Materials and Method

Experimental Model:

The specimens of freshwater fishes, *O. mossambicus* (12 ± 2 cm, 25 ± 1.9 g) of similar size in length and weight were brought to the laboratory from pure brooders, stocked in well aerated tanks containing chlorine free water and acclimated for 10 days. Temperature, pH, and dissolved oxygen of the water were maintained at 27 ± 2° C, 7.1 ± 0.5, and 3.9 ± 0.02 mg/L, respectively. If in any batch, mortality exceeded 5% during acclimatization, that entire batch of fish was discarded. They were fed with commercial fish pallets. 30% Water was renewed every alternate day to provide freshwater, rich in oxygen. Ten well-acclimatized fish (5 male + 5 female) were transferred from the stock to each experimental tank containing 40 L of water exposed to the different concentration of agrochemicals for 14 days. A control group was also maintained in the same condition for the basic test. They were fed with the commercial healthy food during this period and after the study period the fishes were sacrificed. Animal maintenance and experimental procedures were in accordance with the guideline of A.P.H.A., A.W.W.A. and W.P.C.F. (1998).

Experimental design

After the acclimation period of 10 days, sub lethal dose was chosen for the gene expression and hormonal assay studies. The experimental regime was maintained in the laboratory for 14 days with control group. The experiment was performed semi statically with a group of 10 fishes (5 males + 5 females) in experimental aquaria which were divided into 5 groups(n=10), **Group I**:as control, **Group II**: Exposure of Insecticide Imidacloprid (IMI) (0.074mg/L i.e. $1/10^{th}$ of LC_{50}),**Group III** Exposure of Herbicide Pyrazosulfuron Ethyl (PE) (50 mg/L i.e. $1/10^{th}$ of LC_{50}), **Group IV** Exposure of fungicide cymoxanil & mancozeb mixture Curzate (CZ) (4.9mg/L i.e. $1/10^{th}$ of $LC_{50)}$,**Group V** Exposure of micronutrient mixture (MN) (5000mg/L i.e. $1/10^{th}$ of LC_{50}) as summarized in table I. All the groups were kept under continuous observation during the experimental period. After completion of the exposure, fishes were caught very gently using a small dip net, one at a time with least disturbance. They were slowly released in the tough containing 1% clove oil to make it immobile, blotted dry and blood was collected by tail ablation

for hormonal assays. After completion of blood collection, fishes were sacrificed and vital tissues like brain, liver, kidney, gills, thyroid, ovary and testis were dissected out for gene. The tissues were stored at -20^0 C in for further gene expression studies and western blot analysis.

Chemical used	**LC_{50}**	**Sublethal dose (LC_{50}/10)**	**Duration of exposure**	**Species**
Micronutrient mixture (Plant Nutrient)	5000 mg/L	**500 mg/l**	14 days	*O. mossambicus*
Curzate(Fungicide)	49.61 mg/L	**4.96 mg/l**	14 days	
Pyrazosulfuron Ethyl (Herbicide)	500 mg/L	**50 mg/l**	14 days	
Imidacloprid (Insecticide)	0.74 mg/L	**0.074 mg/l**	14 days	

Table I: Agrochemical used with its LC_{50} and duration of exposure for *O. mossambicus*

Gene Expression Study:

Total RNA isolation:

Total RNA was isolated from 100 mg dissected tissues. Tissues were homogenized using homogenizer until tissue was sufficiently disrupted in 500 µl TRIzol reagent (Invitrogen). The homogenate was taken into 2 ml micro centrifuge tubes (Tarsons). After successful homogenization, equal volume of Trizol reagent was added. For complete dissociation of nucleoprotein complexes, homogenized samples were incubated for 5 minutes at room temperature. The incubation was followed by the addition of chloroform and was vigorously shaken for effective mixing of both the solutions. The samples were kept at room temperature for 5 minutes till the aqueous and organic layers were distinct. Thereafter, the tubes were subjected to centrifugation at 12,000x g for 15 minutes at 4°C. The mixture got separated into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. An aliquot of upper aqueous phase was then transferred into a new 1.5 ml micro centrifuge tube using 1000 µl pipette. Precipitation was done by adding 500 µl of isopropanol to the supernatant that was transferred. The samples were kept in -20°C for 10 minutes, centrifuged at 12,000x G for 15 minutes at 4°C. After precipitation the supernatant was discarded without disturbing the pellet and was washed in 1000 µl of 75% ethanol and then 300 µl absolute ethanol was added to the

pellet. Effective mixing was done by gentle inversion and was further subjected to centrifugation at 7,500 x g for 7 minutes at 4°C. The pellet was resuspended by adding 50 µl of DEPC water (Diethylpyrocarbonate) and was incubated at room temperature for 10 mins.

