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ASSESSMENT OF MOLECULAR AND BEHAVIOURAL CHANGES ENCOUNTERED DUE TO POLLUTION MEDIATED ENDOCRINE DISRUPTION IN SOME CHOSEN FISHES IN THE RIVER VAIGAI, SOUTH INDIA

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ABSTRACT

The diversification of chemicals from various point and non-point sources discharged into the environment that can mimic or antagonise the action of hormones. These endocrine-disrupting chemicals (EDCs) can thus interact with physiological systems and cause alterations in development, growth and reproduction in wildlife that are exposed to them. The present study focused on the endocrine sensitivity of the fishes collected from three different contaminated regions of a non-perennial Holly river Vaigai, which flows across Madurai city. The contaminated locales are Sewage confluence area (Point source), Liquid food Effluent area (Point source), and Agricultural runoff area (non-point source). From these three regions, the inhabiting common fish as Oreochromis mossambicus was collected and examined for their hormone (Estrogen) and protein (Vitellogenin) interaction. The controversial results were obtained due to endocrine disruptive chemicals discharged from these contaminated sites when compared to normal reproductive fish physiology.

Key words: *Endocrine disruption, gonadosomatic index, hepatosomatic index, estrogen, testosterone, progesterone, gonadotropin, vitellogenin.*

INTRODUCTION

Endocrine modulation is not new to science. It has been known of as part of herbal and classical medicine, in particular contraception, for centuries. It is a natural mechanism which controls the hormone balance in the body. It can lead to good or bad effects in humans or animals. Endocrine disruption is an alteration of the endocrine system in an uncontrolled or undesired manner without consideration or knowledge of consequences to health. Identification of the possible adverse effects of endocrine disrupting chemicals (EDCs) on reproduction and development in vertebrates is becoming a major focus since 1990s. The National Institute of Environmental Health Sciences ("NIEHS") defines endocrine disruptors as "chemicals that may interfere with the body's endocrine system and produce adverse developmental, reproductive, neurological, and immune effects in both humans and wildlife." Disrupting Hypothalamic-pituitary-gonadal (HPG) axis of the system has a direct impact on the related steroid hormones (estrogens, androgens) (EPA 1998; Huet 2000).

Concerns that endocrine disruption could occur have been raised in respect of including natural and synthetic hormones, environmental contaminants, agricultural chemicals, and industrial chemicals from a variety of point and non-point sources which tends to affect HPG axis by mimicking the steroid hormones (Ankley and Johnson 2004). So far, a limited number of chemical substances have shown weak estrogenic activity. Naturally occurring endocrine active chemicals produced by plants or fungi (phytoestrogens and mycoestrogens) have also been identified. As defined above, endocrine disruption is therefore a mechanism, not an effect. Before a chemical substance can be defined as an "endocrine disruptor", it has to be scientifically proven that it has the ability to produce adverse health effects in humans or wildlife (Piferrer 2001). Regulatory decisions for approval, banning or replacing substances should be based on a science-based risk assessment process. This process takes into account adverse effects and exposure.

Endocrine disruption can occur via multiple mechanisms in adults as well as in developing embryos. These alternations are complex and are not limited to a particular organ or molecular mechanism. Such as contaminants can alter (i) hormone production at its endocrine source (ii) the release of stimulatory or inhibitory hormones from the pituitary or hypothalamus (iii) Hepatic enzymatic biotransformation of hormones (iv) the concentration or functioning of serum-binding proteins altering free hormone concentrations in serum (Guillette et al. 2000). The primary concern in human health to date has been on substances which might mimic the female hormone estrogen, and thereby potentially contribute to a variety of adverse human health effects including reproductive and developmental abnormalities.

In wildlife the concerns focus primarily on potential reproductive dysfunction and abnormalities that might threaten the survival of certain species. Therefore, some of the better studied examples of adverse effects of EDCs in the environment are of aquatic animals especially fishes (Ankley and Giesy 1998; Fairbrother et al. 1999; WHO 2002). Endocrine disruption in fish appears more frequently associated with three types of land use: sewage treatment plants (STPs) (estrogenic effects), pulp and paper mills (antiestrogenic and probably antiandrogenic effects), and areas of high industrial activity/chemical contamination (antiestrogenic and possibly antiandrogenic effects).

Many studies have been reported that many water bodies have been receiving a large amount of input of above said chemicals both synthetic and natural (from diffuse and point sources) acting as androgen and estrogen agonist or antagonist, aromatase inhibitors, also thyroid disruptors (Tyler et al. 1998; Fairborther et al. 1999; Vos et al. 2000). Sumpter & co., in the UK documented the presence of feminized male fish in rivers downstream of municipal waste water treatment plants, and then associated this response with the occurrence of steroidal estrogens and some types of chemicals (alkyl phenols) (Desbrow et al. 1998). This phenomenon was also observed in several other studies where presence of estrogenic chemicals in municipal waste waters found to alter the testes of male fish to produces eggs rather than sperms (Folmer et al. 1996; Kundsen et al. 1996; Larsson et al. 1999; Christiansen et al. 2000). A study conducted at koraiyar, Cauvery River which is a reservoir of both agricultural and sewage waters reported the presence of both male and female secondary sex characteristics in the few *O. mossambicus* fishes due to the cocktail effect of chemicals in the environment that could interrupt or mimic the hormones (Subramanian and Amutha 2006).

Discharge from pulp and paper mills also has direct effect on the fishes with alternation in endocrine functions from the river, noting that the occurrence of the plant by products in the river water has capacity to charge fishes endocrinal functions by altering estrogens and androgens (Larsson et al. 2000; Parks et al. 2001). Native fishes from marine sites also found with possible adverse effects on EDCs which may be related to tetracholorodibenzo-p-dioxin, polychlorinated biphenyls and polycyclic aromatic hydrocarbons (Fairbrother et al. 1999).

These studies have created a greater concern in studying the status of water bodies in various regions. In Canada and Europe, a number of species of freshwater fishes such as *Catostomus catostomus; Coregonus clupea formis; Perca fluvialitilis; Rutilius rutilius* were reported to have altered features in their reproductive development, including reduced gonandal growth, inhibited spermatogenesis as they live downstream to pulp- and paper- mill effluents (Adams et al 1992; Hodson et al. 1992; Dickerson et al. 1998; Karels et al. 1999). Amutha and Subramanian 2012 reported a dosage-dependent increase in the growth (length & weight) and maturation in Cadmium treated *O. mossambicus* through the life-stage experiment. They concluded Cadmium as an estrogen-like growth-promoting substance in early stage of development of *O. mossambicus*.

There are several sensitive biomarkers developed and applied as bio monitoring tool to study the physiological and molecular level changes caused by Endocrine disruptors. Biomarker is a molecule which changes with the biological response, relating to the exposure of the toxic environmental chemicals (Peakall 1994). Vitellogenin is the most widely used biomarker which is normally synthesized in liver of oviparous female fishes but because of the micropollutant in the effluents which tends to act as xenoestrogens thereby enhancing the production Vitellogenin in female fishes even in juveniles and also resulting in secretion of Vitellogenin in blood plasma of male fishes (Sumpter et al. 1999; Angus et al. 2002). An abnormal concentration of vitellogenin is also observed in many cases as they have longer exposure to estrogen through industrial and domestic effluents (Gray et al. 1999; Iwamatus 1999; Kinnberg et al. 2000).

In Tamilnadu, rivers are mostly seasonal except Cauvery the perennial one, during the drought conditions; these rivers hold only small water pockets here and there with/without running or flow. During that period, there is a chance to concentrate the mixed contaminants, which is very dangerous to the aquatic life. Such an exposure not only causes the ill effect during drought conditions but also when the normal flow resumes. Thus the urban and agricultural drainages, threaten the fish population due to its EDC properties, a trace level is enough for the reproductive alterations. The present study investigate the different polluted discharges such as sewage, liquid food industrial effluents, agricultural effluents and Vaigai dam as reference sites of Vaigai River.

