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ORIGINAL ARTICLE

Sublethal Toxic Impact of Chlordecone on Hormonal Parameters and Histological Alterations in Gonads of the Cichlid Fish, *Pseudetroplus maculatus* (Bloch, 1795)

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ABSTRACT
Sublethal toxicity of chlordecone was evaluated by assessing the hormonal parameters and histological alterations in gonads of the cichlid fish, Pseudetroplus maculatus. Chlordecone at 3.5 and 7µg/L was exposed to male and female fishes for 4, 7, 15, and 30 days maintaining control groups without toxicant. At the end of every treatment period, hormonal parameters such as serum levels of thyroid stimulating hormone (TSH), follicle stimulating hormone (FSH), luteinising hormone (LH), testosterone and estradiol (E_2) was evaluated in both male and female fishes and compared to that of the control groups. In males, the concentrations of TSH, FSH, LH and testosterone showed a significant decrease at higher sublethal concentration after chronic exposure whereas E_2 showed significant increase at both sublethal concentrations after chronic exposure when compared with the control groups. In females TSH, LH and testosterone levels decreased significantly at higher concentration of chlordecone exposure after 30 days of treatment along with a significant increase in the level of E_2 without any alterations in the level of FSH when compared with the control groups. Histological examination revealed that chlordecone impairs gonadal growth and differentiation as testis showed degeneration of spermatogenic cells, reduced number of spermatocytes whereas ovary showed vacuolated, atretic vitellogenic oocytes with damaged yolk granules. The severity of gonadal tissue damage increased in time-dependent manner. The results of the study demonstrate that chronic exposure of chlordecone could cause hormonal imbalance and lead to negative changes in gonadal architecture, eventually leading to reduced fertility and reproduction in fish.
Keywords: Chlordecone, *Pseudetroplus maculatus*, hormones, histopathology, gonads

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INTRODUCTION

Over recent years, exposure of several endocrine disrupting chemicals in various environmental compartments poses threat to organisms and have adverse effects on the general health status and reproductive functions in humans, wildlife and aquatic animals [1-2]. Numerous natural and anthropogenic substances released into the aquatic ecosystems possess the ability to disrupt normal hormonal status in fish and other aquatic animals, which in turn could alter several physiological functions. In fish, exposure to endocrine disruptors alter several reproductive and developmental processes [3-4] involving changes in steroid hormones [5], induction of female specific proteins such as vitellogenin in males [6], impaired gonadal development [7], reduced sperm quality [8] and egg production [9], incidence of intersex populations [10] and skewed sex ratio [11].

Chlordecone is a polycyclic chlorinated pesticide widely applied in banana and tobacco fields, ornamental shrubs and citrus trees for the control of various pests including banana root borer, mites, wire worms, slugs, snails and fire ants. It is a stable compound, highly resistant to degradation in the environment [12] and has the potential for trophic transfer through aquatic food webs [13]. Chlordecone has been shown to interact with the estrogen receptors in the uterus of rat and competes with estradiol for binding to the cytoplasmic receptors [14]. Similarly, injection of chlordecone in immature rats increased the estrogen receptor in the uterine nuclear fraction with concomitant decrease of cytosolic estrogen receptors [15].

Moreover, chlordecone exposure at varying doses has been shown to increase progesterone receptors in the uterus of immature rats [16] and resulted in persistent vaginal estrus, anovulation and earlier vaginal opening in adult female rats [17]. Prenatal exposure to chlordecone has been shown to cause thyroid hormone disruption among boys whereas postnatal exposure resulted in thyroid disruption in both sexes [18].

Toxic effects of pollutants in aquatic ecosystem can be well-studied by using fish as a bio-indicator since it is constantly exposed to unfavourable environmental conditions, including chemical contaminants and potential pathogens, thus fishes are regarded as one of the primary risk groups among the aquatic organisms [19]. *Pseudetroplus maculatus* belongs to the broadly distributed class of teleosts cichlid family inhabiting both freshwater and brackish water and are highly sensitive to the changes in environmental conditions. The median lethal concentration of chlordecone in *Etroplus maculatus* determined by probit analysis for 96 h was 35 µg/ L [20]. Previous studies in our laboratory have reported that chlordecone induced oxidative stress in gill, liver, brain and muscle tissues [21-23] and caused genotoxicity which is evidenced by the induction of micronucleus and other nuclear abnormalities in the fish erythrocytes [24]. However, the estrogenic and other endocrine disrupting effects of chlordecone in fish has received minimum attention.

