ECOTOXICOLOGICAL EFFECTS OF POPs ON ARIIDAE *Ariopsis felis (*LINNAEUS, 1766) FROM THREE COASTAL ECOSYSTEMS IN THE SOUTHERN GULF OF MEXICO AND YUCATAN PENINSULA

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Abstract
Persistent Organic Pollutants (POPs) are still used for agricultural and disease vector control, as well as for industrial purposes. In the last decades, various studies have shown that fish are sensitive to the toxicological effects of certain POPs, including a large class of endocrine- disrupting chemicals (EDCs). In the present study, the relationship between POPs and their effects using Vitellogenin gene expression as biomarker of effect in hardhead catfish *Ariopsis felis* (Linnaeus, 1766) from three Ecosystems in the Southern Gulf of Mexico and Yucatan Peninsula are discussed. Contaminant results showed that median concentrations of PCBs, HCHs, DDTs and Chlordanes were higher in Terminos Lagoon with respect to Celestun and Dzilam. In the same way, the Vitellogenin gene expression was clearly over-expressed in fish collected from Terminos Lagoon. Principal Component Analysis showed that Vitellogenin gene expression is related to the concentrations of total DDTs and PCBs, and negatively related to total Drins. Overall, this study represents the first tests exploring changes in molecular diagnostic indicators following exposure of several organic compounds in our country. Vitellogenin gene expressions associated with some endocrine disruptors detected in Terminos Lagoon were measured and we can now report clear changes in fish exposed.

Introduction
Fish have been a particularly popular model for studying the xenobiotic biotransformation and the estrogenic endocrine disruption related to several POPs[1, 2, 3, 4]. Exposure to several classes of chemicals and chemical mixtures is known to alter fish vitellogenesis *in vivo*, including the alkylphenols nonylphenol and octylphenol, the steroidal estrogen ethinylestradiol, the pesticides methoxychlor, chlordane, *o,p´*-DDT and their metabolites[5, 6, 7]. Direct activation of vitellogenesis in hepatic cell culture also occurs in response to some environmental estrogens, including, Aroclor 1254 (a polychlorinated biphenyl mixture), bisphenol A, chlordecone, and lindane.

The goal of the present study was to evaluate the concentration of polychlorinated byphenils (PCBs) and organochlorine pesticides in a representative fish species (*Ariopsis felis*), to assess the exposure and effects of these POPs using Vitellogenin gene expression as a biomarker of effect, and to find the possible relationship between POPs concentrations and biomarker response. This study was carried out during the dry season, a region highly influenced by human activities, such as runoffs of intensive agriculture areas, direct discharges of untreated municipal effluents and chemical release from oil production and refining. The three selected coastal lagoons were: Terminos Lagoon, in the State of Campeche; Celestun, on the border between the states of Campeche and Yucatan, and Dzilam, in Yucatan.

There are no published antecedents for organochlorine pesticides in sediments or fish for Dzilam or Celestun lagoons, but there are a few published papers for Terminos Lagoon and its associated ecosystems, Laguna de Pom and Rio Palizada. Gold-Bouchot *et al*[8, 9, 10] reported pesticide and PCBs concentrations in sediments and biota (oysters, clams, and shrimp) of the lower Palizada river. Levels were relatively low, and were not considered as a cause for concern then. Gold-Bouchot *et al*[11] analyzed pollutant concentrations and biomarkers in the American oyster, *Crassostrea virginica*, finding good correlations between both parameters and thus recommending the use of biomarkers in environmental assessment studies.

Materials and methods
Sixty one male catfish (*Ariopsis felis*), with an average total length of 120.3 cm, were collected during the dry season at three coastal lagoons in the Atlantic coast of Mexico: Terminos Lagoon, Celestun and Dzilam. Catfish were captured at five stations on each lagoon and were selected by their size. Later, fish were killed by a blow on

the head and livers were removed and sectioned in two parts. One of these sections was frozen immediately at 120 °C in liquid nitrogen for molecular analysis, and the other was immediately frozen for organic compound analysis.

