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Expression of estrogenic response genes to different concentration of 17ß-estradiol in male mosquitofish (*Gambusia yucatana*)

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Abstract

The estrogenic effects of endocrine disrupting compounds in fish are not reversible and can reduce populations. Sensitive methods such as Q-PCR, Western blot, microarrays, have been used to determine changes in gene expression and this predict the effects before they become irreversible. The present study was designed to detect the expression of the estrogen receptors vitellogenin and pregnane X indicates that they are potentially useful molecular markers for detecting the presence of endocrine disrupting compounds in the environment. The results demonstrate that the exposure to the hormone 17ß-estradiol induced the expression of estrogenic response genes in male mosquito fish (*G. yucatana*) and this is related to the alterations to the endocrine system caused by the hormone.

Keywords: Gene expression, Biomarkers, Endocrine disrupting compounds (EDCs), Mosquitofish (*Gambusia yucatana*), vitellogenin; Q-PCR.

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Introduction

An endocrine-disrupting compound (EDC) has been defined by the U.S. Environmental Protection Agency (EPA) as "an agent that interferes with the synthesis, secretion, transport, bonding or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behavior" (Kavlock *et al.,* 1996). This means that endocrine disruptors are chemicals, or chemical mixture that interfere with normal hormone function.

Endocrine disruptors are known mainly to alter the hormonal and homeaostatic systems of living system. These systems are primary importance since they are of primary importance since they are involved in the regulation of several significant processes such as metabolism, sexual development, insulin production and utilization, growth, stress response, gender behavior, reproduction and even in fetal development (Kabir *et al.,* 2015).

Different studies have shown that endocrine disruptors are present in the air, water and even in the soil (Kabir *et al.,* 2015). Many 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 EDCs. The exposure route of wildlife to EDCs is also very critical because many of those compounds do not persist in the environment and organism. On the other hand, lots of chemicals persist in the food and habitat of wildlife. Although it is largely depends on the properties and persist nature of EDCs, wildlife is normally exposed to them via air, water, food, soil and sediment, and even via skin absorption (Kidd *et al.,* 2012). Many EDCs are degraded in the environment in various time periods. Some EDCs are found to be highly soluble in water and might be present on water from the level of parts per trillion to parts per billion.

EDCs such as organochlorines and pesticides, as well as plasticizers, pharmaceutics and natural hormones can interact with different receptors, such as the estrogen receptor (ER), vitellogenin (VTG), androgen receptor, and aryl hydrocarbon receptor (Iguchi *et al.,* 2002). Clarifying the molecular basis of EDCs and endogenous estrogens on developing organisms is essential, if we are to understand the linkages between exposure levels, timing exposure, genes responsive to these chemicals, and adverse effects (Iguchi *et al.,* 2007). Various modes of action of chemicals and non-traditional targets of EDCs have been summarized (Tarrant, 2005). Understanding the effects of EDCs 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 approach.

Profiling of transcripts, proteins, and metabolites can help discriminate classes of EDCs and toxicants and clarify modes of action, through systematic efforts to generate mechanistic information, diagnostic and predictive assessments of the risk of

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EDCs and other toxic chemicals will be established in model species for ecological risk assessment. Toxicogenomics an ecotoxicogenomics also provide important information on the basic biology of animals (Iguchi *et al.*, 2007).

DNA microarray methodology has been applied to obtain genome-wide analysis of genes expression stimulated by hormones and/or chemicals (Watanabe and Iguchi, 2003). An understanding of estrogen-responsive genes expression patterns is essential to comprehend the mechanisms of estrogenic chemicals actions on no-target organs (Iguchi *et al.*, 2007).

One of the techniques for monitoring the expression of VTG genes is by determining RNAm, a method that is sensitive to changes in the expression of genes in response to exposure to estrogens and xenoestrogens. It has been demonstrated, in fish, the effects of endocrine disruptors can be detected by the expression of the genes involved in hormone development, before the response can be quantified using conventional analytical methods and even before the effect is visible (Ishibashi *et al.*, 2008; Yamaguchi *et al.*, 2008).

The mosquitofish (*Gambusia yucatana*) is one of the species that have been using for toxicological evaluations. It is widely distributed in the aquatic systems of the Yucatan Peninsula and is easily adapt to the environmental conditions changing (Miller, 2005). Additionally, biomarkers such acetylcholinesterase and glutathione transferase, have been used to determine the quality of the environment (Rendon von Osten *et al.*, 2005, 2006) and also to determine the estrogenic effects of 17ß-estradiol and other chemical contaminants, therefore it is essential to continue these studies to use species in bio-monitoring. In this study we evaluated the expression of genes involved in the VTG production by analyzing RNAm in male mosquitofish (*G. yucatana*) exposed to different concentrations of 17ß-estradiol.

