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Research Article

Xenobiotics Result in Hormonal and Enzymatic Dysregulations in the Red Mussel *Mytilus galloprovincialis* (Lamarck, 1819) (Bivalvia, Mytilidae)

[1]Ibrahim Gaber, [2]Abdallah Ahmad Atallah and [3]Mohamed Elghazaly

[1]Department of Zoology, Faculty of Science, Alexandria University, Alexandria, Egypt
[2]Department of Applied Physics, Faculty of Science, Tafila Technical University, P.O. Box 179, Tafila 66110, Jordan
[3]Department of Zoology, Faculty of Science, Damnhour University, El Behara, Egypt

Abstract

Background and Objective: The contaminants in a marine ecosystem like mercury and synthetic hormones can disrupt the regulation of natural endocrine and reproductive systems of most organisms. This study aims to study the effect of organic and inorganic mercury on the viscera of *Mytilus galloprovincialis* after intracoelomic injection of 17α-ethinylestradiol, 17β-estradiol and Dichlorodiphenyltrichloroethane (DDT) and check the histological changes in the gonads. **Materials and Methods:** Mussels are collected during June-August, 2018 from Ras el tin beach of the Mediterranean Sea of Alexandria, Egypt. This study aims to: test the effect of 17α-ethinylestradiol, 17β-estradiol and DDT on vitellogenin (VTG) synthesis, enzymes dysfunction through intracoelomic injection of methyl mercury in a 0.75 µg/0.1 mL and mercury chloride to a 75 µg/0.1 mL. Gonads are studied histologically in control and treated mussels. Water-administered E2 and EE2 at 120 µL dose induced VTG expression in males 14 days exposure. **Results:** The relative concentration of VTG in the induced groups increases significantly as compared to the control. Alterations in the gonadal tissues and the maturation stages of the mussels are observed. The imposex mussels are characterized by concomitant secondary male sexual characteristics and the female gonad shows testicular structure. Superoxide Dismutase (SOD) activity in mussel digestive glands differed significantly ($p = 0.002$) after 72 hrs of MeHg exposure. **Conclusion:** Significant correlation can be observed between the activities of Glutathione S-Transferases (GST) and Glutathione Reductase (GR) in the digestive glands of mussels treated with MeHg, the enzyme activities of digestive glands treated with $HgCl_2$ and between Superoxide Dismutase-Catalase (SOD-CAT), SOD-GR and GST-GR.

Key words: *Mytilus galloprovincialis*, 17α-ethinylestradiol, 17β-estradiol, DDT, vitellogenin, mercury, enzyme dysfunction, gonad, imposex

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Corresponding Author: Ibrahim Gaber, Department of Zoology, Faculty of Science, Alexandria University, Alexandria, Egypt

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Natural and anthropogenic toxins are dangerous to humans and their offspring[1-3]. These contaminants are absorbed and accumulated in the tissues producing toxic effects[4]. The use of chemicals with an unaccompanied risk assessment in the ecosystem is considered an important potential threat to the health of people, animals and plants[5,6]. The Environmental Protection Agency[7] is established in 1970 to repair the damage already done to the environment and to establish new criteria for the achievement of friendly healthy ecosystems. The quality of human semen declined due to xenoestrogens contamination in Belgium, Denmark, France, Great Britain, south India and Brazil[8-11]. Moreover, Toppari, *et al.*[11] mentioned that testicular cancers are observed in this population. This type of degradation has developed in some animal species[12,13]. The coastal regions are particularly vulnerable to the impact of pollution due to intense human occupation[14-16]. Exposure to xenoendocrine in the aquatic ecosystem causes the appearance of gonadal abnormalities and differentiation towards masculinization or feminization animals[14-16].

A great variety of molecules of anthropogenic origin can disrupt the regulation of the natural endocrine and reproductive systems of most animals and humans[17,18]. The contaminants in marine ecosystem which have endocrine effects are: alkylphenols (used in the formulation of surfactants, detergents, paints, pesticides and in the plastic production), pesticides (Tamron®, commercial DDT which contains several metabolites, the most important are P, P'-DDT and O, P' -DDT considered as estrogenic), Polychlorobiphenyls (PCBs) (comes partly from electrical and chemical industry), phthalates (abundant in the marine ecosystem as di-2-ethylhexyl (DEHP) and di-n-butyl phthalate (DBP), weakly estrogenic), hydrocarbons and oil pills (Polycyclic and halogenated aromatic hydrocarbons disrupt directly cytochromes P 450, they are typically antiestrogenic), synthetic hormones (substances have the ability to mimic estrogens at very low concentrations, the appearance of testicular oocytes is common in the presence of this compound in marine ecosystem), natural phytoestrogens and mycoestrogens (responsible for masculinizing, natural estrogens of animal origin released into the marine environment from farmyard discharges and are also responsible for estrogenic effects)[19-21]. Mercury has a high eco-toxicological risk to marine organisms[22,23]. Methyl mercury and mercury chloride have distinct physicochemical properties and bioaccumulation capacity, varying considerably according to environmental factors and biological models[24,25]. This metal is well known to cause oxidative stress through the production of H_2O_2, inducing a decrease in glutathione (GSH) levels and cause lipid peroxidation[26,27]. The conjugation of GSH with the chemical contaminant, whether spontaneous or catalyzed by GSH-S-transferase (GST), decreases xenobiotics activity and makes these molecules more soluble in water and they can be more easily eliminated[28]. Antioxidant systems including enzymes like superoxide dismutase, glutathione peroxidase, catalase and glutathione S-transferase, are located in different cellular compartments[29,30]. Annelids, crustaceans and bivalves have been used in several toxicological tests in the laboratory.

Biomarkers can be measured to indicate the presence of contamination[31-36]. Vitellogenin is considered a biomarker of estrogen exposure. In general, estrogenic and androgenic receptors are located in the liver or digestive gland to stimulate transcription of a gene sequence particular to the synthesis of a specific protein. Vitellogenesis is stimulated in the liver of mature females by endogenous estrogens (17- β-estradiol) contained in the plasma[37]. The protein is transported by the hemolymph to the ovaries and acts directly on the development of oocytes as a nutrient[38,39]. The unambiguous relationship has been established between the actions of the tributyltin (TBT) and the appearance of a sexual anomaly in gastropod mollusks. The phenomenon known as imposex is the induction of hermaphroditism in females causing them to develop male reproductive organs and consequently leading the species to a population decline[40]. Tributyltin (TBT) induces a rise in testosterone level, the development of the testis and the vas deferens in females and oogenesis is supplanted by the spermatogenesis with sperm production[41,42]. TBT inhibits the conjugation of testosterone and seems to induce the accumulation of androgenic hormones in the plasma. This "storage" of male hormones is responsible for the appearance of masculinizing characters in ovarian tissues[43]. TBT causes long-term sterility of females and extinction of sensitive species[43-46].