Levels of organochlorine pesticides and PCBs in fish were determined according to the procedures described in Sericano *et al*[12] and Wade *et al*[13]. Briefly, freeze-dried liver were extracted with hexane and methylene chloride for 12 hours each in a soxhlet apparatus. Extracts were concentrated in flat-bottomed flasks equipped with three-ball Snyder column condensers and mixed. Extracts were fractionated by alumina:silica column chromatography and two fractions were obtained by sequential elution: aliphatics and aromatics. Lipids were removed by size-exclusion chromatography using a Beckman HPLC. Analytes were separated and quantified by gas chromatography using a Hewlett Packard 5890 Series II gas chromatograph equipped with a 30 m x 0.25 mm HP-5 capillary column working in the splitless mode, and an electron capture detector (ECD) at 325 °C. Individual compounds were identified and quantified using authentic standards from Ultra Scientific®. Quality assurance of the analytical procedures included the addition of internal standards and the analysis of a procedural blank for each set of samples. Recoveries were 87% on average.

For biological monitoring total RNA was extracted from the liver of catfish using the RNAzol method (Trizol REAGENT, Invitrogen) according to the method described by Chomczynski and Sacchi[14]. The purity of RNA samples and their concentration were measured spectrophotometrically, obtaining A260/A280 ratios between 1.8 and 2.0. RNA (30 g per lane approximately). Total RNA was separated electrophoretically in 1.2% formaldehyde-agarose gels; its integrity and quantity were evaluated by staining the gels with ethidium bromide. For analysis of Vitellogenin gene expression, degenerate fish Vtg oligonucleotides were synthesized according to an alignment of the amino acid and nucleotide sequences reported for Vtg from such other species: Sense: 5-GACATGAGCCTTTTCCTTTTG -3; antisense: 5- TTTTTCTTGGGGTTGATTTG -3. Amplification of catfish Vtg fragment was done by the RT-PCR reactions, and the PCR products were resolved by 1% agarose gel electrophoresis.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was performed as described by Zapata *et al*[15]. For reverse transcription, a reaction mixture containing 10 g of total RNA, 400 M dNTPs, 30 ng/ oligo-dT, 0.5 U/l RNAsin, and 1U/ SuperScript™ II Reverse Transcriptase (Invitrogen) in 70 l of 1× first strand buffer was incubated at 37 °C for 1 h. The resulting products were amplified by adding 0.05 U/l *Taq* DNA Polimerase, Recombinant (Invitrogen), and 0.3 g/l each of the above forward and reverse primers with a Termo Hybaid EC 330 (Midicell® Primo™), using a program consisting of 1 cycle of 5 min at 95 °C, 3 min at 80 °C (hot start); 40 cycles of 2 min at 95 °C (denaturation), 1 min at 56 °C (annealing), and 1 min at 72 °C (extension). After 40 cycles, the reaction mixtures were incubated at 72 °C for an additional 7 min to allow complete synthesis. For semiquantitative RT-PCR assays, cDNA was synthesized from 6 g total RNA and 20 pmol of catfish actin primers: The final DNA sequence was obtained in an automatic Genetic Analyzer (ABI PRISM 310) and compared with other Vtg sequences in the GeneBank DNA database.

Results are reported as medians ± inter-quantile range. All statistical tests were non-parametric. Significant differences between medians of various fish groups were determined by a Kruskal-Wallis one-way analysis of variance (ANOVA), using $\alpha = 0.05$. Statistical analyses were done with Statistica Software version 6.1 (Statsoft, Tulsa OK)[16].