Materials and methods.

Mosquitofish were collected from a small and no contaminated pond in the Ria Creek in Campeche, Mexico. Collected fish samples were maintained and cultivated in an artificial pond for three months. Organisms were fed *ad libitum* three times a day with commercial fish food (TretaMin, by Tetra Holding, USA). Fish samples for studies were acclimated for 1 week in 20 L glass aquarium containing 18 L of aerated and dechlorinated water according to Rendon von Osten *et al.* (2005). The stocking rate for the *in vivo* study was 3 fish/aquarium. Fish were not fed for 24 h prior to the experiments and feed was provided only during the test medium renewal period.

A total of 45 fishes were exposed to 17ß-estradiol at nominal concentrations of 500, 1 000, 10 000 and 15 000 ng L^{-1}. Test solutions were prepared by dissolving the appropriated amount of 17ß-estradiol stock solution in ASTM hard water. Two control were included, one with ASTM hard water and another one with ethanol in the experimental

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design. Bioassays were carried out for 14 days in a semi-static test design with test medium renewal each 96 h according to chronic exposure criteria by Orlando *et al.* (2002).

Total RNA was isolated from liver and gonad tissue of male fish according to instructions (Ultra Clean Tissue & Cells RAN Isolation, Mobio Company). RNA was diluted at about 1mg ml^{-1} for RT-PCR or stored at -80º C. The RT-PCR was performed with total RNA extracted from mosquitofish´s livers and gonads. The RT reaction mixture contained 5 µg of total RNA, 1 µL of RNAase inhibitor, 1 µL of oligo (dT) primer and diethylpyrocarbonte-treated water. The reaction mixture was heated at 70º C for 10 min and quickly chilled on ice. After cooling, 4 µL of 5x reaction buffer containing 25 mM $MgCl_2$, 2 µL of deoxynucleotide triphosphate (dNTP´s; 10 mM each), 1 µl of RNAase inhibitor, and 1 µL of ReverTra Ace (BIORAD) were added to a total volume of 20 µL. The reaction mixture was incubated for 60 min at 42º C. The reaction mixture was heated for 5 min to stop the RT.

The PCR reactions was contained 2 µl of the RT reaction mixture as the cDNA template, 5 µL of 10x PCR buffer, 1 µL of Taq polymerase (5 U $µl^{-1}$, BIORAD), 3 µL of 25 mM $MgCl_2$, 1 µL of dNTP´s (10 mM) and 15 pmol of both sense and antisense primers. The primer pair sequences are show in Table 1. The total volume of the reaction mixture was 50 µl. the PCR conditions were as follows: initial denaturation at 94º C for 2 min, 45 cycles of denaturation at 94º C 30 s, annealing at 60º C for 30 S and extension at 72º C for 60 s, and final extension at 72º C for 10 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gels. The gels were stained with ethidium bromide (15 µL of a 10 mg mL^{-1} ethidium bromide solution per 100 ml water). The band densities of amplified products were calculated using Quantity One Software (BIORAD).

Table 1: Sequences of primers pairs used in the RT-PCR study.

Gene name	Primer sequences	References
Estrogen receptor (AB033491)	F: 5´-GTCAGTCGGGTTACTTGCC-3´ R: 5´-CATCACCTTGTCCCAACCTG-3´	Ishibashi *et al.* (2008)
Vitellogenin (AB064320)	F: 5´-TGGAAAGGCTGATGGGGAAG-3´ R: 5´-AACTGCAGGCATGGTGAGCC-3´	Ishibashi *et al.* (2008)
Pregnane X receptor	F: 5´-GAGGAGCAAGAGCACGAATC-3´ R: 5´-ATGAAGCACAGAGGCTGGAG-3´	Yamaguchi *et al.* (2008)
ß-actin (S74868)	F: 5´-AGACCACCTACAGCATC-3´ R: 5´-TCTCCTTCTGCATTCTGTCT-3´	Ishibashi *et al.* (2008)

Significant differences in relative ratios of estrogen-responsive genes and ß-actin mRNA between treatment groups and controls were analyzed by one-way analysis of variance followed by Duncan Multiple Comparison Test. All

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statistical analyses were performed using Statgraphics Centurion XV software.