This study aims to study the effect of organic and inorganic mercury on internal viscera of *Mytilus galloprovincialis* after intracoelomic injection of 17α-ethinylestradiol and 17β-estradiol and the insecticide DDT and to check the histological changes in the gonads.

MATERIALS AND METHODS

Study area: The study was carried out at Microbiology Department, Quality Control Lab, from January, 2018-March, 2019. Approximately 200 adult red mussel *Mytilus galloprovincialis* are collected from Ras El Tin beach of the

Mediterranean Sea of Alexandria, Egypt. The mussels are transported in tanks to the Environmental Toxicology Laboratory-Alexandria University.

Experimental design: *M. galloprovincialis* is divided into four groups with fifteen copies in each aquarium, 100 L: Group 1: control, group 2: mussels exposed to water by EE2 (120 µL E2.L^{-1}), group 3: mussels exposed to water by E2 (120 µL E2.L^{-1}), group 4: mussels exposed to commercial DDT (50 µL L^{-1}). The hemolymph is collected (0.01 mL) by coelom puncture, obtaining an approximate volume of 500 µL per mussel. Then 5 µL of an antiproteolytic solution are added according to the procedure described by Brossa *et al.*[47], centrifuged for 4 min at 10,400×g at 4°C.

Analysis of Vitellogenin (protein blotting): The use of SDS in this process is very important to avoid the interference of the electric charge of the protein during the electrophoretic run, as this detergent is responsible for homogenizing the electrical charge of proteins[48,49].

Determination of calcium concentration: Hemolymph calcium collected from control and treated *M. galloprovincialis* is determined by the selective electrode method measured on the device: AVL 9180 Then, the supernatant of the mixture was filtered through a Whatman filter (Whatman Clifton, NJ, USA).

Enzyme analysis

Experiment design: *M. galloprovincialis* are anesthetized with eugenol (1%) and subjected to an intracoelomic injection of methyl mercury (MeHg) in a 0.75 µg/0.1 mL and mercury chloride to a 75 µg/0.1 mL concentration[50-52] (Table 1-2). After injection, mussels are placed in separate aquaria until the exposure is stipulated at 24, 48, 72, 96, 120 hrs. For the control, mussels are injected with a Phosphate Buffer Solution (PBS) in the same volume that contaminated mussels.

Glutathione reductase (GR): By reducing the GSSG substrate GR oxidizes NADPH, which can be monitored by the decrease in absorbance in the wavelength of 340 nm. NADPH consumption speed, under saturation conditions, expressed in U mg^{-1} protein, the enzymatic activity of Glutathione reductase[53].

Glutathione peroxidase (GPx): It is indirectly accompanied by the disappearance of NADPH. The enzyme when using GSH to degrade organic peroxide such as peroxide tert-butyl (t-BOOH) generates oxidized glutathione (GSSG), which in turn is reduced by glutathione reductase with the oxidation of NADPH.

Glutathione transferase (GST): The determination of total glutathione S-transferase activity is performed according to the assay described by Manduzio *et al.*[54].

Catalase (CAT): CAT is an enzyme that promotes the decomposition of H_2O_2 into H_2O and O. The technique used to measure its activity, which quantifies the rate of decomposition of H_2O_2 by the enzyme, by decreasing the absorbance at 225 nm (0.072 mM cm^{-1}) at 36.5°C.

Glucose 6-phosphate dehydrogenase (G6PDH): G6PDH catalyzes the oxidation of glucose-6-phosphate to 6-phosphogluconolactone which rapidly and spontaneously hydrolyzes to form 6-phosphogluconate. In the catalytic process[54].

Superoxide dismutase (SOD): SOD catalyzes the superoxide radical dismutation reaction in oxygen and hydrogen peroxide. This enzyme is determined according to the method described by Manduzio *et al.*[54] (Table 1).

Table 1: Number of mussels treated with mercury with exposure time/h and used in the enzyme analysis

		Exposure time (hr)				
Treatment	Control	24	48	72	96	120
Methyl mercury	7	8	8	8	8	5
Mercury chloride	7	6	6	6	6	4

Table 2: Number of organs treated with mercury used in the chemical analyses

	Liver	Kidney	Muscle	Ctenidia	Cerebral ganglia
Methyl mercury	17	14	16	20	21
Mercury chloride	18	15	15	18	19
Control	18	11	14	19	12

Chemical analysis: The sample number of tissues from MeHg-treated subjects, $HgCl_2$ and control were used in the chemical analyzes (Tables 1-2). After the pre-established treatment time in this study, mussels are necropsied and the tissues used for the study are separated and weighed in aliquots of app.1.0 g (wet weight)[55,56]. The samples that presented coefficient of variation (CV (%) = (Standard Deviation/Mean)×100) between replicas greater than 10% are again analyzed. The same procedure could not be adopted for the digestive gland, cerebral ganglia and kidney in the function of the small mass of each sample. The detection limit of the calculation method is 17.93 µg kg^{-1}. The calculation followed the methodology described by Manduzio *et al.*[54]:

$$LD = \frac{3.s}{a}$$

where, s is equal to the standard deviation of 10 curve white reads and a is equal to the angular coefficient of the analytical curve.

Histology

Cryomicrotome histology: Frozen organs are cut into thin sections (between 4 and 7 µm) directly on the cryomicrotome (HM SOOM cryostat microtome). Staining protocol is tested on the tissues with Masson trichrome. The cuts are placed in the color bath for 10 min and then rinsed with distilled water (5 min) A drop of glycerine is placed on the slides to fix the lamellae.

Classical histology: Gonads are fixed in ALFAC solution (80% alcohol, formaldehyde and glacial acetic acid) for 16 hrs. Subsequently, they are dehydrated and proceeded the routine processing in histology laboratories[57,58].

Statistical analysis: VTG results are statistically analyzed by ANOVA followed by the Dunnett test. Statistical analysis and graphing are performed using the Graphpad Prism® version 3.0 program. Significant in total Hg concentrations were analyzed using ANOVA one way and afterward the Tukey test[59].