Materials and Methods:

The two common, commercial fishes of river Vaigai, *Oreochormis mossambicus* (Peters 1852), the Mozambique tilapia is deep bodies cichlid fish which can alter their morphological characteristics such as body size at sexual maturity in response to different environmental conditions and *Channa puntatus* (Bloch 1793), the green snake-headed spotted murrel having low total lipid content & adipose tissue (Ghose 2006), hence named Lean Fish were considered for the study.

Matured fishes of both *O. mossambicus* & C. *punctatus* were collected from the river Vaigai (a non-perennial river having water in its run ways) during various seasons such as pre-monsoon, post-monsoon and summer were collected and maintained in cement cistern for acclimization. The periodical sample analysis of both fishes were collected from different effluent drains such as sewage drainages (Arapalayam), liquid-food effluents (Parvai-discharged in Kochadai), Agricultural effluent (Melakkal – Sozhavandan) and Vaigai dam (near Andipatti) and were examined for the following.

Morphological and Anatomical study:

In case of *O. mossambicus*, Fishes that are collected from the area are examined for variations in their external characteristics by venting method i.e. by observing the shape of vent between the anus and anal fin. This provides knowledge on gender of the fishes and also if any abnormabilities observed those fishes chosen for further studies. The morphological changes were monitored either with naked eyes or through microscope. For anatomical studies, the collected fishes were euthanized and immersed in tricaine methanesulfonate. The organs were dissected out as a whole and were weighed immediately for further analysis.

Expolation of GSI and HSI Indices:

The weight of each fish and its gonad was measured to calculate the Gonadosomatic Index likewise the weight of liver was measured to determine its Hepatosomatic Index.

$$\text{Gonadosomatic Index} = \frac{\text{Weight of the gonad}}{\text{Weight of the fish}} \times 100$$

$$\text{Hepatosomatic Index} = \frac{\text{Weight of the gonad}}{\text{Weight of the fish}} \times 100$$

Vitellogenin (Vtg) Assay by ELISA method:

Vtg quantification was performed using a direct enzyme-linked immunosorbent assay (ELISA) as described by Olsen et al. 2005 with a slight modification. Standard Vtg was purified from female, male plasma and juveniles (whole body extract) according to the method developed by Brion et al. 2000. The samples or serial dilutions of standard Vtg were centrifuged at 2,000 g for 3 min, diluted at 1:2 in coating buffer (0.05 M carbonate/bicarbonate, pH 9.6) and incubated in MaxisorpTM microtiter well overnight at 4°C. Non-specific binding was then blocked with 2% (w/v) of BSA in PBS for 1 h at 37°C. After three washes with PBS-Tween-20 0.05% (v/v), plates were incubated with a monoclonal mouse anti-salmon Vtg antibody (BN-5 diluted 1:2000) for 2 h at 37°C, followed by another washing step and incubation for 2 h at 37°C with an anti-mouse IgG secondary antibody labelled with horseradish peroxidase (1:2,000). After 5 washes with PBS-Tween, enzymatic detection was performed using TMB ELISA substrate, and plates were read at 450 nm using a spectrophotometer microtiter plate reader.

Enzyme assay by chemiluminescent method:

Enzyme assay was carried out through Chemiluminescent method for which the serum sample were taken from the collected fishes depending the size of the fish. From juveniles, the whole body extracts were collected and for larger fishes, the blood was collected by either heart puncturing or from their opercular vein. These samples were centrifuged and serum was collected immediately. Within 3 hours of serum collection, the samples were analysed with chemiluminescent immunoassay analytical instrument. This can quantify even very low concentration of complex samples.

Results:

For the periodical sample analysis, both fishes were collected from different effluent discharges such as sewage drainages (Aarapalayam), liquid food industrial effluents (Paravai -discharged in Kochadai), Agricultural effluent (Melakkal-Sozhavandan) and Vaigai dam (Near Aandipatti) in which it was noted that the *O.mossambicus* fishes were found in all the seasons and all the sites whereas the *C.punctatus* was found only at two sites such as liquid food industrial effluents and sewages drainages areas also seen in all the seasons.

Morphological and anatomical variations of both fishes:

The Morphological and anatomical changes examined through venting method revealed that the fishes were morphologically normal but the size of the reproductive papilla size varied such as enlarged female and male papilla even in early sized stages (4 to 5 cms) in *O.mossambicus* and anatomically varied size of testes and ovaries were noted whereas the papillae are merged in *C.punctatus*. Subramanian and Amutha 2006 reported imposex of *O. mossambicus* individuals were observed in the sewage contaminated areas of river Cauvery. The attribute this condition to the presence of androgen-mimicking substance which induced maleness in both sexes leading to imposex formation in female i.e. development of male penis like organ in the lateral line between anus and female gonad. These androgenic substances also have an estimated gonadal enlargement effect in both the sexes (Orlando et al. 2004) whose prolonged exposure created such kind deformation in the early stages of development (Soto et al. 2004) Edmunds et al. studied the complete sex reversal of medaka from male to female through DDT which induced permanent and functional male to female sex reversal acts as a weakly estrogenic compound in transforming an embryo. The egg number varied from one sites to another (Table 2) and also length and weight of the fishes (Table 1). As *O. mossambicus* usually spawns during spring (July to August), pre-monsoon season, at the end of summer season the matured eggs were released for fertilization and new egg maturation starts. This phenomenon was observed in all the areas considered for the study; the egg size was maximal during summer at the end of which spawning takes place. But egg number was very high when compared with reference site around 700 – 800 during summer and a sudden decline in the number of eggs were observed during pre-monsoonal period which was not observed in reference site.

Extrapolation of GSI and HSI indices:

The O.mossambicus both male female GSI and HSI and egg numbers were demonstrated table 1-2. Mozambique tilapia are polygynous (i.e males mate with multiple females) and brood embryos and young fry in their mouth — known as mouth brooding. They are multi-spawner based on the environmental conditions. The GSI and HSI are demonstrated in Table

2. Monthly variations of GSI provide the reasonable indicator of reproductive seasonality for fish. The seasonal timing of reproduction, spawning time is often identified from changes in the Gonadosomatic index which determines reproductive season (Zin et al. 2011). The results obtained showed decreased GSI of the *Oreochromis mossambicus* fishes collected from the three contaminated sites from that of Vaigai river mouth. The reduced GSI is a most important biomarker in determining the Endocrine disruption. In females from Vaigai river mouth, GSI was maximal during summer whereas in contaminated sites it was maximal during pre-monsoon period. In males from Vaigai river mouth, maximal GSI was observed during post-monsoonal and summer seasons whilst in contaminated sites maximum levels were observed during post-monsoonal (SDA & LFIE) and Monsoonal period (AE). Thus a monsoonal or monthly pre-lapsing of spawning period was noted. Cheek et al, 2003 implicate that there was a slight delay in the reproductive spawning of fishes due to feminizing population that has been contaminated by DDT. Similarly Barker et al, 1994 reported the winter flounder fishes taken from Port Harmon (study area) in the May-June time period contained mature gonads, while fish from the reference site had already spawned.

The Hepatosomatic index is the ratio of liver weight to body weight. The liver is a key organ in fish production of vitellogenin, the yolk-precursor (Zin et al. 2011) it is very important because it describes about the fish stored energy and is a good indicator of feeding activity (Zin et al. 2011). Increased HSI in males was noted during pre-monsoon period except in SDA maximal HSI was noted during summer but for females it was during summer. The HSI levels gradually increased from post-monsoon to pre-monsoon period and decreased slightly in monsoon period at the reference. These variations in HSI of both sexes were found to be the same in all three contaminated site except in SDA, the males were found to have the maximal level during summer and gradually decreased.