Results

The RNA and RT-PCR extraction techniques worked well under the set conditions, however, during amplification, the results were unsatisfied with the quantities of cDNA used by Ishibashi *et al.* (2008) and Yamaguchi *et al.* (2008). Therefore, we tested different cDNA concentrations and found that a concentration of 1 µl of RT reaction mixture produced the best gene amplification.

The results of the expression of the estrogen receptor transcripts in liver and gonads are given in Fig. 1. The expression of the gene in the liver was greater than in the gonads for all of the samples with the exception of the negative control where it was greater in the gonads (p>0.05). Females exhibited the highest expression in the liver, significantly different from the other samples (p>0.05); however, in the gonads there were no differences relative to the negative control and the concentration of 10 000 ng L^{-1}. The concentration of 10 000 ng L^{-1} caused the highest expression in both types of tissues compared to the other concentrations used.

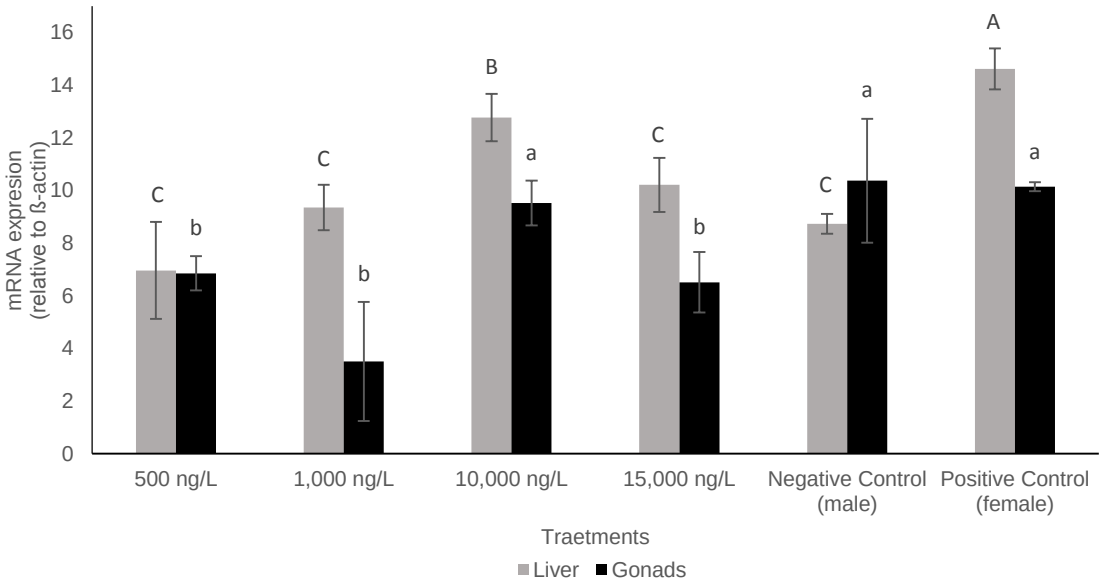


Figure 1: Relative intensity of estrogen receptor transcript expression in the liver and gonads. Bars are the standard deviation of the mean. Values with the same superscript are not statically different (p>0.05).

The greatest expression of the vitellogenin gene was observed in the treatment with 1 000 ng L^{-1}, which was significantly different from the other treatments and the controls (Fig. 2). Females had the greatest expression of this gene with the exception of the treatment with 1000 ng L^{-1} for both types of tissue and the treatment with 500 ng L^{-1} for liver (p>0.05).

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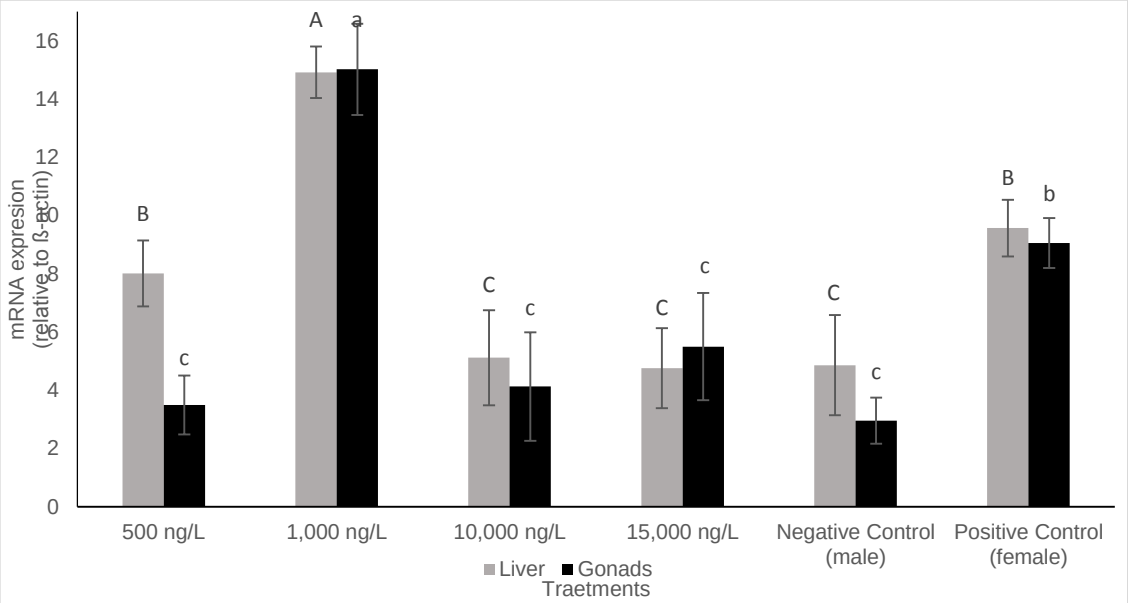