Interplay of various hormones, among which, estrogen plays a vital role in controlling the reproductive functions of fish. Meanwhile an imbalance in the hormonal status could be caused by an exposure to exogenic compounds possessing estrogenic properties thereby leads to adverse effects on reproductive functions. Thus, the present study was aimed to evaluate the sublethal toxic effects of estrogenic compound chlordecone on various hormones such as thyroid stimulating hormone (TSH), follicle stimulating hormone (FSH), luteinising hormone (LH), testosterone and estradiol (E_2) in the circulating blood. Histopathology of gonads is another sensitive tool used to evaluate the degree of endocrine disruption in fish by measuring abnormalities at morphological or at cellular levels [25]. Therefore, gonadal histopathology is also assessed in the present study to predict the reproductive health status of the fish, *Pseudetroplus maculatus*.

MATERIALS AND METHODS

Chemicals

Technical grade organochloride pesticide, chlordecone (Kepone, decachlorooctahydro-1,3,4-metheno-2H-cyclobuta[cd]-pentalen-2-one, 99.9% purity) was obtained from Supelco, USA. Ethanol, xylene, paraffin wax, DPX, eosin and haematoxylin were obtained from Himedia Laboratories, Mumbai, India. Hormone kits were obtained from Diagnostic System Laboratories, Inc. Webster, Texas, USA. All other chemicals were of analytical grade and obtained from local commercial sources.

Fish and experimental design

Adult male and female freshwater cichlid fish, *Pseudetroplus maculatus*, weighing 7±1 g and 7±1.5 cm in length were collected from KKF Nursery, Manjeri, Vaniyambalam, Kerala, India. Fishes were brought to the laboratory with least disturbance and were acclimatized to the laboratory conditions for 15 days prior to experiments. They were maintained in dechlorinated, well-aerated aquarium tanks (40 L capacity) and the health status of the animal was continuously monitored throughout the experiment. The physico-chemical features of the tap water were estimated as per APHA [26] and the water temperature 28±2°C, oxygen saturation 70 to 100%, pH 6.5 to 7.5 was maintained in the range throughout the experiment.

Chlordecone was dissolved in 1% DMSO (dimethyl sulfoxide), therefore used as positive control (vehicle) in the experiment. The median lethal concentration (LC_{50}-96 h) of chlordecone in *Pseudetroplus maculatus* determined by probit analysis was 35 µg/ L [20]. Based on the LC_{50}–96 h value, two sublethal concentrations - 3.5 µg/ L and 7 µg/ L (1/10th and 1/5th of LC_{50}) were selected and exposed to both male and female fishes for 4, 7, 15 and 30 days. Ten animals were maintained in both treatment concentrations, and also in positive and negative control groups.

Post-treatment analysis

At the end of every treatment period, fishes were caught very gently using a small dip net, one at a time with least disturbances. Blood samples were collected from control and treated groups, both sublethal concentrations, for hormone assays. Fishes treated at one-tenth of LC_{50}-96 h of chlordecone concentration were sacrificed for dissecting gonads for histological examinations.

Blood samples were collected from both male and female fishes in separate clean microcentrifuge tubes by cardiac puncture method with the help of syringe. Blood was centrifuged at 1700 g for 15 min at 4ºC and serum samples were collected and stored at -80ºC in microcentrifuge tubes for hormone analysis. Serum levels of TSH, FSH, LH, testosterone and estradiol, were measured by enzyme linked

immunosorbant assay (ELISA) using kits. The assays were done strictly according to the procedure given along with the kits.

Testis and ovary were dissected out from low sublethal concentration (one-tenth of LC_{50}-96 h) and were stored in 10% buffered formalin for 24 to 48 h for histopathological analysis. Gonads were then dehydrated in ascending grades of ethanol and were cleared in xylene until they became translucent. Tissues were transferred to molten paraffin wax for 1 h to remove xylene completely and impregnated with wax. The blocks were cut and sections of thickness 4 to 6 microns were prepared using rotary microtome. The sections were stained with haematoxylin and eosin and mounted in DPX [27]. The slides were examined for structural alterations under light microscope and compared with that of control tissues. Photomicrographs were taken using Cannon shot camera fitted to the Carl Zeiss Axioscope-2 Plus Trinocular Research Microscope.