Quantification of RNA:

Prior to quantification, DNAase (Thermo Scientific) treatment was performed. 1 µg of 10X RNA reaction buffer with $MgCl_2$ was added to the tube and was incubated at 37 °C for 30 min. Then after 20 µl chelating agent EDTA was added in 50 mM concentration and was incubated at 65°C for 10 min in water bath as RNA get hydrolyzed during heating with divalent cations in the absence of a chelating agent. RNA quantification was done by taking A_{260}/A_{280} ratio using Perkin Elmer. This ratio can reveal the presence of contaminants and give evidence of possible degradation. An A_{260}/A_{280} ratio of 1.8 is considered acceptable for RNA. 5µl of template RNA was aliquot and was added to 1.5ml micro centrifuge tube. To the aliquot, 995µl of nuclease free water was added and absorbance was measured at the mentioned ratio against the blank having 1000µl of nuclease free water. The concentration of RNA was done using following standard formula:

1 OD corresponds to 40 µg/mL of RNA).

$$\textit{Amount of DNA (µg/mL) (ng/µL)} = \textit{OD at 260nm} \times 40 \times \textit{dilution factor}$$

Dilution Factor was 200.

cDNA Synthesis:

After the purity check of RNA that was validated using spectrophotometer, the RNA template was reversed transcribe to form cDNA from each tissue. A cDNA kit was employed from Thermo scientific- AB-1453/A. Briefly, fresh nuclease free PCR tubes (Tarsons) were taken, in which 4µl of 5X cDNA synthesis buffer, 2µl dNTP mix, 1µl of RNA primer (oligonucleotides), 1µl of RT enhancer, 1µl of verso enzyme mix, 1-2µl of RNA template (according to the spectroscopic quantification i.e. 1 ng) and the final assay volume was made to 20µl using nuclease free water. The tubes were effectively mix by giving a short centrifuge spin for 30s at around 2000 x g.

The tubes containing the kit mixture was PCR amplified by 2 step reaction process. Firstly, the 1 cycle of cDNA synthesis was carried out at 42^{o}C for 30 mins followed by 1 cycle of inactivation at 95^{o}C for 2mins.

PCR Reaction Mixture:

PCR master mix kit was purchased from Sigma ready mix (Cat no # P4600). Assay volume was made up of 10 µl that was having 5 µl of Master mix (5X concentration), 1.0 µl of Forward primer (10 pmol), 1.0 µl of reverse primer (10 pmol), 100 ng of cDNA and remaining nuclease free water. The master mix was having 2 mM $MgCl_{2,}$ 50 mM KCl, 10 mM Tris HCl, 0.2 mM each dNTPs and 0.5 U Taq polymerase.

Reagents	Assay Volume	Final Concentration
Master Mix (5X) (2mM $MgCl_{2,}$ 50 mM KCl, 10mM Tris HCl, 0.2 mM dNTPs 0.5 U Taq polymerase	5 µl	1X
Forward Primer (100pmol)	1µl	10 pmol
Reverse Primer (100pmol)	1µl	10 pmol
Nuclease free water	3µl	-----

Table II: Depicts the reagents used in a PCR reaction with volume of it used in 10ul assay system.

PCR Conditions for Candidate Genes:

Tissue specific gene expression of key genes of three axis were studied, hypothalamus-pituitary Gonadal axis (HPG), hypothalamus Pituitary interrenal axis (HPI) and Hypothalamus pituitary Thyroid axis (HPT). ABI verity PCR was used to amplify each fragment of cDNA of all the respective genes whose conditions are as follows:

GnRH-I (180bp):

To standardize the expression pattern gradient PCR was performed of various stages. Prior denaturation was carried out at 95^{o}C for 1 minute, followed by the second stage that include denaturation at 95^{o}C for 15 seconds, annealing at 54^{o}C, 55^{o}C, 56^{o}C, 57 ^{o}C,58 ^{o}C,59 ^{o}C,60^{o}C,61^{o}C

of which 60^{o}C was taken into account, as there was no additional bands found in it and extension was carried out at 72^{o}C for 30 seconds. The second stage was repeated for 35 cycles, as this was the optimum cycle's number required to amplify a particular gene. The third stage was only of extension which was carried out at 72^{o}C for 7 minutes and finally samples were cooled down to 4^{o}C.

Stage 1	Stage 2 (35 Cycles)	Stage 3	Tissue/Organ
Denaturation: 95^{o}C for 1 minute	**Denaturation:** 95^{o}C for 15 seconds **Annealing Zones:** 54^{o}C, 55^{o}C, 56^{o}C, 57 ^{o}C,58 ^{o}C,59 ^{o}C, **60^{o}C**, 61^{o}C for 15 seconds **Extension:** 72^{o}C for 30 seconds	**Final Extension:** 72^{o}C for 7 minutes	**Hypothalamus (Brain)**

Table III: Represents the various stages of PCR cycles of GnRH-I. The annealing was standardized at **60^{o}C.**

Kisspeptin-1 (kiss 1 400bp):

For Kiss1 also, gradient PCR was performed of various stages. Prior denaturation was carried out at 95^{o}C for 1 minute, followed by the second stage that include denaturation at 95^{o}C for 15 seconds, annealing at 48^{o}C, 49^{o}C, 50^{o}C, 55 ^{o}C,59 ^{o}C,62 ^{o}C,64^{o}C of which 59^{o}C was taken into account, as there was no additional bands found at that temperature and extension was carried out at 72^{o}C for 30seconds. The second stage was repeated for 35 cycles, as this was the optimum cycle's number required to amplify a particular gene. The third stage was only of extension which was carried out at 72^{o}C for 7 minutes and finally samples were cooled down to 4^{o}C.