RESULTS

Vitellogenin (VTG) detection (protein blotting): The analysis of vitellogenin in the hemolymph of mussels is made by Western blot, which consisted briefly of protein mixture electrophoresis followed by detection using a protein-specific antibody of interest and secondary antibody bound to alkaline phosphatase. Once the membranes are revealed, there is the formation of strong double bands in a region where protein is located with a molecular weight of approximately 250 kDa. The result obtained is quite satisfactory as seen in on the electrophoresis gel (Fig. 1a). *Mytilus galloprovincialis* hemolymph precipitation is performed at the molar ratios of 1.0:2.0, 1.0:1.5 and 1.0:1.0. There are no differences in gel between the ratios. Therefore, the 1.0:1.0 molar ratios are used in all tests. Demonstrating *in vivo* induced VTG analyzed by immunoblotting. Water-administered E2 and EE2 at the 120 µL dose induced VTG expression in males 14 days after exposure, as demonstrated with hemolymph VTG analyzed by protein blotting, followed by staining with CBB (Fig. 1b-c). Such a method consists of precipitation of protein on a bath, eliminating procedures of chromatographic properties and has some molecular properties of VTG, determined by orthophosphate in electrophilic interactions with the added magnesium, thus allowing isolation by centrifugation. The hemolymph of control males analyzed by SDS-PAGE-CBB, did not present the band corresponding to the VTG (≈200 kDa) as shown in (Fig. 1b-c). The hemolymph of females analyzed by SDS-PAGE-CBB presented the band corresponding to the VTG, but with low intensity before induction with E2, indicating that the mussel is in the early stages of vitellogenesis, a fact confirmed by macroscopic analysis of the gonad. This is likely due to the size of the female which is entering its first period of vitellogenesis at the beginning of sexual maturation (Fig. 1d). The electrophoretic pattern of all samples could not be repeated and this is attributed to the freezing of hemolymph which, even if performed only once degraded the VTG. During the 14 days of the experiment, approximately 3 mussels per group, both control and induced. In Fig. 1e, it can be observed that the relative concentration of VTG in the induced groups increased significantly when compared to the control of females. The hormones EE2 and E2 and DDT induced the protein. The hemolymph calcium concentration after the experiment is statistically significant in the treated groups when compared to hemolymph calcium concentration at the beginning of the experiment: Control 2500000 mg mL^{-1}, 17α-ethinyl estradiol 7500000 mg mL^{-1} and 17β-estradiol 7300000 mg mL^{-1}, respectively. Hemolymph calcium concentration was 0.8 and 1.7 mg dL^{-1}, respectively on the first day of the experiment and after 14 days of exposure via water to 17α-ethinyl estradiol (EE2) and 17β-estradiol (E2) (Fig. 1f-g).

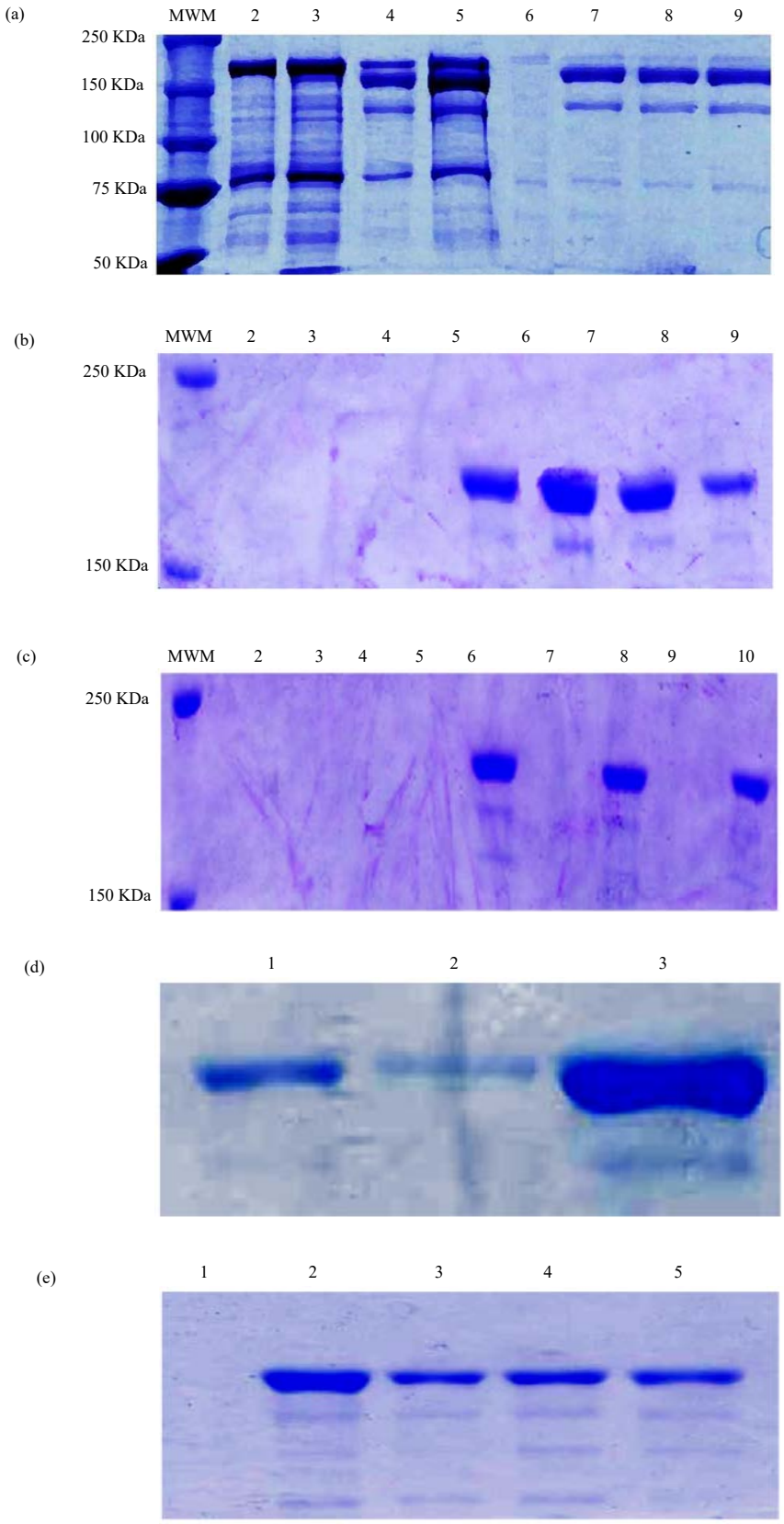

Fig. 1(a-g): Continued

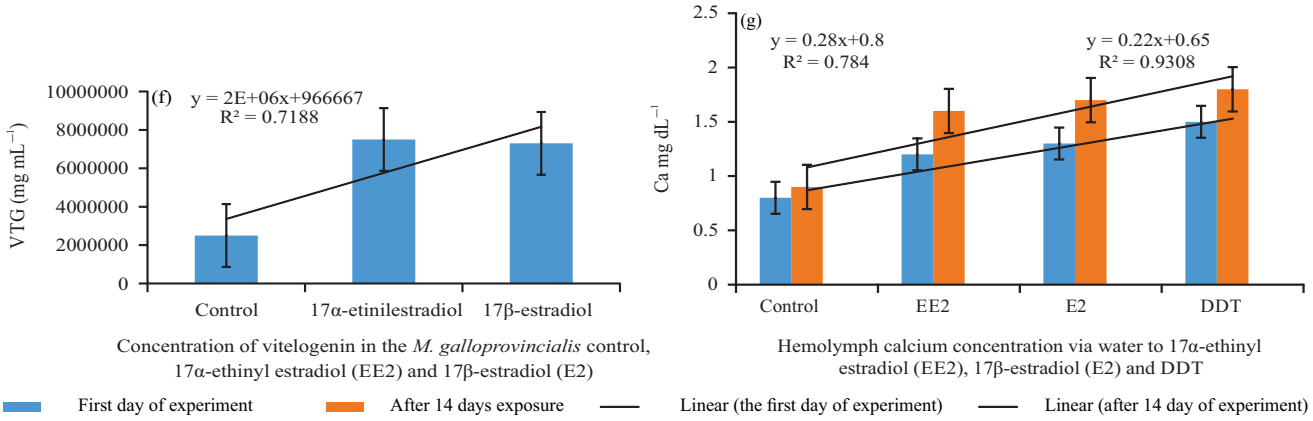

Fig. 1(a-g): (a-e) Electrophoresis analysis of vitellogenin in the hemolymph of mussels and (f-g), Evaluation of concentration of vitellogenin and hemolymph calcium concentration