Statistical analyses

The values of hormonal parameters were expressed as Mean±SD for n = 10 animals/ group. Data were analyzed by using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test using statistical package SPSS 19.0. Differences were considered to be significant at $p<0.05$ against the control groups.

RESULTS

In the present study, fishes exposed to DMSO showed no significant variations in serum levels of TSH, FSH, LH, testosterone and estradiol when compared with solvent-free control group (Figure 1 and 2). Male fish when exposed to chlordecone showed significant ($P<0.05$) decrease in the levels of TSH, FSH, LH and testosterone at higher sublethal concentration after prolonged exposure when compared with the corresponding control groups (Figure 1). The level of E_2 was increased significantly in duration and concentration-dependant manner (Figure 1). In females, serum levels of TSH, LH and testosterone were decreased significantly ($P<0.05$) at one-fifth of sublethal concentration after 30 days of chlordecone exposure whereas the level of FSH remained unchanged throughout the treatment period (Figure 2). Meanwhile, the level of E_2 was increased significantly in duration and concentration-dependant manner when compared with the control groups (Figure 2).

Histological examination revealed control testis having compact seminiferous tubules with well-developed spermatogonia, spermatocytes and mature spermatozoa enclosed in the lumen of testis (Figure 3A and 3B). Chlordecone exposure at one-tenth of sublethal concentration caused degeneration of spermatogenic cells after 4 and 7 days of exposure (Figure 3C and 3D). After 15 and 30 days of chlordecone treatment seminiferous tubules are seen with gross vacuolization, distortion of seminiferous epithelium followed by reduced number of spermatocytes and spermatozoa (Figure 3E and 3F).

Normal histology of ovary showed vitellogenic oocytes characterized by presence of yolk vesicles and yolk granules in the cytoplasm (Figure 4a and 4b). Chlordecone treatment for 4 days showed gross vacuolization in the developing oocytes (Figure 4c). After 7 days of chlordecone exposure, degenerated and loosely packed oocytes with highly proliferated connective tissues was observed (Figure 4d). Exposure to chlordecone for 15 days showed vacuolization and highly reduced oocytes (Figure 4e). Chlordecone when exposed for 30 days showed completely distorted and atretic oocytes having yolk granules scattered in the ovarian cavity (Figure 4f).

DISCUSSION

In recent years, the study of endocrine disrupting compounds (EDCs) on aquatic ecosystem is more prominent because of its estrogenic effects, toxicity and the ability to bioaccumulate in aquatic organisms. Chlordecone, one of the EDCs possessing estrogenic properties, are susceptible to cause adverse reproductive effects in the exposed aquatic organisms. The present study was focused to evaluate the sublethal toxic effects of chlordecone on hormonal parameters and histological alterations in the gonads as bio-indicators to study the estrogenic effects of the compound in the cichlid fish, *Pseudetroplus maculatus*. Fish models are widely used to evaluate the toxic effects of EDCs on aquatic ecosystem. More recently, the study of the impact of EDCs on endocrine system has gained much attention as it serves as the early warning signal to evaluate the adverse reproductive effects of the toxicants. Besides, the measurement of circulating levels of hormones, particularly those involved in the regulation of vital functions such as osmoregulation, energy metabolism, reproduction or growth brings additional information on the toxic stress in fish. It is well understood that the development and functioning of gonads is under the control of steroid hormones such as testosterone and estradiol [28]. In fish, the concentration of these hormones could vary based on the reproductive cycle or breeding seasons [29].