Stage 1	Stage 2 (35 Cycles)	Stage 3	Tissue/Organ
Denaturation: 95^{o}C for 1 minute	**Denaturation:** 95^{o}C for 15 seconds **Annealing Zones:** 48^{o}C, 49^{o}C, 50^{o}C, 55 ^{o}C,**59 ^{o}C**,62 ^{o}C,64^{o}C for 15 seconds **Extension:** 72^{o}C for 30 seconds	**Final Extension:** 72^{o}C for 7 minutes	**Hypothalamus (Brain)**

Table IV: Represents the various stages of PCR cycles of Kiss-1. The annealing was standardized at **59^{o}C.**

Kisspeptin 2 (kiss 2 214bp):

Similar standardization was performed, where gradient PCR was performed of various stages. Prior denaturation was carried out at 95^{o}C for 1 minute, followed by second stage that include denaturation at 95^{o}C for 15 seconds, annealing at 48^{o}C, 50^{o}C, 55 ^{o}C,56 ^{o}C,57 ^{o}C,58^{o}C,59 ^{o}C,60 ^{o}C,61 ^{o}C of which 60^{o}C was taken into account, as there were no additional bands found at that temperature and extension was carried out at 72^{o}C for 30 seconds. The second stage was repeated for 35 cycles, as this was the optimum cycle's number required to amplify a particular gene. The third stage was only of extension, which was carried out at 72^{o}C for 7 minutes and finally samples were cooled down to 4^{o}C.

Stage 1	Stage 2 (35 Cycles)	Stage 3	Tissue/Organ
Denaturation: 95^{o}C for 1 minute	**Denaturation:** 95^{o}C for 15 seconds **Annealing Zones:** 48^{o}C, 50^{o}C, 55 ^{o}C,56 ^{o}C,57 ^{o}C,58^{o}C,59 ^{o}C,**60 ^{o}C**,61^{o}C for 15 seconds **Extension:** 72^{o}C for 30 seconds	**Final Extension:** 72^{o}C for 7 minutes	**Hypothalamus (Brain)**

Table V: Represents the various stages of PCR cycles of Kiss-2. The annealing was standardized at **60^{o}C.**

GtH-Ir (276bp):

Gradient PCR was performed of various stages where Prior denaturation was carried out at 95^{o}C for 1 minute, followed by the second stage that include denaturation at 95^{o}C for 15 seconds, annealing at 51^{o}C, 52^{o}C, 53 ^{o}C,54 ^{o}C,55 ^{o}C,58^{o}C,59 ^{o}C of which 53^{o}C was taken into account, as there were no additional bands found at that temperature and extension was carried out at 72^{o}C for 30 seconds. The second stage was repeated for 35 cycles, as this was the optimum cycle's number required to amplify a particular gene. The third stage was only of extension which was carried out at 72^{o}C for 7 minutes and finally samples were cooled down to 4^{o}C.

Stage 1	**Stage 2 (35 Cycles)**	**Stage 3**	**Tissue/Organ**
Denaturation: 95^{o}C for 1 minute	**Denaturation:** 95^{o}C for 15 seconds **Annealing Zones:** 51^{o}C, 52^{o}C, **53^{o}C**,54 ^{o}C,55^{o}C,58^{o}C,59^{o}C for 15 seconds **Extension:** 72^{o}C for 30 seconds	**Final Extension:** 72^{o}C for 7 minutes	**Ovary and Testis**

Table VI: Represents the various stages of PCR cycles of GtH-Ir. The annealing was standardized at 53^{o}C**.**

GtH IIr (214bp):

Similar standardization was executed, where gradient PCR was performed of various stages. Prior denaturation was carried out at 95^{o}C for 1 minute, followed by the second stage that include denaturation at 95^{o}C for 15 seconds, annealing at 51^{o}C, 52^{o}C, 53^{o}C,54^{o}C,55^{o}C,58^{o}C,59 ^{o}C of which 53^{o}C was taken into account, as there were no additional bands found at that temperature and extension was carried out at 72^{o}C for 30 seconds. The second stage was repeated for 35 cycles, as this was the optimum cycle's number required to amplify a particular gene. The third stage was only of extension which was carried out at 72^{o}C for 7 minutes and finally samples were cooled down to 4^{o}C.

