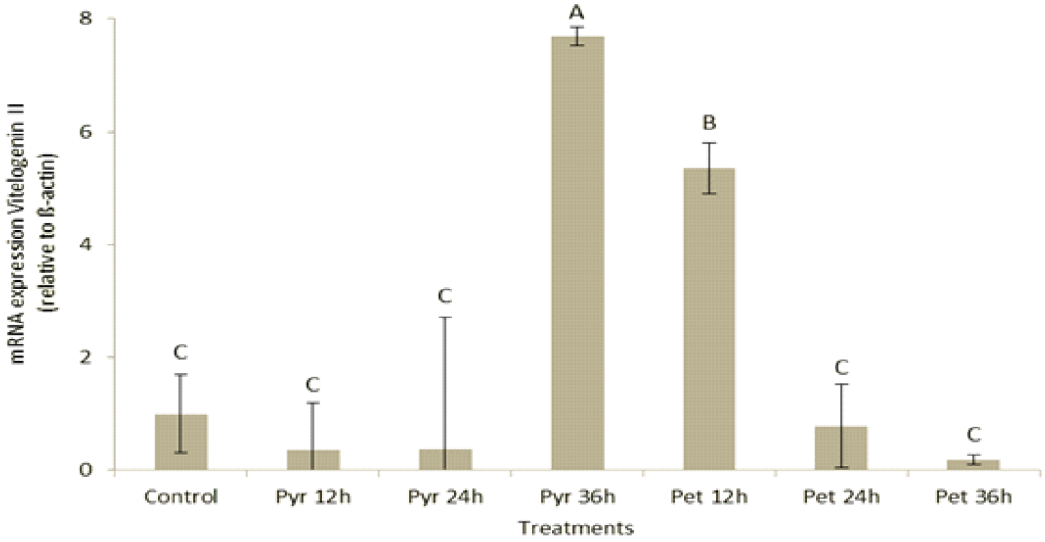

Fig 2: Mean and standard deviation of the relative intensity of Vitellogenin II receptor transcript in the gonads of Black Mollies fishes exposed to Petrogenic (Pet) and Pyrogenic (Pyr) hydrocarbon. Values with the same superscript are not statistically different (p>0.05).

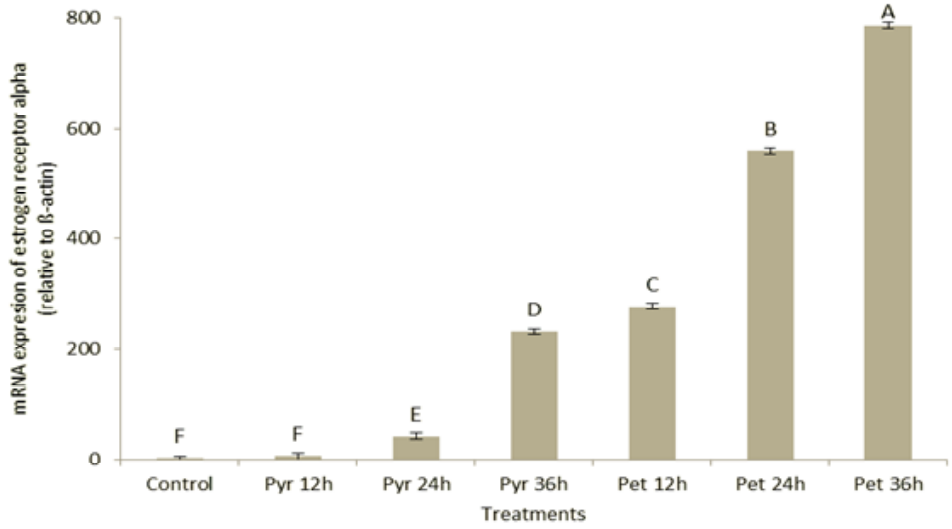

Fig 3: Mean and standard deviation of the relative intensity of Estrogen Receptor α transcript in the gonads of Black Mollies fishes exposed to Petrogenic (Pet) and Pyrogenic (Pyr) hydrocarbon. Values with same superscript are not statistically different (p>0.05).

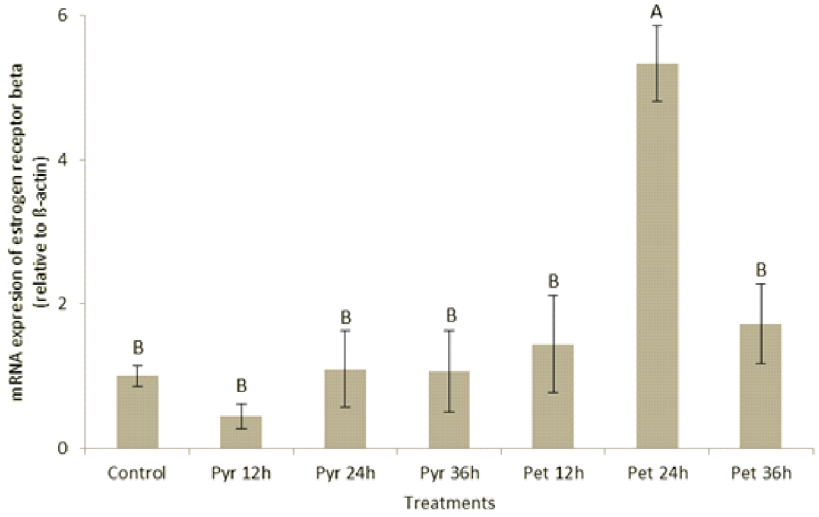

Fig 4: Mean and standard deviation of the relative intensity of estrogen receptor ß transcript in the gonads of Black Mollies fishes exposed to Petrogenic (Pet) and Pyrogenic (Pyr) hydrocarbon. Values with the same superscript are not statistically different (p>0.05).

effect of hydrocarbons. The VTG is synthesized in response to stimulation by estrogenic chemicals that bin to the ER´s forming ER complexes that then bin to the estrogen response elements of the target genes to regulate their expression (Watanabe *et al*., 2009). A recent *in vitro* study with primary cultured zebrafish hepatocytes suggested that hydrocarbons have a common estrogenic effect but may interact differently with ER isoforms, perhaps contributing directly to differential the ER and the VTG gene transcription (Maradonna *et al*., 2013). Several studies have reported the induction of the VTG synthesis or transcription in the fish or cultured male fish hepatocytes upon exposure to hydrocarbons (Carnevali *et al*., 2010; Uren-Webster *et al*., 2010; Maradonna *et al*., 2013). Although the induction of the VTG could depend on species sensitivity, exposure time, and concentrations, these studies demonstrate the estrogenic activity of hydrocarbons (Wang *et al*., 2013). In our study the expression of the vitellogenin-related genes in the Black Molly fish was observed only when exposure was to petrogenic hydrocarbon and not to pyrogenic hydrocarbon, probably due to the nature of the compounds. Kitana *et al*. (2007) detect that low-level of polycyclic hydrocarbon may disrupt endocrine function in males of *Chrysemis picta* by an increase in the plasma concentration of vitellogenin; they explain that hydrocarbons exert their effects via aryl hydrocarbon receptor, and this gene regulate the function of the other genes that effect the development of the gonads. Rochman *et al*. (2014) concluded that petroleum products alter the function of endocrine system in aquatic animals and a permanent effect could be observed; in this work we concluded that the same effect occur in Black Molly fish.

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