Figure 1 Effect of chlordecone on serum hormone levels in male fish

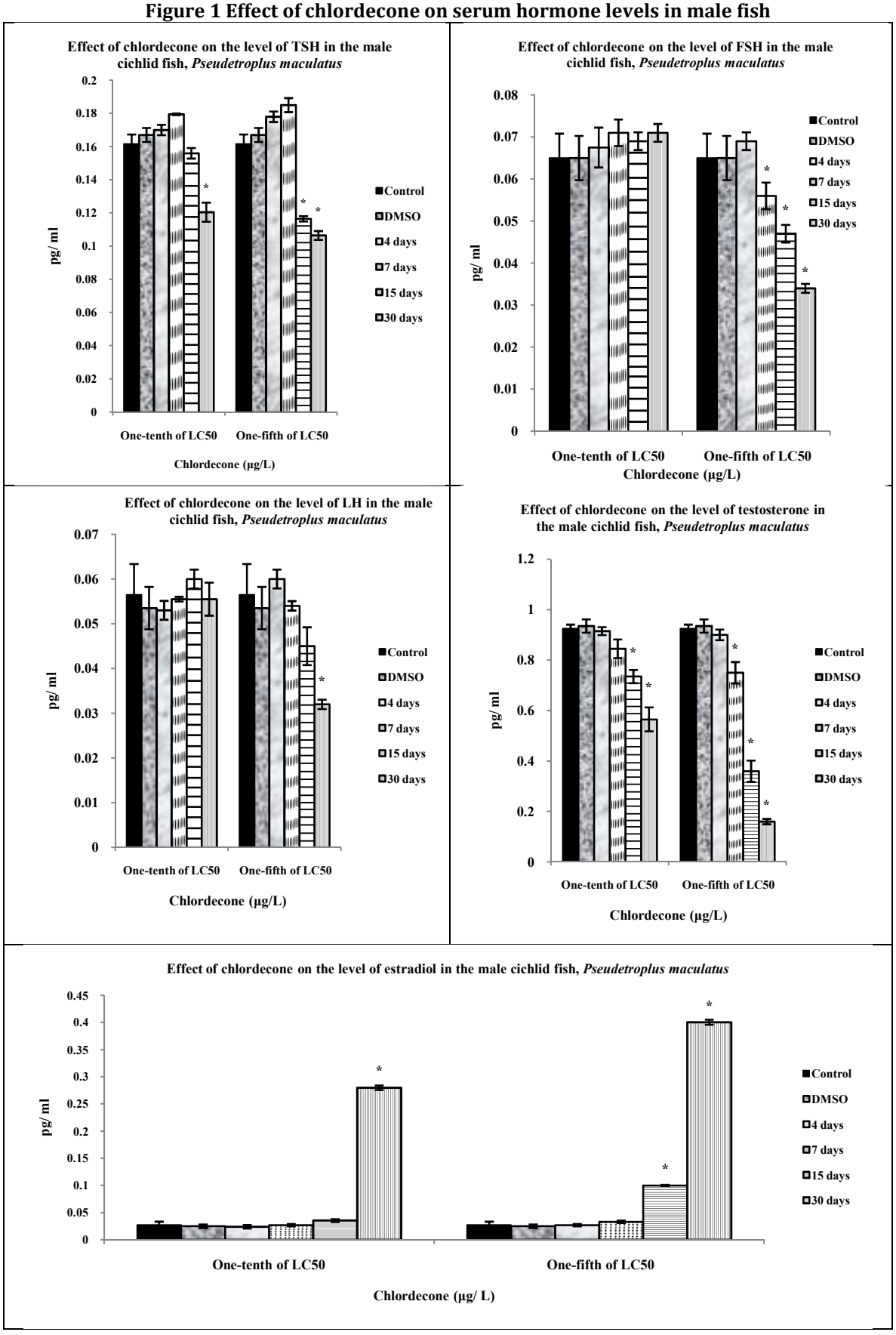


Figure 2Effect of chlordecone on serum hormone levels in female fish

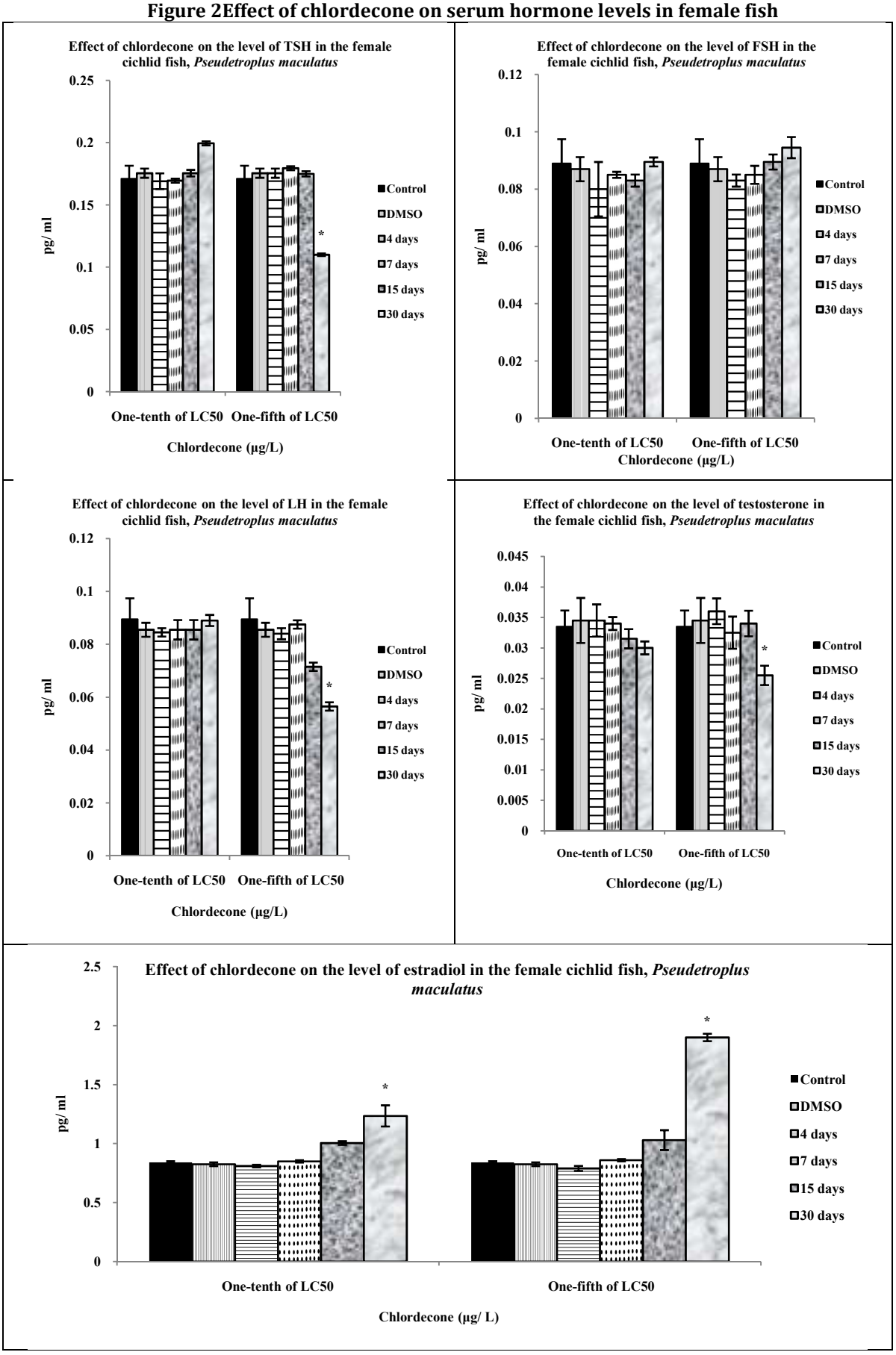


Figure 3 Photomicrographs of histological sections, stained with H&E in the testis of *Pseudetroplus maculatus*. A-Control; B-DMSO-treated; C-Chlordecone-treated for 4 days; D-7 days; E-15 days; F-30 days showing degeneration of spermatogenic cells, reduced number of spermatocytes and complete atresia