Stage 1	Stage 2 (35 Cycles)	Stage 3	Tissue/Organ
Denaturation: 95^{o}C for 1 minute	**Denaturation:** 95^{o}C for 15 seconds **Annealing Zones:** 51^{o}C, 52^{o}C, **53^{o}C**,54 ^{o}C,55^{o}C,58^{o}C,59^{o}C for 15 seconds **Extension:** 72^{o}C for 30 seconds	**Final Extension:** 72^{o}C for 7 minutes	**Ovary and Testis**

Table VII: Represents the various stages of PCR cycles of GtH-IIr. The annealing was standardized at 53^{o}C**.**

***ER-I* (85bp)**

In case of ER I, gradient PCR was performed which was carried out at 95^{o}C for 1 minute, followed by the second stage that include denaturation at 95^{o}C for 15 seconds, annealing at 51^{o}C, 52^{o}C,53^{o}C,54^{o}C,55^{o}C,58^{o}C,59^{o}C of which 53^{o}C was taken into account, as there were no additional bands found at that temperature and extension was carried out at 72^{o}C for 30 seconds. The second stage was repeated for 35 cycles, as this was the optimum cycle's number required to amplify a particular gene. The third stage was only of extension which was carried out at 72^{o}C for 7 minutes and finally samples were cooled down to 4^{o}C.

Stage 1	Stage 2 (35 Cycles)	Stage 3	Tissue/Organ
Denaturation: 95^{o}C for 1 minute	**Denaturation:** 95^{o}C for 15 seconds **Annealing Zones:** 51^{o}C, 52^{o}C, **53^{o}C**,54 ^{o}C,55^{o}C,58^{o}C,59^{o}C for 15 seconds **Extension:** 72^{o}C for 30 seconds	**Final Extension:** 72^{o}C for 7 minutes	**Ovary, Testis and Brain**

Table VIII: Represents the various stages of PCR cycles of ER-I. The annealing was standardized at **53^{o}C.**

ER-II (192bp):

In case of ER II, gradient PCR was performed which was carried out at 95^{o}C for 1 minute, followed by the second stage that include denaturation at 95^{o}C for 15 seconds, annealing at 51^{o}C, 52^{o}C, 53 ^{o}C,54 ^{o}C,55 ^{o}C,58^{o}C,59^{o}C of which 52^{o}C was taken into account, as there was no additional bands found at that temperature and extension was carried out at 72^{o}C for 30 seconds. The second stage was repeated for 35 cycles, as this was the optimum cycle's number required to amplify a particular gene. The third stage was only of extension which was carried out at 72^{o}C for 7 minutes and finally samples were cooled down to 4^{o}C.

Stage 1	Stage 2 (35 Cycles)	Stage 3	Tissue/Organ
Denaturation: 95^{o}C for 1 minute	**Denaturation:** 95^{o}C for 15 seconds **Annealing Zones:** 51^{o}C, **52^{o}C**, 53 ^{o}C,54 ^{o}C,55 ^{o}C,58^{o}C,59^{o}C for 15 seconds **Extension:** 72^{o}C for 30 seconds	**Final Extension:** 72^{o}C for 7 minutes	**Ovary, Testis and Brain**

Table IX: Represents the various stages of PCR cycles of ER-II .The annealing was standardized at **52^{o}C.**

AR-I (73 bp):

For AR I, gradient PCR was performed which was carried out at 95^{o}C for 1 minute, followed by the second stage that include denaturation at 95^{o}C for 15 seconds, annealing at 51^{o}C, 52^{o}C, 53^{o}C,54^{o}C,55^{o}C,58^{o}C,59^{o}C of which 54^{o}C was taken into account, as there were no additional bands found at that temperature and extension was carried out at 72^{o}C for 30 seconds. The second stage was repeated for 35 cycles, as this was the optimum cycle's number required to amplify a particular gene. The third stage was only of extension which was carried out at 72^{o}C for 7 minutes and finally samples were cooled down to 4^{o}C.

Stage 1	Stage 2 (35 Cycles)	Stage 3	Tissue/Organ
Denaturation: 95^{o}C for 1 minute	**Denaturation:** 95^{o}C for 15 seconds **Annealing Zones:** 51^{o}C, 52^{o}C, 53^{o}C,**54 ^{o}C,**55 ^{o}C,58^{o}C,59^{o}C for 15 seconds **Extension:** 72^{o}C for 30 seconds	**Final Extension:** 72^{o}C for 7 minutes	**Ovary, Testis and Brain**

Table X: Represents the various stages of PCR cycles of AR-I .The annealing was standardized at **54^{o}C.**

AR-II (150 bp):

For AR II, gradient PCR was performed which was carried out at 95^{o}C for 1 minute, followed by the second stage that include denaturation at 95^{o}C for 15 seconds, annealing at 51^{o}C,52^{o}C,53 ^{o}C,54^{o}C,55^{o}C,58^{o}C,59^{o}C of which 55^{o}C was taken into account, as there were no additional bands found at that temperature and extension was carried out at 72^{o}C for 30 seconds. The second stage was repeated for 35 cycles, as this was the optimum cycle's number required to amplify a particular gene. The third stage was only of extension which was carried out at 72^{o}C for 7 minutes and finally samples were cooled down to 4^{o}C.