Figure 2: Relative intensity of vitellogenin transcript expression in the liver and gonads. Bars are the standard deviation of the mean. Values with the same superscript are not statically different (p>0.05).

Females also had greatest expression of the pregnant X receptor gene in both types of tissue with respect to the other treatments, with the exception of the gonad samples for the treatments with 500 and 10000 ng L^{-1}, which were not statistically different from the other sample (Fig. 3). The 1000 ng L^{-1} treatment was associated with the lowest expression of the gene in both types of tissue.

Discussion

The steroid hormones that circulate in the organisms' plasma are essential to reproduction and sexual differentiation. The estrogenic hormones increasingly dumped in the environment are contaminants and detecting them requires efficient, sensitive methods, such as using molecular biomarkers. The species used as an indicator in this study was the mosquito fish (*G. yucatana*), a poecilid that has been used to evaluate toxicity biomarkers such as acetylcholinesterase (AChE) and glutathione S-transferase (GST) (Rendon von Osten *et al*.,, 2005), along with several response parameters such as vitellogenin concentration, sperm count, the gonadosomatic and gonadopodial indices (Dzul-Caamal, 2007).

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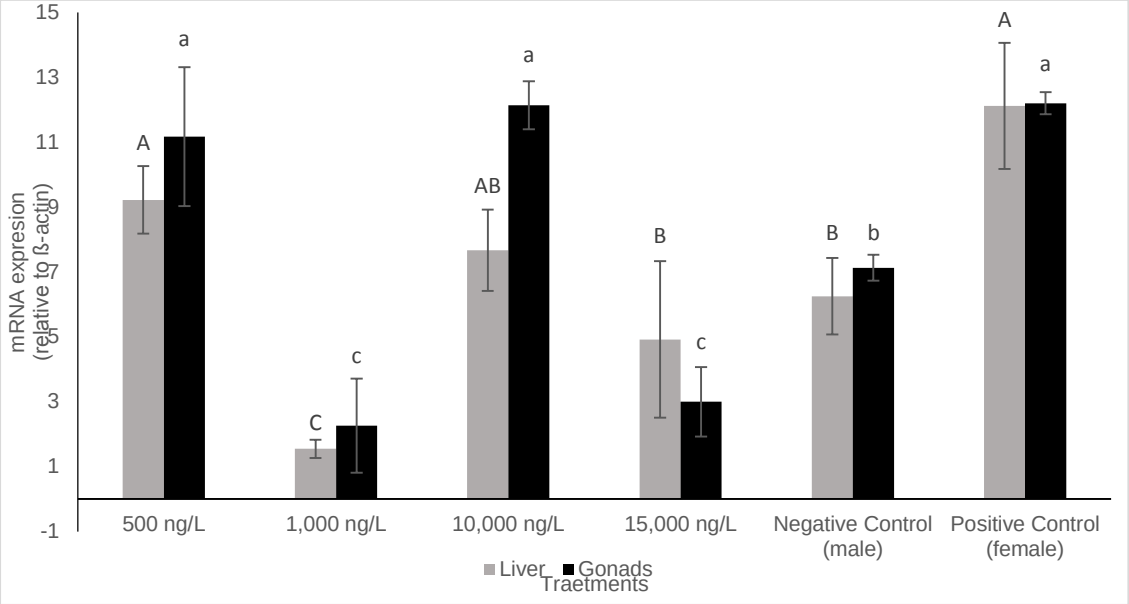


Figure 3: Relative intensity of pregnane X receptor transcript expression in the liver and gonads. Bars are the standard deviation of the mean. Values with the same superscript are not statically different (p>0.05).