a : 4% acrylamide stacking gel and 8% separation gel. Coloring by Coomassie Brilliant Blue. Mussel hemolymph collected before and after 14 days of 17β-estradiol (E2) 120 µL induction. E2.L-1. and 17α-Ethinyl estradiol (EE2) 15 µL EE2.L-1 and DDT administered by water.

Lane 1 : molecular weight marker (MPM),
Lane 2 : 15 µL of control hemolymph without precipitation,
Lane 3 : 15 µL of 17β-estradiol (E2) induced hemolymph one-day exposure,
Lane 4 : 15 µL of 17α-ethinyl estradiol (EE2) 15 µL EE2.L-1 induced hemolymph one-day exposure,
Lane 5 : 15 µL DDT induced hemolymph one-day exposure,
Line 6 : 15 µL of 17β-estradiol (E2) induced hemolymph 14 days exposure,
Lane 7 : 17α-ethinyl estradiol (EE2) 15 µL EE2.L-1 induced hemolymph14 days exposure,
Lane 8 : 15 µL DDT induced hemolymph 14 days exposure,
Lane 9 : 15 µL of protein precipitate redissolved in 1 M NaCl after precipitation of the 17β-estradiol (E2) hemolymph in a solution of Na2EDTA.2H2O added $MgCl_2$. generating the following molar ratios.
Finals Lane 9 : [EDTA] ÷ [Mg++] = 1.0 ÷ 2.0.

b : 4% acrylamide stacking and 8% separation gel. Staining by Coomassie Brilliant Blue. Hemolymph was collected after 14 days of 17β-estradiol (E2) induction 120 µL $E2.L^{-1}$ and 17α-ethinyl estradiol (EE2) 120 µL $EE2.L^{-1}$. DDT 120 µL administered by water.

Lane 1 : Molecular weight marker (MPM),
Lanes 2 to 5 : 15 µL of control hemolymph,
Lane 6 : 15 µL of 17β-estradiol (E2) induced hemolymph,
Lane 7 : 15 µL of 17α-ethinyl estradiol (EE2) . induced hemolymph,
Lane 8 : 15 µL of induced hemolymph DDT,
Lane 9 : 15 µL of 17α-ethinyl estradiol (EE2) 17β- estradiol-induced hemolymph. Protein precipitate redissolved in 1 M NaCl after hemolymph precipitation in Na2EDTA.$2H_2O$ solution added with $MgCl_2$ at molar ratio from 1.0: 1.0.

c : 4% acrylamide stacking gel and 8% separation gel. Coloring by Coomassie Brilliant Blue. Hemolymph was collected before and after 14 days of induction by 17β-estradiol (E2) 120 µL $E2.L^{-1}$ and 17α-ethinyl estradiol (EE2) 120 µL $EE2.L^{-1}$. administered by water.

Lane 1 : molecular weight marker (MPM).
Lanes 2 to 4 : 15 µL of control hemolymph,
Lane 5 : 15 µL of hemolymph before induction by 17β-estradiol (E2),
Lane 6 : 15 µL of 17β-estradiol (E2) induced hemolymph,
Lane 7 : 15 µL of hemolymph before 17α-ethinyl estradiol induction (EE2),
Lane 8 : 15 µL of 17α-ethinyl estradiol (EE2) 17β- estradiol-induced hemolymph,
Lane 9 : 15 µL of hemolymph before induction by 17β-estradiol (E2) .
Lane 10 : 15 µL of 17β-estradiol (E2) induced hemolymph, Protein precipitate redissolved in 1 M NaCl after hemolymph precipitation in $Na^2EDTA.2H_2O$ solution added with $MgCl_2$ at a molar ratio from 1.0: 1.0.

d : 4% acrylamide stacking gel and 8% separation gel. Coloring by Coomassie Brilliant Blue. Hemolymph collected before and after 14 days of induction by 17β-estradiol (E2) 120 µL $E2.L^{-1}$ administered by water.

Lane 1 : 15 µL of hemolymph before induction by 17β-estradiol (E2).
Lane 2 : 15 µL of hemolymph before induction by 17α-ethinyl estradiol (EE2) 120 µL $EE2.L^{-1}$.
Lane 3 : 15 µL of hemolymph after induction by 17β-estradiol (E2). Protein precipitate redissolved in 1 M NaCl after hemolymph precipitation in Na2EDTA.$2H_2O$ solution added with MgCl2 at a molar ratio from 1.0: 1.0.

e : Denaturing electrophoresis in Polyacrylamide Gel for mussels hemolymph vitellogenin analysis in groups of EE2. E2 and DDT. 4% acrylamide stacking gel and 8% separation gel. Coloring by Coomassie Brilliant Blue. Hemolymph collected after 14 days of 17β-induction estradiol (E2) and 17α-ethinyl estradiol (EE2) and exposure to the pesticide DDT (50 µL.L-1) administered via water.

Lane 1 : 15 µL of hemolymph following exposure to DDT.
Lane 2 : 15 µL of hemolymph following induction by 17β-estradiol (E2),
Lane 3 : 15 µL of hemolymph following induction by 17β-estradiol (E2).
Lane 4 : 15 µL of hemolymph following induction by 17α-ethinyl estradiol (EE2),
Lane 5 : 15 µL of hemolymph following induction by 17α-ethinyl estradiol (EE2). Protein precipitate redissolved in 1 M NaCl after plasma precipitation in $Na_2EDTA.2H_2O$ solution added with $MgCl_2$ at molar ratio from 1.0: 1.0. 1g. hemolymph calcium concentration was evaluated on the first day of the experiment and after 14 days of exposure via water to 17α-ethinyl estradiol (EE2) and 17β-estradiol (E2).

f : Vitellogenin conc. of *M. galloprovincialis* control, 17α-ethinyl estradiol (EE2) and 17β-estradiol (E2).

g : Hemolymph calcium concentration was evaluated on the first day of the experiment and after 14 days of exposure via water to 17α-ethinyl estradiol (EE2) and 17β-estradiol (E2)