Stage 1	Stage 2 (35 Cycles)	Stage 3	Tissue/Organ
Denaturation: 95^{o}C for 1 minute	**Denaturation:** 95^{o}C for 15 seconds **Annealing Zones:** 51^{o}C, 52^{o}C, 53^{o}C,54 ^{o}C,**55 ^{o}C**,58^{o}C,59^{o}C for 15 seconds **Extension:** 72^{o}C for 30 seconds	**Final Extension:** 72^{o}C for 7 minutes	**Ovary, Testis and Brain**

Table XI: Represents the various stages of PCR cycles of AR-II. The annealing was standardized at **55^{o}C.**

For GR, gradient PCR was performed which was carried out at 95^{o}C for 1 minute, followed by the second stage that include denaturation at 95^{o}C for 15 seconds, annealing at 51^{o}C, 52^{o}C, 53^{o}C, 54^{o}C, 55^{o}C, 58^{o}C, 59^{o}C of which 59^{o}C was taken into account, as there were no additional bands found at that temperature and extension was carried out at 72^{o}C for 30 seconds. The second stage was repeated for 35 cycles, as this was the optimum cycle's number required to amplify a particular gene. The third stage was only of extension which was carried out at 72^{o}C for 7 minutes and finally samples were cooled down to 4^{o}C.

Stage 1	**Stage 2 (35 Cycles)**	**Stage 3**	**Tissue/Organ**
Denaturation: 95^{o}C for 1 minute	**Denaturation:** 95^{o}C for 15 seconds **Annealing Zones:** 51^{o}C, 52^{o}C, 53^{o}C,54 ^{o}C,55^{o}C,58^{o}C,**59^{o}C** for 15 seconds **Extension:** 72^{o}C for 30 seconds	**Final Extension:** 72^{o}C for 7 minutes	**Brain, Liver, Gills, Kidney, Thyroid, Ovary, Testis**

Table XII: Represents the various stages of PCR cycles of GR-I. The annealing was standardized at **59^{o}C.**

TSH –βr (150 bp):

TSH *–βr* PCR was performed which was carried out at 95^{o}C for 1 minute, followed by the second stage that include denaturation at 95^{o}C for 15 seconds, annealing at 51^{o}C, 52^{o}C, 53^{o}C, 54^{o}C, 55^{o}C, 58^{o}C, 59^{o}C of which 54^{o}C was taken into account, as there were no additional bands found at that temperature and extension was carried out at 72^{o}C for 30 seconds. The second stage was repeated for 35 cycles, as this was the optimum cycle's number required to amplify a particular gene. The third stage was only of extension which was carried out at 72^{o}C for 7 minutes and finally samples were cooled down to 4^{o}C.

Stage 1	Stage 2 (35 Cycles)	Stage 3	Tissue/Organ
Denaturation: 95^{o}C for 1 minute	**Denaturation:** 95^{o}C for 15 seconds **Annealing Zones:** 51^{o}C, 52^{o}C, 53^{o}C,**54 ^{o}C**,55^{o}C,58^{o}C,59^{o}C for 15 seconds **Extension:** 72^{o}C for 30 seconds	**Final Extension:** 72^{o}C for 7 minutes	**Thyroid**

Table XIII: Represents the various stages of PCR cycles of TSH- *βr* .The annealing was standardized at **54^{o}C.**

MAPK-2 (149bp):

MAPK-2 PCR was performed which was carried out at 95^{o}C for 1 minute, followed by the second stage that include denaturation at 95^{o}C for 15 seconds, annealing at 51^{o}C, 52^{o}C, 53^{o}C, 54^{o}C, 55^{o}C, 58^{o}C, 59^{o}C of which 53^{o}C was taken into account, as there were no additional bands found at that temperature and extension was carried out at 72^{o}C for 30 seconds. The second stage was repeated for 35 cycles, as this was the optimum cycle's number required to amplify a particular gene. The third stage was only of extension which was carried out at 72^{o}C for 7 minutes and finally samples were cooled down to 4^{o}C.

Stage 1	Stage 2 (35 Cycles)	Stage 3	Tissue/Organ
Denaturation: 95^{o}C for 1 minute	**Denaturation:** 95^{o}C for 15 seconds **Annealing Zones:** 51^{o}C, 52^{o}C, **53^{o}C**,54 ^{o}C,55^{o}C,58^{o}C,59^{o}C for 15 seconds **Extension:** 72^{o}C for 30 seconds	**Final Extension:** 72^{o}C for 7 minutes	**Brain**

Table XIV: Represents the various stages of PCR cycles of MAPK-2 .The annealing was standardized at **53^{o}C.**

PKC PCR was carried out at 95^{o}C for 1 minute, followed by the second stage that include denaturation at 95^{o}C for 15 seconds, annealing at 51^{o}C, 52^{o}C, 53^{o}C, 54^{o}C, 55^{o}C, 58^{o}C, 59^{o}C of which 55^{o}C was taken into account, as there were no additional bands found at that temperature and extension was carried out at 72^{o}C for 30seconds. The second stage was repeated for 35 cycles, as this was the optimum cycle's number required to amplify a particular gene. The third stage was only of extension which was carried out at 72^{o}C for 7 minutes and finally samples were cooled down to 4^{o}C.