Estimation of Gonadial hormones by chemiluminescent assay:

Estrogen activity:

Estradiol (E_2) is the naturally occurring steroid sex hormone which is produced by conversation of androstenedione by enzyme aromatase triggers the formation of secondary sexual characters in females (Nelson and Randy 2005). It has been exerted out in urine by mammals and humans (Hanselman et al. 2003) and is also used in hormone replacement therapies. The levels of E_2 has notified as a biomarker in endocrine disruptive effect due to its natural wide occurrence. *O.mossambicus* estradiol activities are more in sewage drainages and gradually decreased in agricultural discharges, liquid food industrial effluents and very low level in Vaigai dam respectively. Male and juveniles also hike the estradiol synthesis collected from sewage drainages and agricultural discharges. An elevation in the level of E2 was observed in all the 3 contaminated sites especially in sewage drainaged area (SDA), even the juveniles collected from SDA were showing very high elevation in estradiol levels upto

150µg/mL during summer. In the reference site i.e. Vaigai River mouth, the Estradiol levels were normal and varied minimal in accordance with their spawning period i.e. during the month of July to August (Pre monsoon and monsoon seasons). While comparing with the reference site, all the three contaminated sites showed maximal elevation of Estradiol. During summer, an elevation in estradiol levels was also monitored. In sewage and agricultural effluents, the estradiol synthesis was in an unrespectable manner because seasons hike their activity (Fig-1). In *C.punctatus* females showed higher estrogenic activity during summer collected from both sewage and liquid food effluent regions then gradually decreased in other seasons (Fig-6). Thus, inferring that the estrogen-like substances present in this area interferes with the normal reproductive cycle as reported by Amutha and Subramanian 2006. They say that Endocrine disruptors doesn't allow the binding of hormone with that of the protein present in the blood thus increasing the chance of availability of the hormone to the cell, thereby increasing the feminization/masculization of fishes. This can also be exerted by the agonistic mimicking or through antagonistic blocking of the hormonal precursors.

Testosterone activity:

Testosterone is the steroid sex hormone from androgen group secreted by gonads which is the principal male sex hormone and plays a major role in secondary sexual character formation in males (Nelson and Randy 2005). This hormone is administered as 1-testosterone, for hormonal treatment. The testosterone levels of *O.mossambicus* was higher in male during all season collected from sewage drainages, agricultural discharges, liquid food industrial effluents and Vaigai dam respectively. In sewage drainage, juvenile showed higher elevation of testosterone when compared to other sites of juveniles. In the case of female, the higher testosterone levels were observed in sewage and agricultural areas. Monsoon showed higher elevation of testosterone in all sites for male *O.mossambicus* (Fig-2). In *C.punctatus*, only during summer season the hike of testosterone level was noted when compared to other seasons in all sites. The female fishes showed very low level testosterone secretions. The sewage sites show higher testosterone than the liquid food industrial effluent areas (Fig-7).

In the reference site, the testosterone levels peaked at summer and gradually decreased in pre monsoon and monsoon period and increased gradually during post monsoon season.(Fig-2) The level of testosterone didn't vary much from the levels of testosterone at reference site. Only SDA showed a significant elevation of testosterone that to during monsoon season which means that sewage effluent also containing androgenic substance that is washed more during the rains into the river. This disrupts the Hypothalamus-pituitary-gonadal axis and causes elevation of testosterone might be leading to enlargement of Penis. As sewage effluent has been reported to contain a wide range of chemicals that can be either estrogenic or anti-androgenic activity in nature. Studies on androgenic effect of EDCs on fishes due to sewage effluents were not widely studied.

Progesterone activity:

Progesterone a steroid hormone produced by corpus luteum of the ovaries, adrenal glands and placenta during pregnancy (Nelson and Randy 2005) involved in biosynthesis of other steroids in fishes such as androgens (testosterone and 11-ketotestosterone), cortisol, estradiol-17b (E2), and 17a, 20b-dihydroxyprogesterone (17a, 20b-P) (Nagahama 1994) and also in embryogenesis. In the present investigation in river Vaigai, the reference site (Vaigai river mouth) showed normal progesterone levels in females and in juveniles and males. The progesterone levels peaked at SDA in juveniles, females and males. Unlike SDA, AE showed very minimal variation from that of the reference site. In the case of LFIE site, unusual variations in female and Juveniles were observed. In *C.punctatus*, females showed higher activity collected from sewage when compared to other site. In the case of male, higher level of progesterone was noted in the sewage drainages. During monsoon period, an elevation level of progesterone was observed in both male and female (Fig-8).

Gonadotropins (GTH I and GTH II) activity:

The GTH I act as follicle stimulating hormones (FSH) of mammals for the stimulation the sex hormone synthesis and control. In *O.mossambicus*, female and male showed the elevated level than juveniles. The higher activity was observed in Vaigai dam, sewage effluent regions, agricultural drainages and liquid food industrial effluents regions respectively (Fig-4). In agricultural effluents, female and male showed higher activity during pre-monsoon period whereas the sewage sites showed higher activity of GTH I during summer seasons (Fig-4). In the case of juveniles, higher elevation of GTH I was noted in fishes collected from sewage drainages. GTH II acts as luteinizing hormones (LH) of mammals. The elevation level was observed in both sexes collected from all the sites but slight changes occurred seasonally (Fig-5). The sewage juveniles showed higher elevation of GTH II than agricultural drainages juveniles and gradually lowered in liquid food industrial effluent site and very low in Vaigai dam (Fig-5).

In *C.punctatus*, the GTH I level was increased during summer season than gradually decreased other seasons in both male and females. The higher elevation of GTH I and GTH II in both sexes collected from liquid food industrial effluents. Among the sexes male showed higher elevation when compared to female (Fig 9-10).

Vitellogenin Assay by ELISA methods:

Vitellogenin (VTG) is a female-specific phospholipoglycoprotein of 200-700kDa which is synthesized in the liver of oviparous vertebrates such as fish, in response to circulating estrogens and transported by the bloodstream to the ovary where it is taken up oocytes, cleaved in the final egg-yolk proteins lipovitellin and phosvitin and deposited as yolk granules or platelets (Wallace 1985). The plasma of adult *C.puntatus* and *O.mossambicus* & the whole body extracts of *O.mossambicus* juveniles were taken for analysis. The vitellogenic activities were higher during summer seasons when compared to other seasons because liver synthesized Vitellogenin in both fishes. The *C.puntatus* females synthesized higher Vitellogenin than that of male. They showed a higher production during summer season; both females and males from LFIA showed elevation in all seasons unlike SDA which elevated only during summer and pre-monsoon periods. *C. punctatus* males showed very low level of Vitellogenin activity when compared to *O. mossambicus* male. The reduced GSI, increased HSI, higher elevation in the levels of estradiol and gonadotropins (GTH I and GTH II) confirms the endocrine disrupting activity in fishes in contaminated sites. This has caused the enlargement of both male and female papillae of *O. mossambicus* and merged papillae in *C. punctatus*. Testosterone and progesterone does not potentially create any modulation in fish morphology. Vitellogenin levels were high in all the contaminated sites even juveniles and males produced Vitellogenin in their plasma this confirms that there is feminization of male fishes occurring to a certain extent in the sites of study. Seasonal variations in *O. mossambicus* showed that there is pre-lapse occurring in the sewage contaminated area due to the increased concentration of exogenous substance present in small packets of water available during summer. Of both the fishes studied, *C. punctatus* seems to be resistant to the contaminant than that of *O. mossambicus*.