Histology: Of the mussels analyzed 35 are females and 25 are males. Optical light microscopy analysis showed that gonads are located and develop within the animal's body, around the periphery of the viscera, which makes it difficult to weigh and excise only the gonadal tissue. Possible alterations in the gonadal tissues and the maturation stages of the mussels are observed: Rest stage in which rapid regeneration of the reserve tissue occurs. Mature gametes are absent (Fig. 2a), gonial cells multiplication which is considered as early gametogenesis with previtellogenic oocytes and a great number of oogonia (Fig. 2b), the progression of gametogenesis in which active development but not yet mature gametes, gonad maturation in which large follicles are present and the connective tissue of stroma is minimal. The previtellogenic oocyte is linked to the germinal epithelium by a stalk (Fig. 2c), vitellogenesis cells in female follicles, in male follicles, thinner layer of germ cells and spermatocytes and a great number of spermatids and sperm (Fig. 2d-e), gamete release which is follicle emptying phase with gamete elimination, some residual gametes may remain (Fig. 2f), restoration stage in which interfollicular spaces begin to be filled by connective tissue (Fig. 2g), resorption proceeds and the entire gonad consist of connective tissue cells, major degradation of follicular structures accompanied by hemocytes (Fig. 2h). Atresia follicle and voids are not considered as a developmental stage in the oogenesis but rather as structures derived from the referred process. In males, spermatogenesis is vigorously affected in mussels treated with E2, EE2 and DDT compared to the control group (Fig. 2i-l), This malformation involves vacuoles formation inside the testicular follicles, necrosis of germ cells and deformity of spermiogenesis respectively compared to the control group. In ovaries treated with E2, EE2 and commercial DDT, there are many atretic gonial cells with a granulomatous reaction. Moreover, Imposex female mussels presented ovarian follicles in the anterior, middle and posterior portions of the gonad (Fig. 2m). Some mussels showed testicular tumours especially in the germinal epithelium (Fig. 2n). The non-vital oocytes are degraded by hemocytes (Fig. 2o).

Imposex: The imposex mussels of *Mytilus galloprovincialis* are characterized by the presence concomitant of secondary male sexual characteristics (penis) and female gonads. Penis length in imposex mussels varied between 2.03 and 8.41 mm. histologically, the penis presented the same structure as in males. The female gonad of yellowish or light brown coloration is observed located at the distal end of the visceral mass. Histological analysis showed that imposex mussels presented ovarian follicles in the anterior, middle and posterior portions of the gonad (Fig. 2p). The imposex females studied (N = 20) presented their mature ovaries, observing mostly vitellogenic oocytes at the histological level (Fig. 2q). The mussel that presented the longest penis (8.53 mm) also showed a light brown gonad which allowed us to classify it as an imposex mussel, however histological analysis of female gonad showed testicular structure (Fig. 2r).

Accumulation of total mercury in various tissues: Mean mercury concentrations and standard deviation of mussels treated with methyl mercury, mercury chloride and non-mercury administered mussels (controls) are presented in Table 3. The data show a significant increase in the total mercury in all tissues relative to controls. The decreasing distribution of mercury in tissues treated with the methyl mercury is cerebral ganglia>kidney>liver>muscle>ctenidia. In regarding mussels treated with mercury chloride, the distribution is liver>ctenidia>cerebral ganglia muscle kidney (Table 3). In control mussels the decreasing order of accumulation is kidney>liver>ctenidia>muscle>cerebral ganglia (Table 3). However, the averages of mercury concentration in the control mussels are close and not differing significantly between tissues. By comparing the accumulation of mercury between the organs treated with mercury chloride, the digestive gland the only organ that showed a significant difference to other organs. In mussels treated with methyl mercury, the kidney, digestive gland and cerebral ganglia significantly differentiated from the ctenidia and muscle (Table 3).

Digestive gland: For mercury chloride-treated mussels, exposure times of 24 and 96 h presented higher accumulation of Hg (68720 and 44541 μg kg^{-1}, respectively, not significantly differentiating between them. Significant differences ($p<0.05$) are observed between the other times of exposure between $HgCl_2$ and control samples, they obtained concentrations ranging from 52±19 μg kg^{-1}. Mean mercury concentrations (μg kg^{-1}) in digestive system control and treated with $HgCl_2$ (a) and MeHg (b), respectively, for up to 96 h. Hg accumulation in MeHg-treated mussels is increasing with exposure time, reaching the highest concentration after 96 h (774 μg kg^{-1}) (Table 4-6).