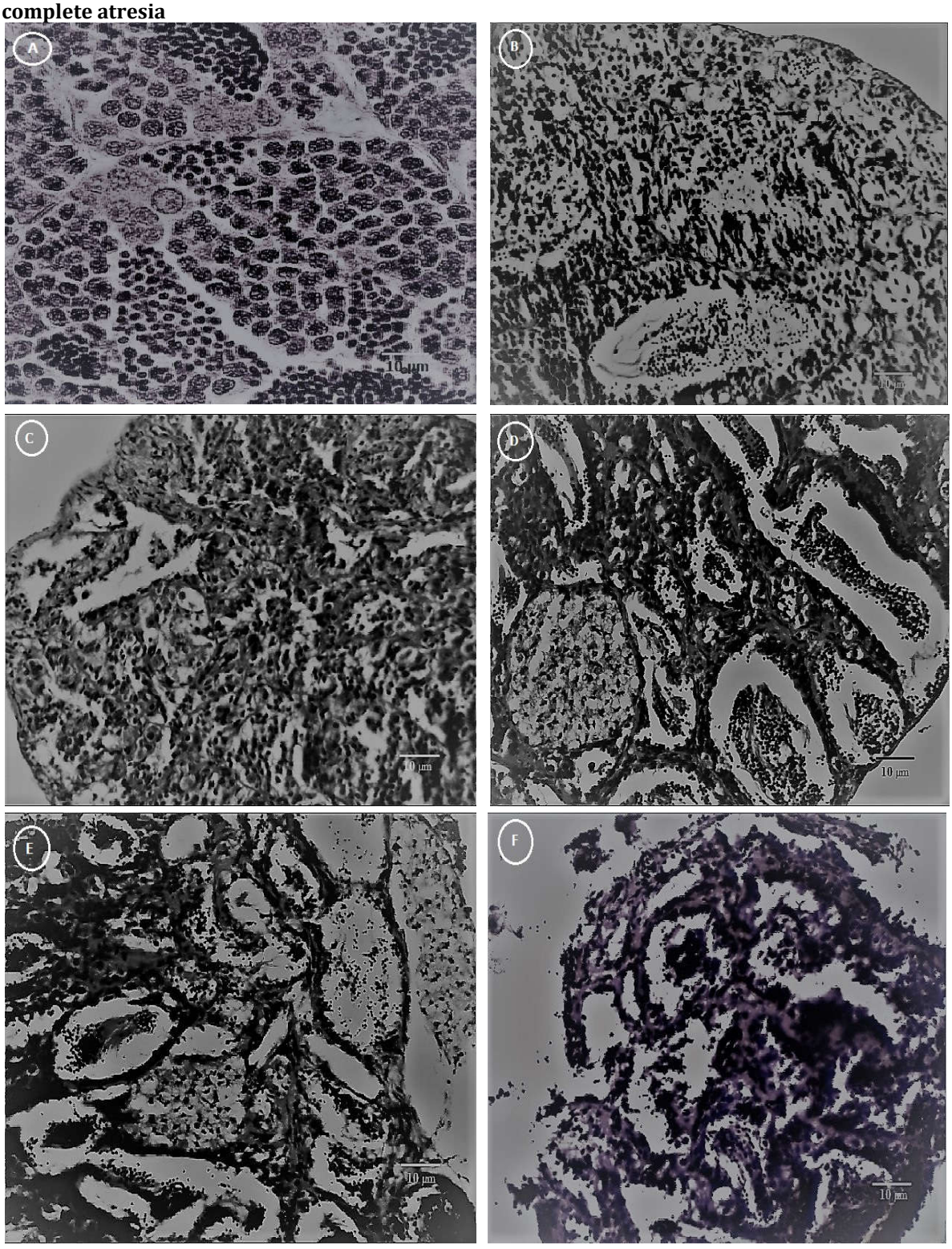
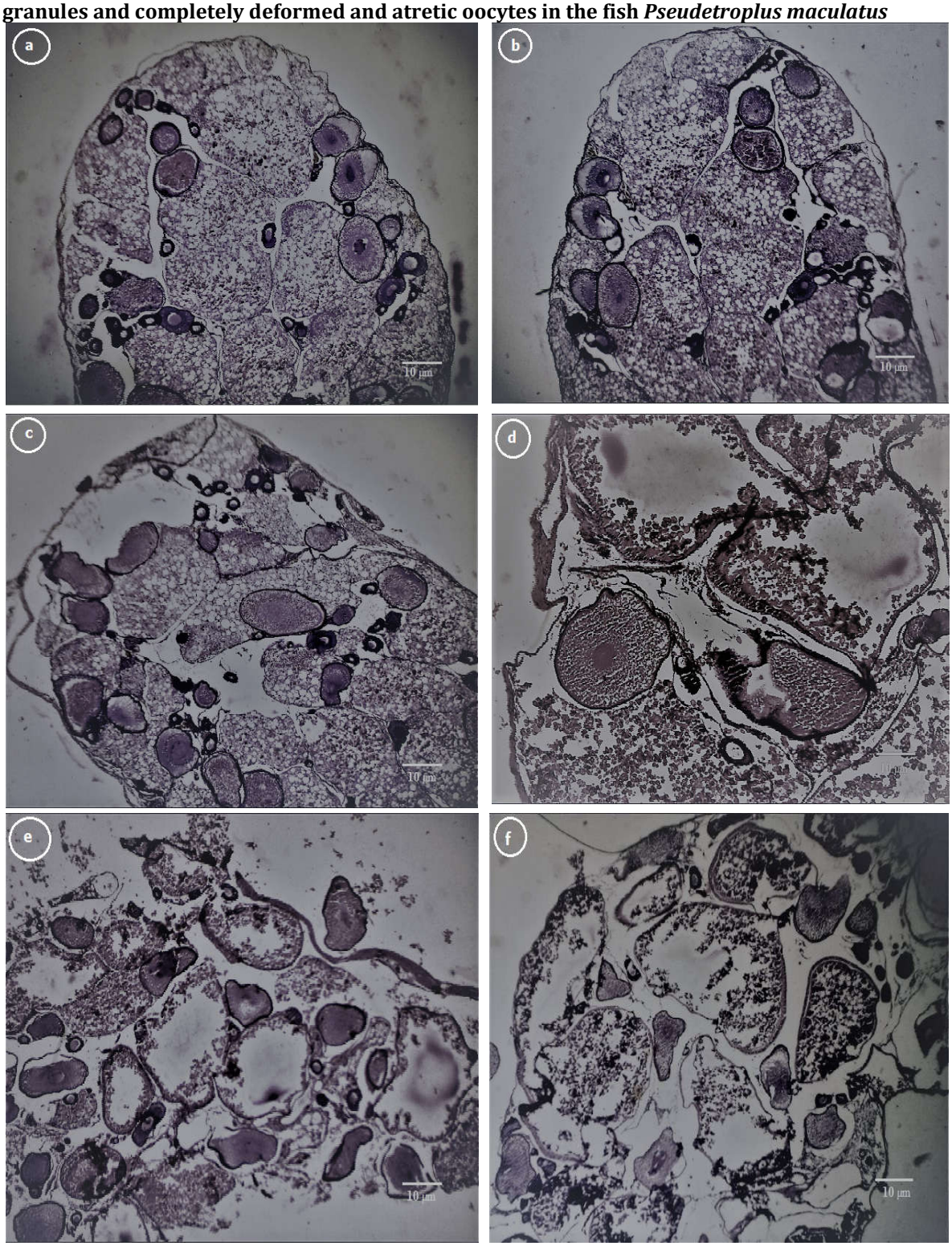