Stage 1	**Stage 2 (35 Cycles)**	**Stage 3**	**Tissue/Organ**
Denaturation: 95^{o}C for 1 minute	**Denaturation:** 95^{o}C for 15 seconds **Annealing Zones:** 51^{o}C, 52^{o}C, 53^{o}C,54 ^{o}C,**55^{o}C**,58^{o}C,59^{o}C for 15 seconds **Extension:** 72^{o}C for 30 seconds	**Final Extension:** 72^{o}C for 7 minutes	**Hypothalamus**

Table XV: Represents the various stages of PCR cycles of PKC .The annealing was standardized at **55^{o}C.**

18sRNA (102bp)

18sRNA PCR was carried out at 95^{o}C for 1 minute, followed by the second stage that include denaturation at 95^{o}C for 15 seconds, annealing at 50^{o}C, 52^{o}C, 53^{o}C, 54^{o}C, 55^{o}C, 58^{o}C, 59^{o}C of which 50^{o}C was taken into account, as there were no additional bands found at that temperature and extension was carried out at 72^{o}C for 30 seconds. The second stage was repeated for 35 cycles, as this was the optimum cycle's number required to amplify a particular gene. The third stage was only of extension which was carried out at 72^{o}C for 7 minutes and finally samples were cooled down to 4^{o}C.

Stage 1	Stage 2 (35 Cycles)	Stage 3	Tissue/Organ
Denaturation: 95^{o}C for 1 minute	**Denaturation:** 95^{o}C for 15 seconds **Annealing Zones:** 51^{o}C, 52^{o}C, 53 ^{o}C,54 ^{o}C,**50 ^{o}C**,58^{o}C,59^{o}C for 15 seconds **Extension:** 72^{o}C for 30seconds	**Final Extension:** 72^{o}C for 7 minutes	**Brain, Liver, Gills, Kidney, Thyroid, Ovary, Testis**

Table XVI: Represents the various stages of PCR cycles of 18s rRNA .The annealing was standardized at **50^{o}C.**

Primer Designing:

Primers were designed using NCBI Primer 3 designing software and the optimum conditions that were taken into consideration were:

1. GC content was kept 40-60 %.
2. T_m of both the primers was having ±1^{o}C difference.
3. The sequence homology was kept minimums thus it was not palindrome to each other hence not allowing inter and intra molecular interactions with one own self or to each other.
4. The stability of the primer was also checked by calculating the ΔG value of 5 bases from 3' end.
5. Specific annealing temperature for minimum false product formation.

$$T_a\ Opt = 0.3\ x(T_m\ of\ primer) + 0.7\ x(T_m\ of\ product) - 14.9$$

Where,

T_m of primer is the melting temperature of the less stable primer-template pair.

T_m of product is the melting temperature of the PCR product.

6. After the successful designing of the primer the amplicon/product length was again validated using the given formula:

$$Product\ length = (Position\ of\ antisense\ primer - Position\ of\ sense\ primer) + 1$$