Discussion

This study exposed that *O. mossambicus* captured from sewage drainage area along with liquid food industry effluents, Vaigai River mouth showed maximum estradiol (E2) activity during summer other than premonsoon, post monsoon and monsoon but in *O. mossambicus* captured from Vaigai River mouth showed some different result from other study site like juvenile exhibited estradiol activity almost same in all seasons, but in case of female it was premonsoon, monsoon showed maximum activity, simultaneously male has showed maximum activity during monsoon.In continuous exposure of heavy metal cadmium which shown the elevated level of all reproductive hormones even juveniles(Amutha and Subramanian 2013). A very high elevation in levels of E2 was observed during the summer season when the temperature is very high in Madurai causing increased natural evaporation of water, the resulting concentrated water has high estrogen-like substance in a small pool of water where the fishes survive. As the body gets exposed to increasing amount of xenobiotics with are endocrine disrupting in nature causes this elevation leading to impairment in the sexual organs (gonads) of *O. mossambicus*. In all these contaminated sites, SDA showed maximal impact on O. mossambicus followed by Agricultural effluent and liquid food industry effluents. Many report showed that there were presence of intersex and imposex in fishes due to direct or indirect contact with the sewage wastewaters that are either treated or untreated. The observation report says that Estradiol present in the sewage waters lead to intersex formation through estradiol-like substance consequentially leading to feminization of male fishes in the habitat (Jobling et al. 2002; Pait and Nelson 2002; Amutha and Subramanian 2006; Jobling et al. 2006; Kidd et al. 2007; Hinck et al. 2009).

The simultaneous occurrence o f other abnormal biological responses, such as suppression of primary and secondary sex characteristics, significant increases in sex steroids in serum and testicular atrophy, were also observed in male fish (Lu et al.2010; Vajda etal.2011). Moreover, elevated serum 17b-estradiol (E2) concentrations and Aggravated sperm DNA damage were also simultaneously observed in demersal male flat fish collected in California (Rempel et al. 2006). These phenomena can directly and/or indirectly reduce the rate of survival, growth (generally during early-life stages), and reproductive success (e.g., fertility, hatching success) of individuals and have an impact on the population level (Hutchinson et al. 2006). The feminization of male fish downstream waste water discharges has mainly been demonstrated by the presence of elevated levels of female yolk protein (vitellogenin) in the blood of male or juvenile fish or eggs (or oocytes) in the testes (intersex) (Jobling and Tyler 2003). In concluding the results of present study, combined use of biological responses and chemical tools will help in validating the efficacy of biomarkers chosen to detect the endocrine disruptive effect. The combined levels of hormonal response and Vitellogenin in the fishes (both *O. mossambicus* and *C. punctatus*) collected from three different contaminated sites (Sewage Drainage area; Liquid food industrial effluent and Agricultural effluent area) showed a significant variation from that of the reference site (Vaigai river mouth). The reduced GSI, increased HSI, higher elevation in the levels of estradiol and gonadotropins (GTH I and GTH II) confirms the endocrine disrupting activity in fishes in contaminated sites. This has caused the enlargement of both male and female papillae of *O. mossambicus* and merged papillae in *C. punctatus*.

In testosterone activity it has shown some variable results in all study sites like in sewage drainage site male, female showed maximum testosterone activity during monsoon but juvenile has showed maximum activity during premonsoon season. Side by side the maximum testosterone activity of *O. mossambicus* from liquid food industry effluents has showed maximum activity like, in male it was in summer, in female it was in summer, premonsoon and in case of juvenile it has showed maximum activity during post monsoon and summer season but in case of agricultural effluents male showed maximum activity during pre and post monsoon season, in case of female and juvenile the activity was almost same in all season, simultaneously the *O. mossambicus* from the Vaigai River mouth showed the maximum testosterone activity in case of male it was recorded during summer but in case of juvenile and female the activity was recorded almost same in all season. Progesterone activity of *O. mossambicus* from sewage drainage site showed result like, in male the monsoon showed maximum activity although there was no significant difference in progesterone activity while in case of juvenile it was in premonsoon and in female summer, post monsoon season showed significant activity respect to other season. Female *O. mossambicus* from liquid food industrial effluent showed maximum activity during monsoon but in case of male and juvenile there was no significant difference in progesterone activity in all seasons. Simultaneously in agricultural effluents female showed optimum activity during summer and monsoon season but there was no significant difference in progesterone activity recorded in all other seasons, same as in Vaigai River mouth female showed almost equal amount of progesterone activity throughout all seasons but at the same time male and juvenile activity was recorded almost nil in all seasons. The estradiol level in case of *C. punctatus* female from sewage drainage and liquid food industry effluent showed optimum activity during monsoon season, other side male also showed very less but maximum activity during monsoon. Testosterone activity in *C. punctatus* male showed maximum activity during summer collected from both sewage and liquid food industrial effluents but sewage site showed more activity and it was found that testosterone activity almost nil in all season. Female *C. punctatus* progesterone from both two sites showed maximum activity during monsoon season. Zachary et al. 2012 studied the exogenous progesterone administered to fathead Minnow in the form of P4 They found that exogenous P4 is a endocrine active substance having negative impacts on the fecundity of the fishes from a point source and significant decrease in fertilization was also observed. As progesterone by itself does not directly act on any particular process of fish reproduction. They also hypothetically conclude that P4 acts as a primary intermediate in one or more steps of Hypothalamus-pituitary-gonadal axis after that it gets converted into one/ more biologically important fish steroids. Studies on progesterone-like substance activity pattern reported their nullification action on fish reproduction i.e. they neither masculinizes females nor increase or decrease the masculinity of males, thus concluded that exogenous progesterone were not converted into androgen. (Miura et al. 2007; Nagahama et al. 2008; Scott et al. 2010) Exogenous progesterone like substance activity were been reported to be cancelled out by Antiprogestin substance, it binds with progesterone receptors or by interrupting the progesterone control mechanism. Pesticides such as alachlor, endosulfan and kepone are reported to have this effect. (Vonier et al.1996; Amutha and Subramanian, 2012). Testosterone and progesterone does not potentially create any modulation in fish morphology. Vitellogenin levels were high in all the contaminated sites even juveniles and males produced Vitellogenin in their plasma this confirms that there is feminization of male fishes occurring to a certain extent in the sites of study. Seasonal variations in *O. mossambicus* showed that there is pre-lapse occurring in the sewage contaminated area due to the increased concentration of exogenous substance present in small packets of water available during summer. Of both the fishes studied, *C. punctatus* seems to be resistant to the contaminant than that of *O. mossambicus*.

GTH I activity of *O. mossambicus* from sewage drainage site exhibited optimum activity like, in case of male it was recorded during monsoon, female and juvenile showed maximum activity during post monsoon season but there was no such significant activity was recorded in juvenile. *O. mossambicus* male during premonsoon, juvenile during post monsoon and female showed almost same activity during all season when collected from liquid food industry effluents, but *O. mossambicus* from agricultural wastes showed result like; both male female it was in post monsoon season and juvenile showed almost same activity in all season. Simultaneously *O. mossambicus* male from Vaigai River mouth showed the maximum activity during all season, in case of female it was recorded that post monsoon showed the optimum activity but in case of juvenile it was recorded that there was no significant difference in progesterone activity. GTH II activity of male and juvenile *O. mossambicus* from sewage drainage system was recorded almost similar in all season, but for female post monsoon was optimum for GTH II activity, in other side male during monsoon, female and juvenile during premonsoon season showed optimum activity while they have captured from liquid food industrial effluents. Side by side male and juvenile from agricultural effluents showed maximum activity during pre monsoon, and female showed during summer season but male from Vaigai River mouth showed during premonsoon, female showed maximum activity during post monsoon and summer, juvenile showed almost same activity during all season. GTH I activity in case of female *C. punctatus* showed maximum activity during summer when they have captured from liquid food industries effluent but male and female from both two sites showed almost same activity. GTH II activity in both male and female showed optimum activity during pre monsoon season when samples collected from liquid food industrial effluent. GSI & HSI % was found high during monsoon in *C. punctatus* captured from sewage and agricultural fields but in case of liquid food industrial effluent, other than monsoon and in Vaigai River mouth during summer and pre monsoon showed maximum GSI & HSI %.