Figure 4 Photomicrograph showing A-Control; B-DMSO-treated; C- Chlordecone-treated for 4 days; D-7 days; E-15 days; F-30 days showing vacuolated, vitellogenic oocytes with damaged yolk granules and completely deformed and atretic oocytes in the fish *Pseudetroplus maculatus*



In male fish, 11-ketotestosterone is the main androgen hormone which is secreted from Sertoli cells and is responsible for reproductive behaviour and expression of secondary sexual characters, spermatogenesis and sperm maturation in most of the teleosts [30]. However, 17β-estradiol is synthesised from the follicular cells which stimulate liver to synthesize the egg yolk precursor protein vitellogenin, which is then transported to ovaries and incorporated into the developing oocyte in female fish [30]. Usually, in males the level of estradiol is relatively low when compared with the female fish. However, EDCs released into the surrounding water has been shown to upregulate the activity of an important enzyme aromatase, which converts the male hormones to female hormones resulting in an increased level of estradiol [31]. In the present study chlordecone exposure at two sublethal concentrations increased the level of estradiol in both male and female fishes with concomitant decrease in the level of testosterone in the fish, *Pseudetroplus maculatus*. The estrogenicity of chlordecone in male fish was very well proven by 10-fold and 16-fold increase in the level of estradiol after 30 days of treatment when the concentration of chlordecone was doubled. It has been reported that exposure to atrazine in male gold fish increased the level of estradiol in dose and time-dependent manner with suppression of testosterone level [32]. Meanwhile the level of testosterone in males was found decreased significantly at both sublethal concentrations after chlordecone exposure and in females the decrease was noted only at higher concentration after 30 days. Thus, the alterations in sex steroid hormones in fish indicate the estrogenicity of chlordecone and its impact on steroidogenesis.