Accession No.	Gene Name	Sequence	Tm	Species	Amplicon Size (bp)
NM_001113489	Kiss1	FP:5'CTCAGGGGAACAGACACTCG3'	59^{0}C	*Daneo rerio*	400
		RP:5'GCAAATACCTCAGAGAGGACCA 3'			
NM_001279468.1	Kiss 2	FP:5':GGATCCCAGCCTCTGCTTTT3'	60^{0}C	*O.niloticus*	214
		RP:5':TCAGGTGGGTACCTCCAGTT3			
AB104861.1	GnRH1	FP:5'CGCCATTTCCTCTCCAGCTTA3'	60^{0}C	*O.niloticus*	180
		RP:5'CGCTACTCCAACAGAGGTCG3'			
AB042422,	GTH Ir	FP:5'ACCTGCTGGAGAGTATCGGT3'	60^{0}C	*O.mossambicus*	276
		RP:5' AGGCGGTGGAATGGATCTTG3'			
AB042423,	GTH IIr	FP: 5'AAATGCTCCCCAAAGCCAGA3'	60^{0}C	*O.mossambicus*	214
		RP:5'GCCAGTCTGTGGCTGATTGT3'			
NM_001279770.1	ER-α (Type I)	FP:5'GGAGGTATGCGTAAGGACCG3'	53^{0}C	*O.niloticus*	85
		RP: 5'GCAGGTCTTTGGCTGGTTTG3'			
NM_001279774.1	ER-β (Type II)	FP:5'CAATGTCATGCATGGGTTGTCT3'	52^{0}C	*O.niloticus*	198
		RP:5'TCCATGTTGGGGTTGCATCA3'			
AF497908	18srRNA (Internal Control)	FP:5′-TATTGTGCCGCTAGAGGTGAA-3′	51^{0}C	*O.mossambicus*	102
		RP:5′-CCTCCGACTTTCGTTCTTGA-3′			
AF080247	Glucocorticoid Receptor (GR)	FP:5'TTTCGGTAATTGGTTGCTGATGAT-3'	59°C	*O.niloticus*	113bp
		RP: 5'-AGTGCTCCTGGCTGTTTCTAAGT-3'			
XM_003453648.2	TSH-βr	FP:5'ACCACCATCTGCATGGGATT3'	53^{0}C	*O.niloticus*	150
		RP:5'AGGGTTGGCTTCAATGGGAC3'			
NM_001279613.1	AR-Ir	FP:5'GTAATGCGCCGTGAACAGTG3'	54^{0}C	*O.niloticus*	73^{0}C
		RP:5'GCACCACACGCCCCAAAAA3'			
NM_001279615.1	AR-IIr	FP:5'CACACGACCACAAGCAACAG3'	55^{0}C	*O.niloticus*	115
		RP:5'TCGCACAGCGTGAAGTTTTG3'			
XM_005452298	MAPk2	FP:5'CTGATCCCAGTTCAGCCGTT3'	60^{0}C	*O.niloticus*	149
		RP:5'ACTTCTACACCAGCACGACG3'			
XM_013277412.1	PKC	FP:5'CAGATGTGTCTGGGGCGGG3'	60^{0}C	*O.niloticus*	242
		RP:5'TCTGGAGAGGCGGAAAGTCT3'			

Table XVII: Depicts the primer sequence with its annealing temperature and product size.

SDS PAGE:

Hypothalamus was dissected from the brain tissue from each experimental group and was prepared in laemmli SDS (Laemmli 1970) sample buffer, further 10% homogenate were for total protein estimation assayed using Bradford method (Bradford 1976). Equal amount was loaded on to 15% SDS PAGE gels. These gels were further used for western blot analysis.

	Final Resolving Gel Percentage (Volume in ml)					
	5%	7%	9%	10%	12%	**15%**
Stock Solution						
1M Tris pH 8.8	3.75	3.75	3.75	3.75	3.75	**3.75**
20% SDS	0.05	0.05	0.05	0.05	0.05	**0.05**
40% Acrylamide	1.25	1.73	1.69	2.50	3	**3.75**
H_2O	4.85	4.40	3.81	3.73	3.2	**1.25**
10% Ammonium Persulfate	100 µL	100 µL	100 µL	100 µL	100 µL	**100 µL**
TEMED	10 µL	10 µL	10 µL	10 µL	10 µL	**10 µL**

Table XVIII: SDS PAGE resolving gel percentage, which was used for the separation of bands on total protein isolated.15% resolving gel was used for the separation of kisspeptin bands.

Requirements for Stacking Gel Preparation	
	Volume in ml
Stock Solution	
1M Tris pH 6.8	0.63
20% SDS	0.05
40% Acrylamide	0.83
H_2O	3.4 µL
10% Ammonium per sulfate	50 µL
TEMED	5 µL

Table XIX: List of chemicals required for preparation of SDS-PAGE stacking gel

Western Blot study:

30 µg of total protein (Bradford 1976) was resolved on 15% SDS-PAGE Tris-glycine gels and transferred to nitrocellulose membranes. Non-specific binding was blocked by incubating the membranes in 5% BSA and 0.1% Tween in Tris-buffered saline (TBS, pH 7.4) for 1 h at room temperature. The blots were subsequently incubated with primary polyclonal antibodies (raised in rabbit 1:1000 Dilution) for kisspeptins were kindly gifted from Prof. Parhar of Jeffrey Cheah school of Medicine and health sciences, Monash University, Malaysia, overnight at 4°C, with gentle agitation. Blots were washed with TBS containing 0.1% Tween (TBS-T) (4 × 15 min) and then incubated with respective anti-rabbit secondary antibodies conjugated with HRP (horse radish peroxidase) for 2 h at room temperature with gentle agitation. After four washes with TBS-T and one wash with TBS; specific bands of immunoreactive proteins were visualized using enhanced chemiluminescence (ECL) reagent (Millipore) in Chemidoc (Alliance Model 4.7).