In vertebrates, Vtg dimmers are sequestered via receptor-mediated endocytosis into the growing oocytes where further site-specific cleavages occur to yield smaller yolk proteins (Wahil 1988). The Vtg protein is secreted from the cell through the secretory pathway before it enters circulation and is taken up by the growing oocytes. This makes blood plasma a natural target for Vtg analysis. Plasma Vtg concentrations are normally an indication of the maturational status of female fish (Mommsen and Walsh 1988; Arukwe and goksyr 2003). Several studies demonstrated that even male fish caught in rivers and streams had high levels of plasma Vtg (Purdom et al. 1994; Jobling et al. 1998) caused by chemicals acting like estrogens present in the environment. Vtg induction in fish has become an accepted measure of xenoestrogenic potency of chemicals, effluents and discharges. The simultaneous occurrence o f other abnormal biological responses, such as suppression of primary and secondary sex characteristics, significant increases in sex steroids in serum and testicular atrophy, were also observed in male fish (Lu et al.2010; Vajda etal.2011). Moreover, elevated serum 17b-estradiol (E2) concentrations and Aggravated sperm DNA damage were also simultaneously observed in demersal male flat fish collected in California (Rempel et al. 2006). These phenomena can directly and/or indirectly reduce the rate of survival, growth (generally during early-life stages), and reproductive success (e.g., fertility, hatching success) of individuals and have an impact on the population level (Hutchinson et al. 2006). The feminization of male fish downstream waste water discharges has mainly been demonstrated by the presence of elevated levels of female yolk protein (vitellogenin) in the blood of male or juvenile fish or eggs (or oocytes) in the testes (intersex) (Jobling and Tyler 2003). In concluding the results of present study, combined use of biological responses and chemical tools will help in validating the efficacy of biomarkers chosen to detect the endocrine disruptive effect. The combined levels of hormonal response and Vitellogenin in the fishes (both *O. mossambicus* and *C. punctatus*) collected from three different contaminated sites (Sewage Drainage area; Liquid food industrial effluent and Agricultural effluent area) showed a significant variation from that of the reference site (Vaigai river mouth). Vitellogenin activity in case of *C. punctatus* sowed maximum activity during summer and premonsoon season after captured from sewage site, while *C. punctatus* form both liquid and agricultural effluent region showed very less vitellogenin activity throughout all season but *C. punctatus* from liquid food industrial effluents showed almost higher and equal amount of vitellogenin activity throughout all seasons. in both males and females and reduced the egg production and developed antifertility after the first spawning, which includes degeneration of gonads in adults.Similar results were reported by Nanda et al. 2002. Thus, sewage contaminated site shows more potent threat than other contaminated to fishes that to during summer season. This effect caused the initial feminization of *O. mossambicus* fishes in Vaigai River. The presence of VTG in male fish, for example, is known to be negatively correlated with testicular growth and maturation(Jobling et al 1996) but here the vitellogenin activity in *O. mossambicus* female from sewage and liquid food industries effluent showed maximum activity but in case of male or juvenile production of vitellogenin was significantly lower than female, simultaneously female from agricultural effluents showed less vitellogenin production than other site and in Vaigai River mouth female showed equal activity during all seasons but male or juvenile did not show any vitellogenin activity. In vertebrates, Vtg dimmers are sequestered via receptor-mediated endocytosis into the growing oocytes where further site-specific cleavages occur to yield smaller yolk proteins (Wahil 1988). The Vtg protein is secreted from the cell through the secretory pathway before it enters circulation and is taken up by the growing oocytes. This makes blood plasma a natural target for Vtg analysis. Plasma Vtg concentrations are normally an indication of the maturational status of female fish (Mommsen and Walsh 1988; Arukwe and goksyr 2003). Several studies demonstrated that even male fish caught in rivers and streams had high levels of plasma Vtg (Purdom et al. 1994; Jobling et al. 1998) caused by chemicals acting like estrogens present in the environment. Vtg induction in fish has become an accepted measure of xenoestrogenic potency of chemicals, effluents and discharges.Thus, the anthropogenic contaminants in the environment, such as persistent bio-accumulative contaminants and other toxic contaminants (Kavlock et al. 1996; Munns et al. 1997; Nacci et al. 2002), may disturb the wild life population and may also affected their prolonged existence .

Conclusion:

From the above study it was clear that, season as well as place with different nature and quantity of endocrine disrupting chemicals contamination will play an important role on the activity of the hormone secreted from endocrine system, not only that vitellogenin level of activity will also increase due to elevated level of endocrine disrupting chemicals toxicity.

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Fig-1 Estrodiol (E2)activities in whole body extract(Juveniles) and serum(Adults) of *O.mossambicus* during different seasons from different drainages of River Vaigai

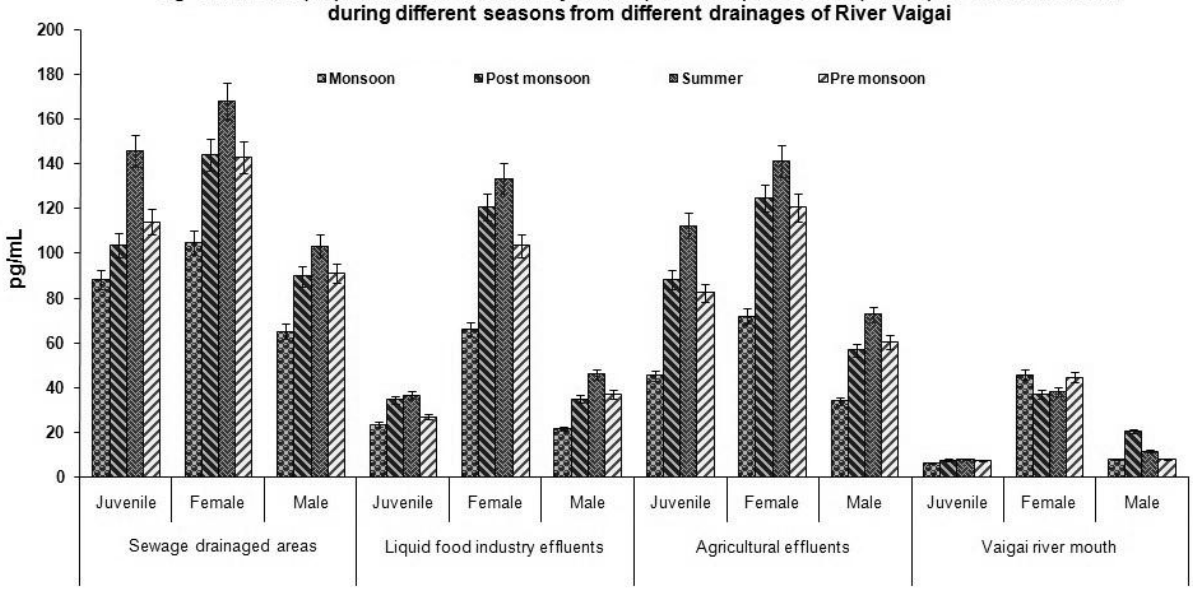

Fig-2 Testosterone activities in whole body extract(Juveniles) and serum(Adults) of *O.mossambicus* during different seasons from different drainages of River Vaigai