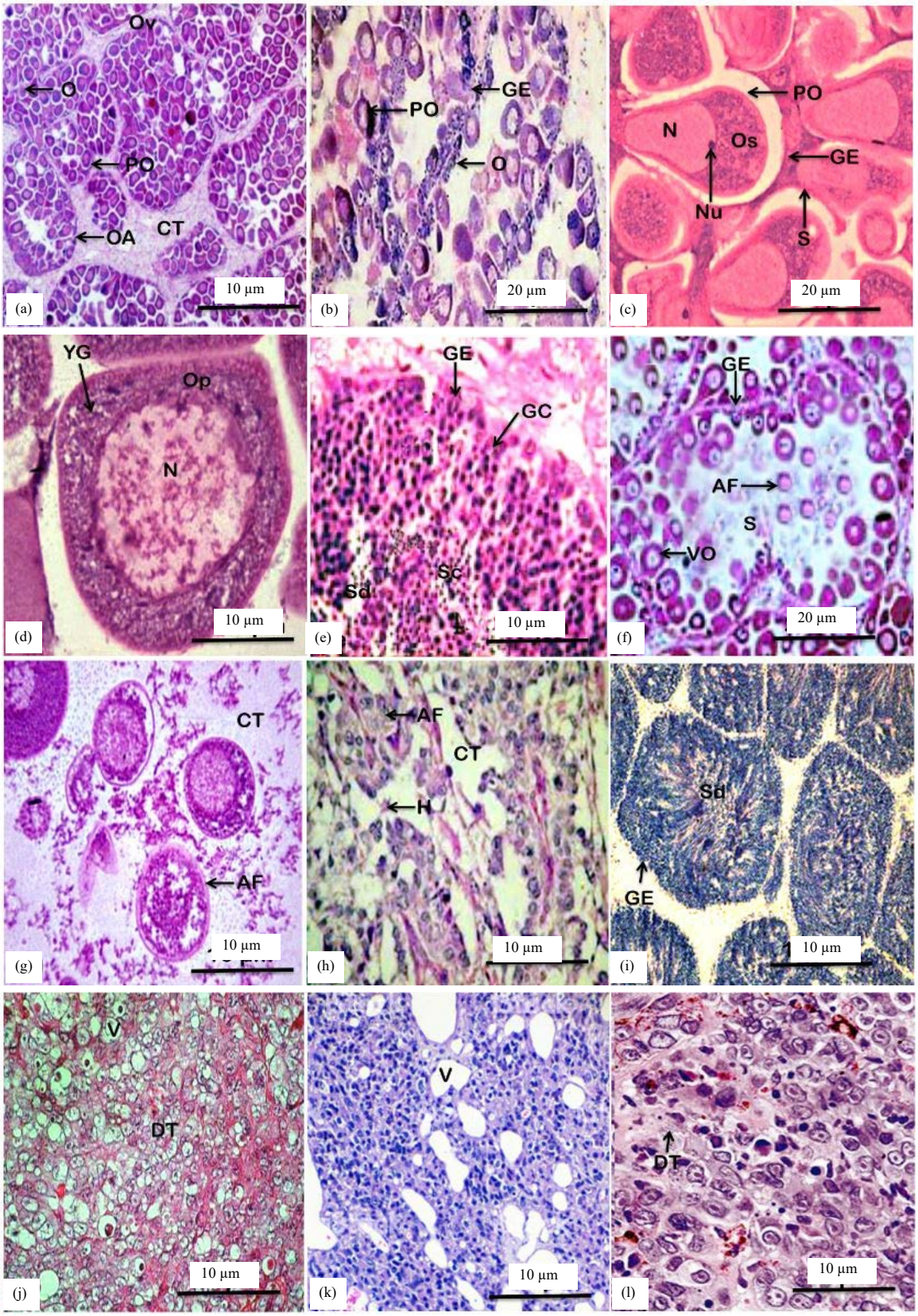

Fig. 2(a-r): Continued

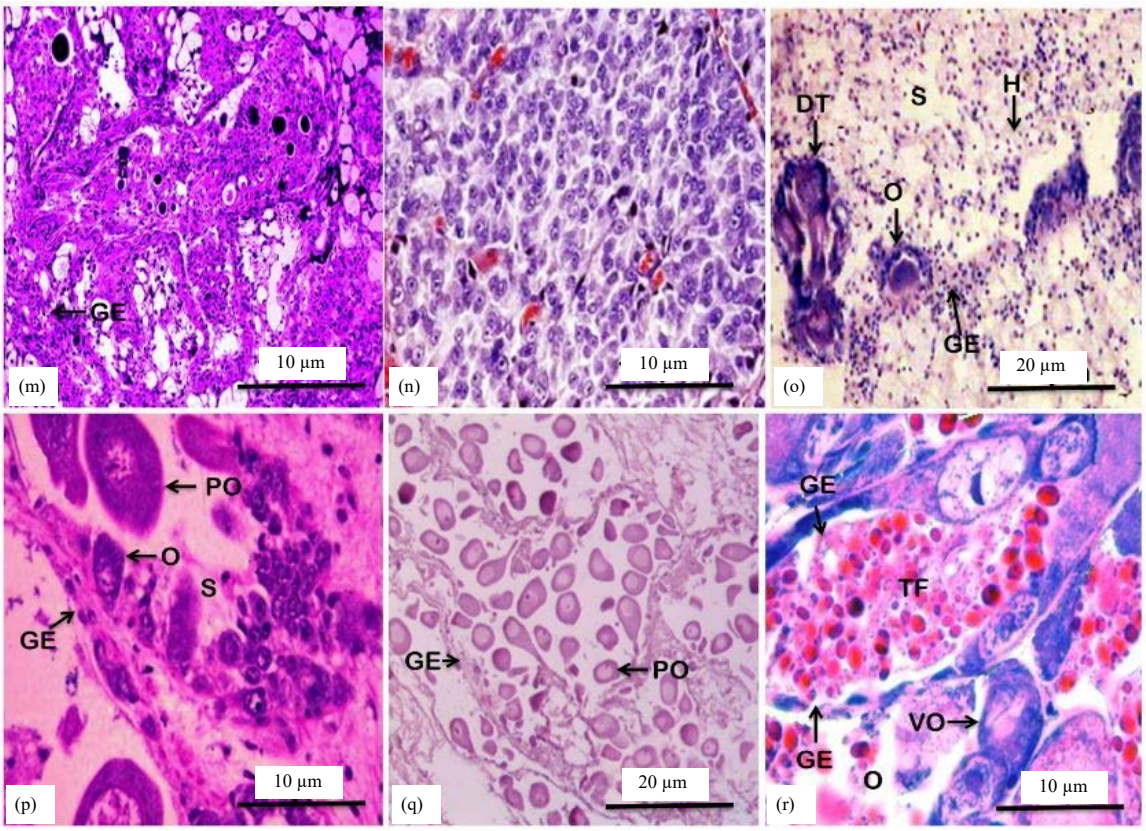

Fig. 2(a-r): Histological study of the gonad of *Mytilus galloprovincialis*

a: Rest stage of the ovary in which rapid regeneration of the reserve tissue occurs. Mature gametes are absent, b: Gonial cell multiplication in which the ovary is considered as in early gametogenesis with small follicles and a great number of gonial cells, c: Gonad maturation in which large follicles are present and the connective tissue of stroma is minimal, d: Vitellogenesis cells in female follicles, e: In male follicles. a thinner layer of germ cells and spermatocytes and large numbers of spermatids and sperm, f: Gamete release which is follicle emptying phase with gamete elimination. Some residual gametes may remain, g: Restoration stage in which interfollicular spaces begin to be filled by connective tissue, h: Resorption proceeds and the entire gonad consist of connective tissue cells. Major degradation of follicular structures is accompanied by hemocytes, i-l: In males: Spermatogenesis is vigorously affected in mussels treated with E2. EE2 and the commercial DDT compared to the control group, m: Some mussels showed testicular necrosis especially in the germinal epithelium, n: Imposex female mussels presented ovarian follicles in the anterior, middle and posterior portions of the gonad, o: The non-vital oocytes are degraded by hemocytes, p: The imposex mature ovaries contained mostly vitellogenic oocytes, q: The imposex females presented their mature ovaries, observing mostly vitellogenic oocytes at the histological level, r: Imposex female mussels showed testicular structure inside the ovary. AF: Atresia follicle, CT: Connective tissue, DT: Dead tissue, GC: Germ cells, GE: Germinal epithelium, H: Hemocyte, IS: Interfollicular spaces, MO: Mature oocyte, N: Nucleus, Nu: Nucleolus, O: Oogonium, Op: Ooplasm, Ov: Ovary, OA: Ovarian acinus, PO: Previtellogenic oocyte, RG: Residual gamete, S: Sperm, Sd: Spermatid, Sc: Spermatocyte, S: Stalk, TF: Testicular follicle, TN: Testicular necrosis, V: Vacuole, VO: Vitellogenic oocyte, YG: Yolk granules

Cerebral ganglia: The storage of Hg in this tissue presented behavior differentiated from the different forms of mercury. The mussels administered with $HgCl_2$ showed an oscillation in Hg accumulation in the cerebral ganglia. In mussels treated with alkylphenol (DDT), higher concentrations are evidenced after 24 hrs (2699 µg kg^{-1}), after this time a decrease in these concentrations and after 72 hrs (3962 µg kg^{-1}) a further increase is noticed.