In addition to sex steroid hormones, TSH, LH and FSH also play major role in reproduction, growth and development in fish. Thyroid hormone is mainly involved in growth, development and metabolism in teleost fishes [33] and also exerts relationship with reproduction as well as modulation of reproductive cycle [34]. Thyroid stimulating hormone (TSH) is a glycoprotein hormone family produced by adenohypophysis of teleost fish and appears to be primarily under the control of hypothalamus [35]. TSH stimulates the thyroid gland to produce thyroxine (T_4) and tri-iodothyronine (T_3), which have a wide range of biological effects in physiological processes of vertebrates [33, 36]. Hence TSH is routinely used to evaluate homeostasis in fish and also used as potential biomarkers to determine the impact of toxicants in thyroid gland of fish [37]. The information on the effects of environmental pollutants on the thyroid system of fish remains scanty. Earlier report in gold fish have suggested that thyroid hormone work synergistically with gonadotropin to control ovarian development by increasing ovarian sensitivity to gonadotropic stimulation [38]. Besides, expression of steroidogenic enzymes and steroid receptors in goldfish are also mediated by thyroid hormones [39]. The present results suggest that male fish showed a slight increase in the level of TSH after 4 and 7 days of chlordecone exposure, followed by significant decrease after 15 and 30 days at both sublethal concentrations. However, the level of TSH in female fish showed significant decrease only at higher concentration after 30 days of chlordecone exposure. Thus, chlordecone induced reduction in the level of TSH is correlated with the reproductive and developmental impairments in the fish.

FSH and LH released into the blood stream as a result of stimulatory action of gonadotropin releasing hormone (GnRH) act on gonads, where they stimulate the synthesis of sex steroid hormone. In male FSH is known to play a significant role in early stages of spermatogenesis, whereas LH is primarily involved in later stages of maturation [40]. In female fish, FSH and LH are involved in follicular growth and oocyte maturation [41]. The present results showed no alterations in the level of FSH in female fish, whereas significant decrease was observed at higher concentration in time-dependant manner in male fish which reflects alteration in spermatogenesis is mainly due to toxicant exposure. The level of LH decreased significantly at higher concentration after 30 days of chlordecone exposure in both sexes and this could be due to pituitary dysfunction in LH release as a result of chlordecone exposure. Therefore, hormonal parameters proved as the most sensitive tool in monitoring the sublethal toxicity of chlordecone in fish.

Histological changes in gonads are the endpoint used in the present study to evaluate morphological damage induced by chlordecone. Histology of gonads offers general profile for gonad development and is cost effective tool to validate the health status of fish population. Histological studies of gonads in the control and solvent-treated group showed normal histoarchitecture. Exposure to one-tenth of sublethal concentration of chlordecone for 4 and 7 days caused degeneration of spermatogenic cells. The severity of damages increased after 15 and 30 days of chlordecone treatment which showed seminiferous tubules with gross vacuolization, distortion in seminiferous epithelium and reduction in the number of spermatocytes and spermatozoa. Similar results were observed on studying the effect of endosulfan in freshwater cyprinid fish, *Cyprinion watsoni* [42]. Histology of ovary when exposed to chlordecone for 4 days showed gross vacuolization in the developing oocytes. Highly degenerated and loosely packed oocytes with proliferated connective tissues were observed after 7 days of chlordecone exposure. Exposure to chlordecone for 15 days showed vacuolization and highly reduced oocytes and when the

treatment period is extended for 30 days showed completely distorted and atretic oocytes with yolk granules scattered in the ovarian cavity. Similar observation as atretic follicles and vacuolization in the ovary has been reported in the fish, *Labeo rohita* when exposed to sublethal dose of endosulfan for 30 days [43]. Thus the present histological study clearly illustrates the sublethal toxic effects of chlordecone in gonads of the fish, *Pseudetroplus maculatus*.

CONCLUSION

In the present investigation both hormonal and histopathological endpoints showed sublethal gonadotoxic impact of chlordecone in the fish *Pseudetroplus maculatus*. Thus the changes induced by chlordecone reflect the estrogenic effect of the toxicant and this could seriously lead to severe consequences on reproductive potential of the fish population.

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The authors have declared that no competing interest exists.

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