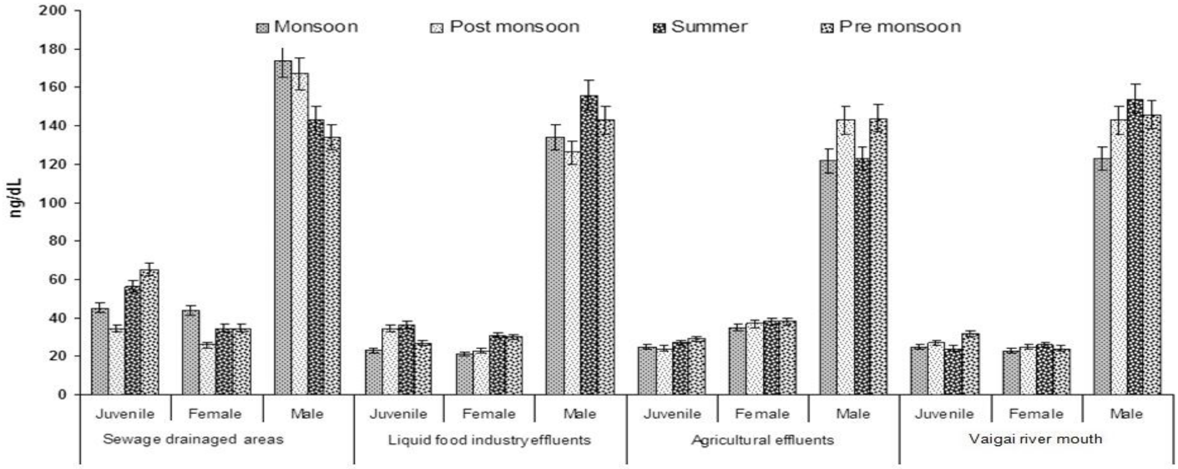

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Fig-3 Progesterone activities in whole body extract (Juveniles) and serum(Adults) of *O.mossambicus* during different seasons from different drinages of River Vaigai

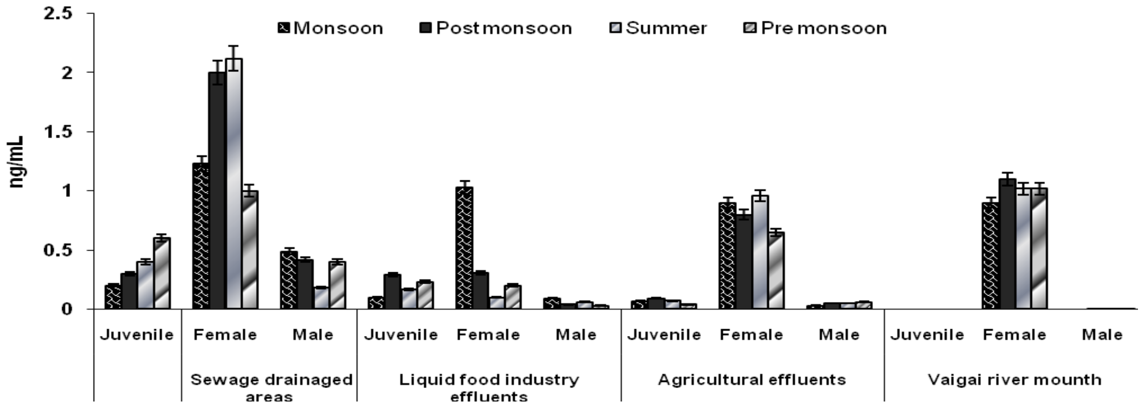


Fig-4 GTH I activities in whole body extract(Juveniles) and serum(Adults) of *O.mossambicus* during different seasons from different drainages of River Vaigai

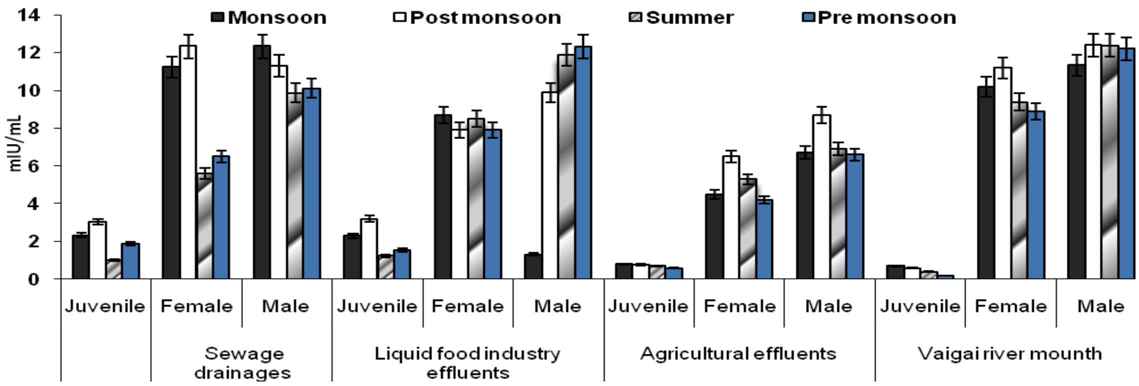
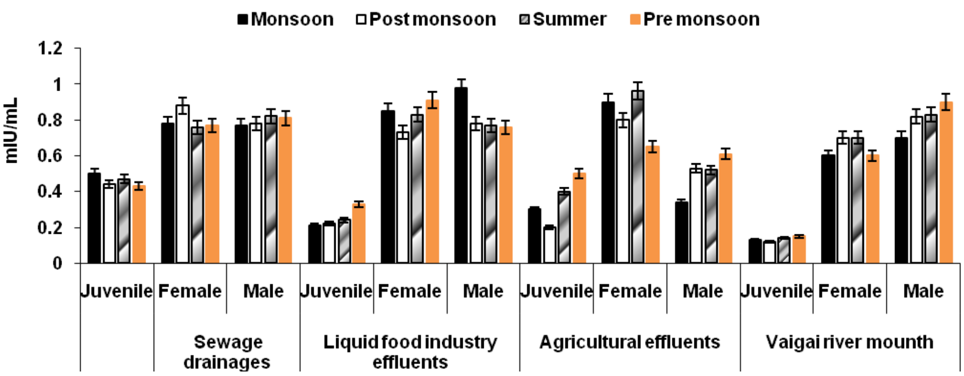


Fig-5 GTH II activities in whole body extract(Juveniles) and serum(Adults) of *O.mossambicus* during different seasons from different drainages of River Vaigai



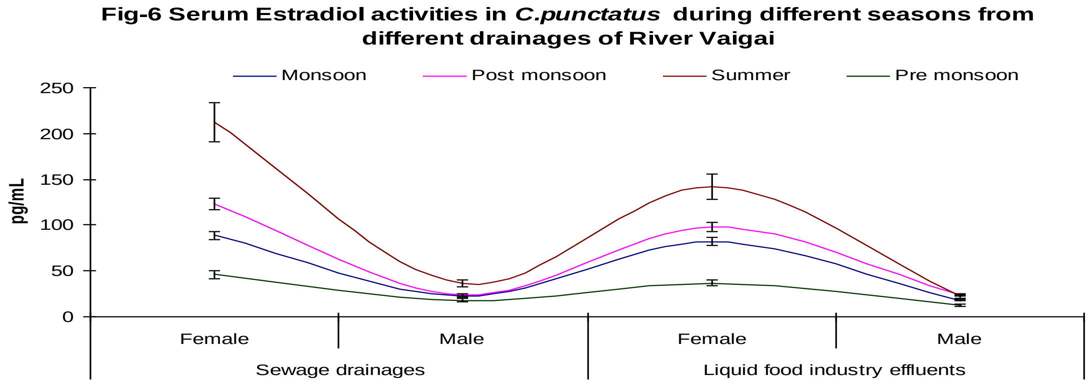


Fig-6 Serum Estradiol activities in C.punctatus during different seasons from different drainages of River Vaigai
Monsoon
Post monsoon
Summer
Pre monsoon
pg/mL
250
200
150
100
50
0
Female
Male
Female
Male
Sewage drainages
Liquid food industry effluents