No significant difference in cerebral ganglia Hg accumulation is observed in $HgCl_2$-treated mussels between 24 and 72 hrs, 24 and 96 hrs and 48 and 96 hrs. Regarding the concentration of Hg in the control mussels all the time of exposure are significantly different from the concentrations in the contaminated organisms. Hg accumulation in methyl mercury treated mussel cerebral ganglia is increasing to exposure times, as occurred in the digestive system treated with methyl mercury (Table 3). No significant differences in Hg accumulation are observed in cerebral ganglia treated with MeHg between 24 and 48 hrs, as well as between 72 and 96 hrs. Concerning control mussels all times of exposure are significantly different.

Table 3: Accumulation of mercury (μg kg^{-1}) in different tissues treated with methyl mercury and mercury chloride compared to control

Treatment	Liver	Kidney	Muscle	Ctenidia	Cerebral ganglia
Methyl mercury	391.0±215[a]	473.0±791[ab]	140.0±50[cd]	118.0±37[d]	541.0±372[be]
Mercury chloride	47173.0±20300[a]	452.0±193[b]	872.0±334[b]	2583.0±1670[b]	2080.0±1581[b]
Control	63.0±18	92.0±44	54.0±15	60.0±17	53.0±30

Different letters mean significant differences (p<0.05), mean±SD

Table 4: Correlation of enzymes analyzed in digestive glands of mussels treated with methyl mercury

Enzyme	Control	24	48	72	96	120
SOD (U mg^{-1} protein)	12.5±2.77	11.2±2.9	10.1±2.2	9.1±2.1*	8.2±2.1	9.1±1.2
GST (mU mg^{-1} protein)	152.0±26.4	132.0±12.7	139.0±36.3	132.0±33.2	107.0±22.3	118.0±12.8
CAT (U mg^{-1} protein)	450.0±111	612.0±120	530.0±88.1	521.0±80.7	491.0±32.4	547.0±75.5
GR (mU mg^{-1} protein)	42.7±19.3	28.8±19.6	40.2±20.1	34.0±12.1	22.7±8.2	32.6±8.2
G6PDH (mUmg^{-1} protein)	31.2±10.2	41.4±22.1	48.1±16.1	32.1±11.8	49.1±23.8	35.2±28.7
GPx (mU mg^{-1} protein)	61.6±16.3	61.7±17.2	61.9±12.4	77.4±26.5	49.2±18.8	68.3±27.1

Mean values±standard deviation. *Difference from control (p<0.05). SOD (p = 0.005) and GR (p = 0.015) activities in mussel digestive glands treated with $HgCl_2$ are inhibited after the total treatment period (120 hrs)

Kidneys: Mean Hg concentrations observed in treated mussel kidneys with $HgCl_2$ after 24, 48 and 96 hrs of exposure are similar (1454, 1504 and 1368 μg kg^{-1}, respectively). The highest concentrations are observed after 72 hrs of the experiment (3138 μg kg^{-1}). There is no significant difference in Hg accumulation in the mussel kidneys administered with $HgCl_2$ between 24 and 48 hrs, 24 and 96 hrs and 48 and 96 hrs. Significant differences between exposure times and kidneys of treated and untreated mussels with mercury (control) (Table 2). In renal tissues of MeHg-treated mussels, higher concentrations of Hg values are observed after the end of the experiment (96 h-663 μg kg^{-1}), as observed in mercury-treated trait digestive glands and cerebral ganglia. No significant differences are observed between 24 and 72 h, 24 and 96 hrs and 72 and 96 hrs of exposure. Significant differences are observed when comparing the MeHg administered kidneys and the control mussel Kidneys (Table 3).

Muscle: Oscillation in muscle Hg concentration is common in both chemical forms. Mean Hg titre (μg kg^{-1}) in control and treated muscle with $HgCl_2$ (a) and MeHg (b), respectively, for up to 96 hrs. In $HgCl_2$ treated mussels, there is a peak in Hg accumulation after h (1333 μg kg^{-1}) of treatment. This exposure time differentiated significantly from the others. In mussels treated with MeHg, the variation of the accumulation of Hg over exposure time is low (one day -145 μg kg^{-1}, two days-155 μg kg^{-1}, three days-130 μg kg^{-1}) (Table 2 and 3).

Reductions in Hg accumulation after 96 hrs of treatment are observed in mussels treated with MeHg and $HgCl_2$. Significant differences in Hg concentrations in mussel muscles treated with MeHg are observed only at the final exposure time 96 hrs in comparison to other times. Significant differences are also observed between mussel muscles treated with MeHg and muscles of control subjects, in all exposure times.

Ctenidia: The highest average concentrations of Hg in ctenidia treated with MeHg and $HgCl_2$ are observed at baseline (24 hrs). In ctenidia treated with $HgCl_2$, the mean is 4940 μg kg^{-1}. For ctenidia treated with MeHg, this average is 140 μg kg^{-1}) (Table 2 and 3).

Enzyme analysis: SOD activity in mussel digestive glands differed significantly (p = 0.002) after 72 hrs of MeHg exposure to the control mussel digestive gland 9.1±2.1 whereas in control was 12.5±2.77. The total level of enzymes analyzed in mercury chloride-treated and control digestive glands at different times were CAT 1.03, GST 1.01, G6PDH 1.02, GR 1.04, GPx 1.05 and SOD 1.01 which were significantly different value p<0.05 (Table 4 and 5). A positive and significant correlation can be observed between the activities of the GST and GR in the digestive glands of mussels treated with MeHg. Significant positive correlations are observed between the enzyme activities of digestive glands treated with $HgCl_2$. Significant correlations (p<0.05) are present between SOD-CAT, SOD-GR and GST-GR. (Zero hour of treatment with $HgCl_2$ SOD (U mg^{-1} Protein) was 10.1±3.2, GST(mU mg^{-1} protein) was 139±21.5, CAT (U mg^{-1} protein) was 451±104, GR (mU mg^{-1} protein) was 34.7±17.1, G6PDH (mU mg^{-1} Protein) was 31.2±10.1 and GPx (mU mg^{-1} protein) was 59.2±12.9. After five days treatment with $HgCl_2$ SOD (U mg^{-1} protein) was 6.6±1.2, GST(mU mg^{-1} protein) was 129±21.8, CAT (U mg^{-1} protein) was 412±41.7, GR (mU mg^{-1} protein) was 17.1±8.9, G6PDH (mU mg^{-1} protein) was 42.7±9.2 and GPx (mU mg^{-1} protein) was 51.6±9.4 (Table 6).