Results and discussion

The results of organochlorine pesticides and PCBs concentrations in liver of Catfish are presented in Figures 1 and 2. For convenience, pesticide and PCB concentrations were grouped according to chemical class, which has the advantage of decreasing the number of non-detects in the database. The median concentrations of HCHs, Drins and PCBs in catfish for each lagoon are given in Figure 1. Median concentrations of PCBs and HCHs seem higher in Terminos Lagoon, and Celestun has the lowest concentration of Drins. Only for Drins the difference is statistically significant using a non-parametric Kruskal-Wallis ANOVA test ($H_{2, 61}=7.7$; $P=0.021$). Of particular importance is Lindane (γ-HCH), which has an overall mean concentration of 6.9 ng/g (almost identical to that of the α isomer, 6.2 ng/g), about one third of total HCHs with a median concentration of 22.6

ng/g. It is important to say that some of the HCH levels are related to lindane, whose register is to be revoked by the Mexican Ministry of Health in the near future. In Figure 2 the median concentrations of Chlordanes and DDTs is presented. Median concentrations of both DDTs types are higher in Terminos Lagoon than in the other two lagoons studied. The differences between lagoons is statistically significant for both total DDTs ($H_{2, 61}$=20.1; P=0.0000) and total Chlordanes ($H_{2, 61}$=7.8; P=0.024) using a non-parametric Kruskal-Wallis ANOVA test.

Vitellogenin gene expression in catfish livers was evaluated by RT-PCR. The Vitellogenin gene is over-expressed in Terminos Lagoon, the lagoon with the highest concentrations of organochlorine pollutants. The differences are highly significant using a non-parametric Kruskal-Wallis ANOVA test ($H_{2, 61}$=52.5; P=0.0000). HCHs concentrations in Terminos Lagoon are the highest, but the other chlorinated compounds reported here are in the range of concentrations found in other costal regions like Florida, Chetumal Bay, and the Mesoamerican Barrier Reef System[17, 18]. To display graphically the correlation structure of the data, a Principal Component Analysis was run on the correlation matrix. Figures 3 and 4 show the results for the principal component axis 1 versus principal component axes 2 and 3, respectively. These two graphs represent 50.4 and 48.1 % of total variance respectively. In Figure 4 it can be clearly seen that Vitellogenin gene expression is related to the concentrations of total DDTs and PCBs, and negatively related to total Drins. It is known that *o,p'*-DDT is estrogenic[19] and also some PCBs[20], but there are no reports on any possible anti-estrogenic effects of Drins.

In general, tissue samples from Terminos lagoon have higher concentrations of Vitellogenin and organic contaminants than fish collected from Celestun and Dzilam lagoons. The production of Vitellogenin in male catfish *Ariopsis felis* might be related to an individual contaminant or a combination of several contaminants that are present in the water column or in sediments. Some of the compounds that have been detected in the tissue extracts are suspected endocrine disrupters. Their presence, however, does not imply a direct correlation with Vitellogenin induction. Further studies are necessary to determine if these pollutants are related to Vitellogenin production in male catfish.

Overall, this study represents the first approach to explore the changes in molecular diagnostic biomarkers following the exposure to several organic compounds. Vitellogenin gene expressions associated with some endocrine disruptors detected in Terminos Lagoon were measured and we can now report clear changes in exposed fish. Future studies will be focused to conduct ecologically realistic experiments with aquatic stressors at environmentally relevant concentrations under controlled conditions. Data obtained through this study will also provide technical expertise and knowledge aimed to develop national research strategies in POPs related projects. Furthermore, results from this study will provide a significant scientific input to support policy making and regulation of chemicals, particularly for POPs, in Mexico.