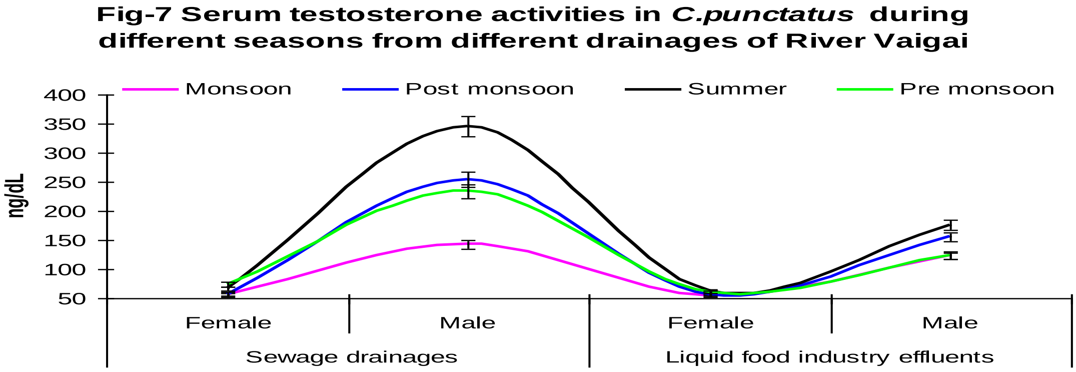


Fig-7 Serum testosterone activities in C.punctatus during different seasons from different drainages of River Vaigai
Monsoon
Post monsoon
Summer
Pre monsoon
ng/dL
400
350
300
250
200
150
100
50
Female
Male
Female
Male
Sewage drainages
Liquid food industry effluents

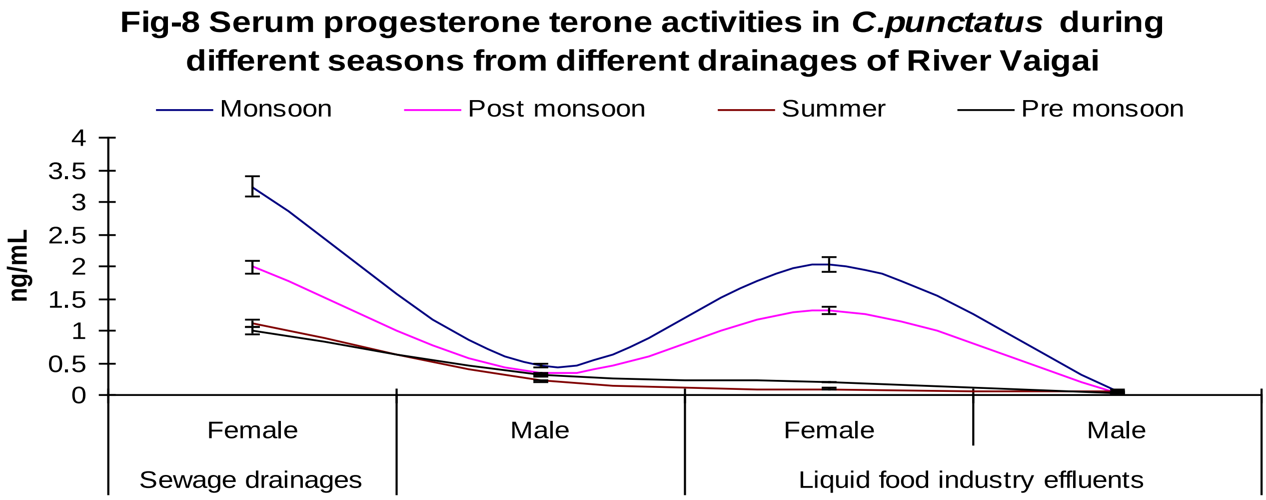


Fig-8 Serum progesterone terone activities in C.punctatus during different seasons from different drainages of River Vaigai
Monsoon
Post monsoon
Summer
Pre monsoon
ng/mL
4
3.5
3
2.5
2
1.5
1
0.5
0
Female
Male
Female
Male
Sewage drainages
Liquid food industry effluents

Fig-10 Serum GTH-II activities in *C.punctatus* during different seasons from different drainages of River Vaigai

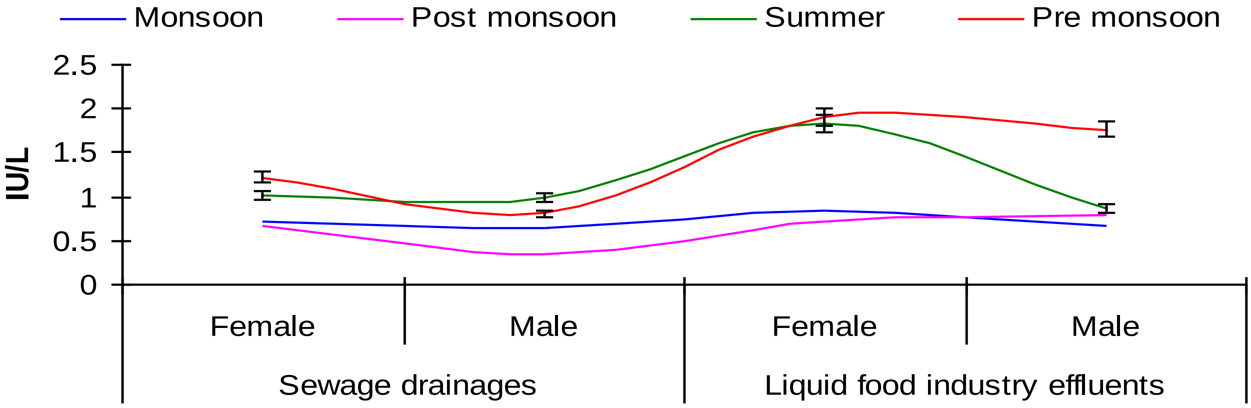


Fig-11 GSI and HSI for female *C.punctatus* during different seasons and sites of Vaigai river

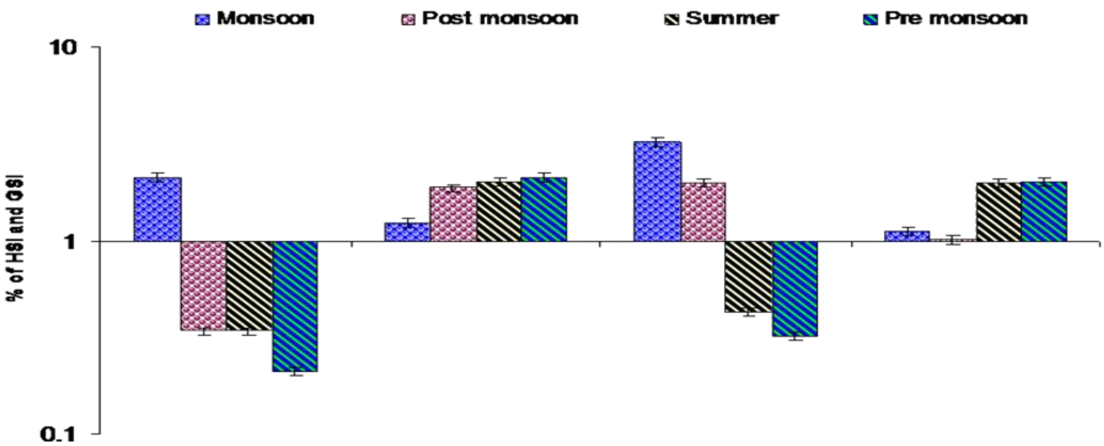


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Fig-12 Plasma(Adult) and whole body extract vitellogenic activies on (Juveniles) of *O.mossambicus* during different seasons from different drainages of River Vaigai