In this study survival rate was low (<48), which could be a result of exchange causing chemical stress in the exposed organisms and causing their death (Auró and Ocampo, 1999). After being exposed to 17ß-estradiol, the mosquito fish showed no apparent change in their behavior and were physically healthy with characteristics similar to reported by Schoenfuss *et al.* (2001).

Exposure to 17ß-estradiol stimulated the induction of vitellogenin and the expression of the genes involved in it, allowing us to infer that this compound causes changes in the endocrine system of the fish similar to those reported by Lori (2007) for Nile tilapia (*Oreochromis mossambicus*) under 17ß-estradiol concentrations of 5 µg g^{-1}. Jin *et al.* (2008) the vitellogenin genes were observed to express at 17ß-estradiol concentrations of 10 to 1 000 ng L^{-1} in zebra fish (*Danio rerio*) liver, authors reported significant differences starting 50 ng L^{-1} and the greatest expression at 1 000 ng L^{-1}. In the present study the concentration of 15 000 ng L^{-1} for male mosquito fish was comparable to the expression observed in female mosquito.

Increasing 17ß-estradiol in natural fish population can cause a decrease in their reproductive efficiency because this hormone interacts with the estrogen receptors and can cause a decrease in the gonadosomatic index. Dzul-Caamal (2007) also observed a decreasing in this index at concentrations of 500 to 15 000 ng L^{-1} of 17ß-estradiol in male *G. yucatana* fish. In the present study 17ß-estradiol was observed to have an estrogenic effect on male mosquito fish and induce the expression of the vitellogenin gene, as observed by Yamaguchi *et al.* (2005).

The family of estrogen receptor can be induced by xenoestrogens and produce different responses: phosphorylation of signals regulated by

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kinases, abnormal cell growth and the increased expression of genes (Albanito *et al.*, 2009). Studies by Hawkins *et al.* (2000) and, Liang and Fang (2012) mentioned that the estrogen receptors participate in the regulation of vitellogenesis and have been isolated in teleost fish; these receptors have been proposed to be sensitive to variations in estrogen levels. The present study revealed that the estrogen receptor expressed to different degrees at all of the concentrations of 17ß-estradiol used, which is in agreement with the results of Yamaguchi *et al.* (2005); these authors observed gene expression at concentrations between 10 and 10 000 ng L^{-1}. In contrast, Choi *et al.* (2004) studied the effects of the induction by 17ß-estradiol on estrogen receptor in male olive flounder (*Paraclichthys olivaceus*), and observed that gene expression was dose-dependent.

Yamaguchi *et al.* (2008) observed in *Oryzias latices*, pregnant X receptor (PXR) is expression in several different types of tissue, though mainly in the liver and intestine. Furthermore, PXR participates as xenoestrogen mediator, similar to our findings, given that the gene expressed in both tissues. Steven *et al.* (2002) mentioned that in females during gestation this gene is for protection of the fetus, but in males it can be express in the presence of high levels of exogenous steroids. The females used in our study were gestating and therefore PXR expressed in them, and in the males as protection against the presence of 17ß-estradiol, like studies of Xie *et al.* (2000).

A decrease in the intensity of gene expression was observed when the fish were exposed to a concentration of 1 000 ng L^{-1} of 17ß-estradiol, which might have been the result of stress. In this regard, Palstra *et al.* (2010) reported that when organisms make an effort and are under stress there is a decrease in the levels of expression of estrogen-dependent genes as consequence of directing energy to their muscles as strategy to allow then to flee quickly and/or prevent muscular atrophy. It is worth mentioning that a malfunction was observed in gene expression, and this might have been due to the prolonged exposure (15 days) to 17ß-estradiol (Dzul-Caamal, 2007). Yamaguchi *et al.* (2008) mentioned that when organisms are exposed to xenobiotics in a controlled fashion, gene expression should be evaluated over a period no longer than 96 h because after that length of time metabolic adaptation to the contaminant occurs.

In conclusion, we found that our results demonstrate the exposure to the hormone 17ß-estradiol induced the expression of estrogenic response genes in male mosquito fish (*G. yucatana*) and this is related to the alterations to the endocrine system caused by the hormone. In addition, the mosquito fish has been shown to be a good indicator for studies of gene expression using more sensitive techniques such as Q-PCR and exposure trials in the environment to verify usefulness of this species as bio-indicator.

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