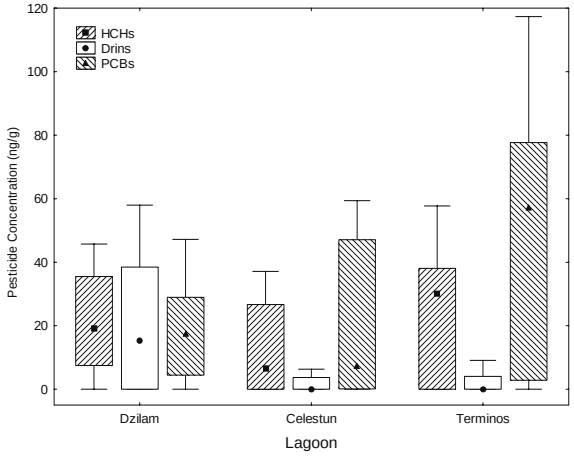

Fig. 1 Median concentrations (± 1 interquartile range) in fish (*Ariopsis felis*) of total HCHs, total Drins and total PCBs for each lagoon.

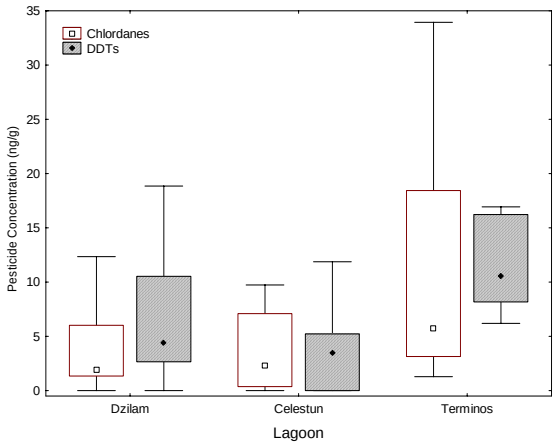

Fig. 2 Median concentrations (± 1 interquartile range) in fish (*Ariopsis felis*) of total DDTs and total Chlordanes for each lagoon.

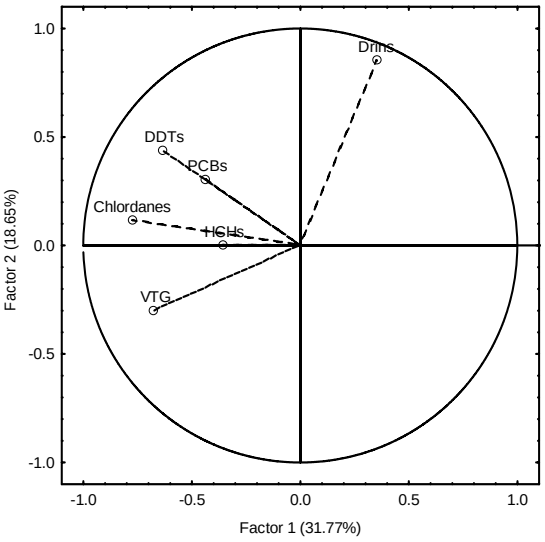

Fig. 3 Principal component axes 1 and 2 (50.4 % total variance), based on the correlation matrix of the data.

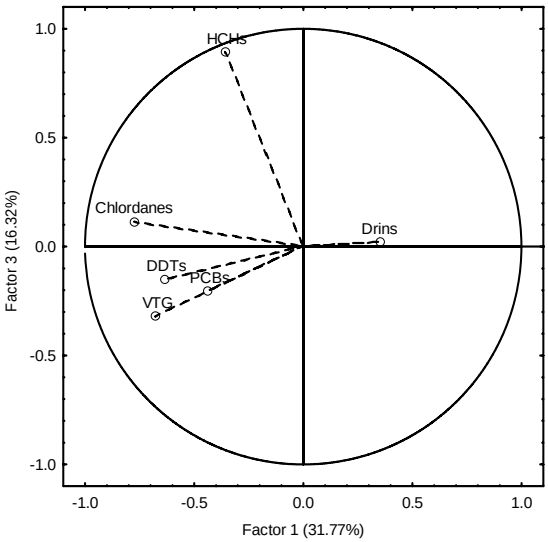

Fig. 4 Principal component axes 1 and 3 (48.1 % total variance), based on the correlation matrix of the data.

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