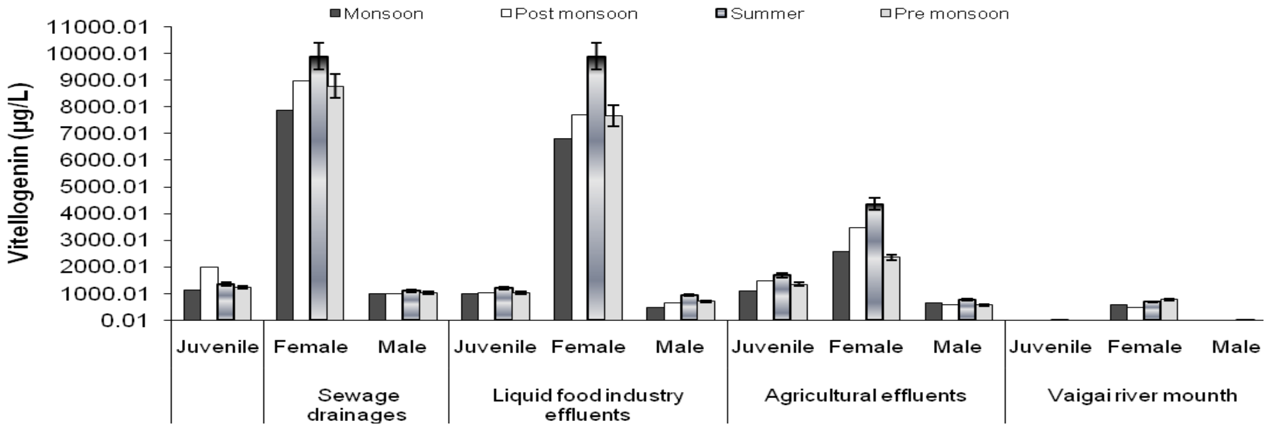

Fig-13 Plasma vitellogenic concentrations of male and female Channa punctatus during different seasons from different drainages of River Vaigai

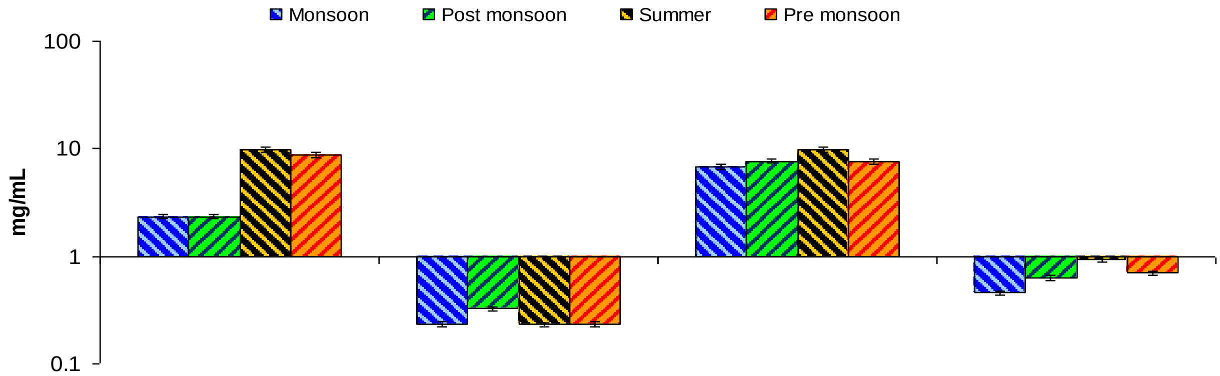

Table-1 Length and weight relationships of *O.mossambicus* collected from various polluted sites of Vaigai tributaries(n = 10 -for each sex and seasons.

Seasons	Sewage drainages				Liquid food industry effluents				Agricultural effluents				Vaigai river mouth			
	Male		Female		Male		Female		Male		Female		Male			Female
	Length(cm)	Weight(g)	Length(cm)	Weight(g)	Length(cm)	Weight(g)	Length(cm)	Weight(g)	Length(cm)	Weight(g)	Length(cm)	Weight(g)	Length(cm)	Weight(g)	Length(cm)	Weight(g)
Monsoon	11.23±2.34	25.12±4.12	12.24±3.56	29.33±3.45	10.88±3.31	26.62±2.99	12.13±3.11	29.21±2.32	11.11±2.33	26.15±3.22	12.67±2.12	30.12±3.12	10.53±2.32	25.34±2.43	11.87±3.22	27.12±4.11
Post Monsoon	10.32±2.11	25.88±2.12	12.61±3.11	30.2±2.24	10.32±2.45	27.45±3.11	11.89±2.34	29.2±3.51	10.12±2.89	26.2±3.22	11.98±3.22	28.87±3.56	10.32±3.45	27.98±3.11	11.20±3.22	29.23±2.15
Summer	11.03±1..32	27.87±3.22	12.32±3.56	30.11±2.33	10.12±2.44	26.78±3.23	12.34±3.11	30.24±2.43	10.33±2.32	28.22±2.34	12.33±2.43	31.22±2.99	10.87±2.98	28.43±3.44	12.02±3.11	30.24±3.66
Pre Monsoon	10.21±2.31	26.11±2.12	11.34±2.46	28.13±3.37	10.01±3.22	27.21±3.66	12.22±3.22	29.11±4.21	10.04±3.29	26.21±3.26	12.15±2.87	29.18±3.21	10.31±3.23	27.21±3.01	12.24±3.01	29.51±3.21

Table-2: Hepato-somatic index (HSI) and Gonado-somatic index (GSI) of the *O.mossambicus* collected from various polluted sites of Vaigai tributaries (n = 10 - for each sex and seasons)

Vaigai river sites	Seasons	Sex	GSI(%)	HSI(%)	Egg number and Size (μM)
Sewage drainages	Monsoon	Male	1.33	16.21	376.23± 68.21 (443) μM)
		Female	18.22	18.10	
	Post monsoon	Male	2.12	12.21	432.34± 47.13 (554 μM)
		Female	21.11	17.67	
	Summer	Male	1.02	17.12	636.34± 52.41 (754 μM)
		Female	18.21	28.96	
	Pre monsoon	Male	1.01	16.23	243.46± 88.76 (267μM)
		Female	24.67	16.34	
Liquid food industry effluents	Monsoon	Male	2.11	19.45	614.11± 77.12 (458 μM)
		Female	18.98	24.56	

	Post monsoon	Male	2.22	17.87	771.33± 58.15 (577 µM)
		Female	16.77	25.34	
	Summer	Male	1.32	17.99	857.78± 98.41 (798 µM)
		Female	19.21	27.89	
	Pre monsoon	Male	1.09	22.23	643.46± 44.64 (341)µM)
		Female	22.76	18.97	
Agricultural effluents	Monsoon	Male	2.12	18.32	766.34± 48.33 (234)µM)
		Female	23.12	20.32	
	Post monsoon	Male	1.23	12.34	655.44± 52.21 (432)µM)
		Female	21.21	24.24	
	Summer	Male	1.09	21.89	761.78± 66.12 (786)µM)
		Female	18.98	26.78	
	Pre monsoon	Male	1.34	24.31	567.76± 47.22 (167)µM)
		Female	20.21	18.98	
Vaigai river mouth	Monsoon	Male	1.45	20.24	475.33± 70.43 (123) µM)
		Female	18.98	21.34	
	Post monsoon	Male	2.12	17.67	532.34± 36.29 (452 µM)
		Female	22.56	18.65	
	Summer	Male	2.09	18.17	536.34± 52.41 (754 µM)
		Female	28.44	22.13	
	Pre monsoon	Male	1.78	21.56	567.41± 66.11 (112)µM)
		Female	20.12	23.35	