Table 5: Total levels of enzymes analyzed in mercury chloride-treated and control digestive glands at different times (h)

Enzyme	CAT	GST	G6PDH	GR	GPx	SOD
CAT	1.03					
GST	0.12	1.01				
G6PDH	0.21	0.15	1.02			
GR	0.04	0.52*	0.38	1.04		
GPx	0.12	0.24	-0.25	0.10	1.05	
SOD	0.14	0.36	-0.17	0.28	0.35	1.01

*Significantly different value $p<0.05$

Table 6: Correlation of enzymes analyzed in digestive glands treated with $HgCl_2$

	Duration (days)					
Enzyme	0	1	2	3	4	5
SOD (U mg^{-1} protein)	10.1±3.2	11.3±1.4	9.1±1.2	11.1±1.9	8.1±1.1	6.6±1.2*
GST(mU mg^{-1} protein)	139.0±21.5	130.0±29.1	128.0±21.3	111.0±22.1	137.0±21.7	129.0±21.8
CAT (U mg^{-1} protein)	451.0±104	627.0±139	557.0±41.4	603.0±217	139.0±27.2	412.0±41.7
GR (mU mg^{-1} protein)	34.7±17.1	21.2±8.2	18.4±7.2	25.6±7.2	20.1±7.8	17.1±8.9*
G6PDH (mU mg^{-1} protein)	31.2±10.1	30.3±7.9	47.8±13.5	55.1±6.8	47.2±6.9	42.7±9.2
GPx (mU mg^{-1} protein)	59.2±12.9	68.4±12.8	72.1±0.6	61.9±11.0	63.9±20.1	51.6±9.4

Mean values±standard deviation. *SD values regarding control ($p<0.05$)

DISCUSSION

In this study the VTG selective precipitation method with EDTA: Mg_{2+} is used for the mussel *M. galloprovincialis* by varying the final molar ratio between EDTA- and Mg^{++} in precipitation. Hemolymph VTG induction is verified in the experiment performed with the mussels in groups E2 and EE2 as expected. Only small amounts of synthetic hormone are needed to induce the synthesis of VTG *in vivo*. E2 plays an important role in the development, growth, sexual differentiation and reproduction. Vitellogenesis begins with the production of E2, which stimulates the synthesis and release of VTG by the liver, which is transported by hemolymph to the oocytes in the ovary, where it is cleaved into two primary components, the phosphorite and vitelline. The commercial DDT after 14 days did not induce the synthesis of VTG. One of the possible causes would be the time of exposure or insufficient concentration of the substance. How much histological changes of the male gonads of the groups exposed to 17α-ethinyl estradiol (EE2) and 17β-estradiol (E2), there are no changes in the sperm lineage cells when compared to the control group, this indicates that the concentrations used did not cause microscopic alterations in the male mussel gonads of the different groups.

Most recently, structural changes in the female gonads are recorded[60]. The same result is obtained also by other authors performing experiments with the Sydney rock oyster, *Saccostrea glomerata*[61,62]. Adults of *Mytilus edulis* exposed for 10 days to habitat concentrations of 17β-estradiol results in the induction of VTG whereas ripe mussels of the same species do not show significant change[63]. Similar study was reported with *Elliptio complanata*, *Dreissena polymorpha* and *Mytillus edulis*[64]. For the accomplishment of these experiments the hormone chosen is endogenous (E2) and synthetic hormone (EE2), substances known for their estrogenic or anti-estrogenic activity that comes constantly being found in sewage treatment plants in countries at different concentrations[65]. Due to the importance of substances with the potential to deregulate the endocrine system it is necessary to continue with studies on the detection of methodologies and their actions on the reproductive system. Usually, mussels can accumulate methyl mercury and alkylphenol in their tissues from the marine ecosystem. Concentrations of these chemicals in mussels are higher than non-filter feeders[64]. The same result is obtained by Ciocan *et al.*[65] after exposure to EE2 in *Saccostrea glomerata* for 15 days. Metals sequestered by cytosolic proteins such as metallothioneins or stored in lysosomes are considered detoxified rather than more bioavailable to cellular targets[66,67]. However, the link metallothionein-mercury is rapidly disrupted[68,69] and thus this metal is again made available to the bloodstream. Inhibition of the activity of the enzymes analyzed is also observed. By Dellali *et al.*[70] in *Ruditapes decussatus* and *Mytilus galloprovincialis* treated with the same mercury concentration after 70 days of trophic exposure and by Pfeifer *et al.*[71] in *Mytilus* sp. contaminants by mercury. Both previously mentioned authors state that these enzyme inhibitions result in a stressful situation oxidative. Al- Farraj *et al.*[72] compare enzyme activity in the barnacle *Amphibalanus amphitrite* from Alexandria, Mediterranean Sea treated with different metals (Zn, Ni, Co, Cd, Cu, Sb, Al, Fe^{+2}

and Fe^{+3}) and conclude Hg is the metal that has the greatest effect on decreasing GST activity. Similar results were recorded in bivalve impacts in freshwater and marine ecosystems[73]. The determination of the stages of gonadal development in mussels is based on the classification adopted by Neff *et al.*[74]. The collected mussels characterize the following stages: gametogenesis, gamete repletion, partial spawning and total spawning. Accordingly stages of the reproductive cycle cannot be determined as static phases but must be viewed as dynamic and continuous processes[75]. According to Kopecka-Pilarczyk[76], some abiotic factors can synchronize the reproductive cycle of mollusks such as temperature and salinity, the former being the most important. Thus, it can be concluded that there is a need for longer exposure to obtain morphological effects on mussels. No significant difference is observed in the activity of GST in the methyl mercury and alkylphenol-treated mussels and the control, but a significant positive correlation is observed between GR activity (inhibited significantly) and GST for mussels treated with methyl mercury and alkylphenol. Despite the lack of significance in CAT activity in livers treated with Hg to livers of control individuals, it is possible to observe a significant positive correlation between CAT activity and SOD, in mussels treated with alkylphenol (DDT). These enzymes have an important role in the detoxification of radicals in nonreactive molecules; while SOD catalyzes the conversion of superoxide anions ($O2^{-}$) in peroxide of hydrogen (H_2O_2) CAT metabolizes these peroxide molecules formed in O_2 and H_2O.

CONCLUSION

There is no relationship between VTG induction and increased hemolymph calcium concentration. Both synthetic hormone EE2 and E2 induce VTG. The commercial DDT does induce VTG synthesis. Regarding the histology of male and female gonads, there is no significant change between groups. Mussels respond similarly to the organic and inorganic Hg, by inhibiting the activity of SOD. Significant inhibition of GR in mussels treated with alkylphenol allows the use of its activity as a tool for specific biochemical biomarkers. EE2, E2 and DDT induce imposex in female mussels and dysfunctional the testis.

SIGNIFICANCE STATEMENT

This study discovers the relationship between VTG induction and hemolymph calcium concentration in addition explains the role of synthetic hormone EE2 and E2 on gametogenesis that can be beneficial for evading pollution stress in marine ecosystem.

This study will help the researcher to uncover the critical areas of anthropogenic activity that many researchers were not able to explore. Thus a new theory on bio-monitoring marine pollution may be arrived at.

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