Hormonal Assays:

Estradiol (E_2) Hormonal Assay:

Principle:

The estradiol assay is based on the competition between estradiol and an estradiol acetylcholinesterase (AChE) conjugate (Estradiol Tracer) for a limited amount of Estradiol Antiserum. Because the concentration of the Estradiol Tracer is held constant while the concentration of estradiol varies, the amount of Estradiol Tracer that is able to bind to the Estradiol Antiserum will be inversely proportional to the concentration of estradiol in the well. This antiserum-estradiol complex binds to mouse monoclonal anti-rabbit IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of Estradiol Tracer bound to the well, which is inversely proportional to the amount of free estradiol present in the well during the incubation i.e.

$$\text{Absorbance} \propto [\text{Bound Estradiol Tracer}] \propto 1/[\text{Estradiol}].$$

Procedure:

Plasma was separated and circulating steroid levels were measured by Cayman ELISA kit (Cat #582251). Each sample was assayed in triplicates were 100 µl of ELISA buffer was added to all the wells, followed by addition of 50 µl of estradiol standard to each well. Estradiol standard was made using serial dilution from the stock solution (400ng/ml) in 8 tubes.50µl of sample was added to each triplicate trailed by 50µl AChE tracer in each well except for blank and TA (total activity). Finally 50µl of estradiol antiserum was added to each well expect for the well of TA and NSB (Non-Specific binding). The plate was incubated at room temperature on orbital shaker for 1 hr, and was developed using Ellman's reagent. The standard curve and sample concentration was determined using the following formula:

$$\text{logit}(B/B0) = \ln [B/B0/(1 - B/B0)]$$

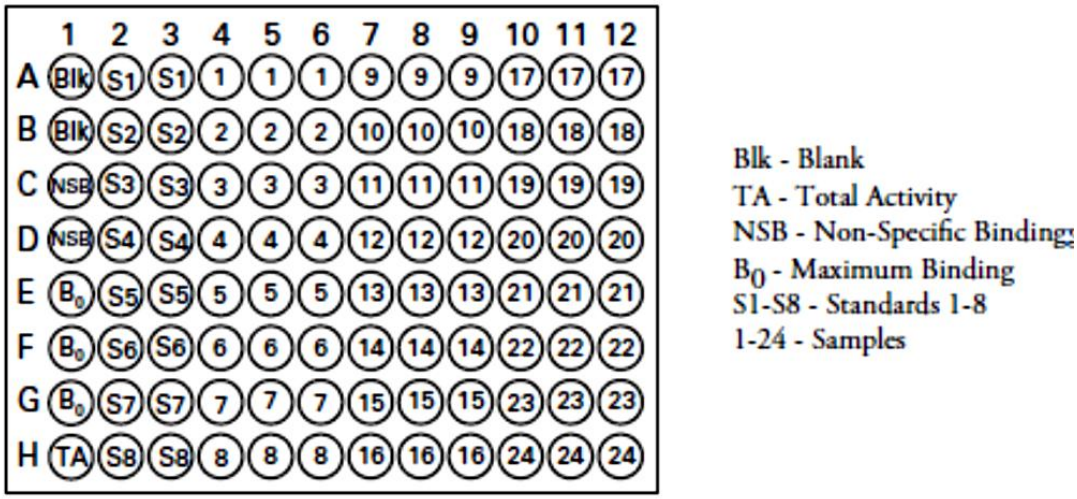

Figure X: Schematic representation of experiment carried out on ELISA plate for E_2

B/B0 (Sample or Standard Bound/Maximum Bound)

B0- Maximum binding

B-Sample or Standard Bound

11-keto Testosterone hormonal assay:

Principle:

This assay is based on the competition between 11-KT and 11-KT acetylcholinesterase (AChE) conjugate (11-KT tracer) for a limited number of 11-KT-specific rabbit antiserum binding sites. The concentration of the 11-KT tracer is held constant while the concentration of 11-KT varies,that is why the amount of 11-KT racer that is able to bind to the rabbit antiserum will be

inversely proportional to the concentration of 11-KT in the well. This rabbit antiserum-11- KT (either free or tracer) complex binds to the mouse monoclonal anti-rabbit IgG antibody that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color, determined spectrophotometrically, is proportional to the amount of 11-KT tracer bound to the well, which is inversely proportional to the amount of free 11-KT present in the well during incubation i.e/

$$\text{Absorbance} \propto [\text{Bound 11-KT Tracer}] \propto 1/[\text{11-KT}]$$

Procedure:

Plasma was separated from the blood and circulating steroid levels were measured by Cayman ELISA kit (Cat # 582751). Each sample was assayed in triplicates were 100 µl of ELISA buffer was added to all the wells, followed by addition of 50 µl of 11-keto testosterone standard to each well. 11-keto testosterone standard was made using serial dilution from the stock solution (400ng/ml) in 8 tubes.50µl of sample was added to each triplicate trailed by 50µl AChE tracer in each well except for blank and TA (total activity). Finally 50µl of 11-keto testosterone antiserum was added to each well expect for the well of TA and NSB (Non-Specific binding). The plate was incubated at room temperature on orbital shaker for 1 hr, and was developed using Ellman's reagent. The standard curve and sample concentration was determined using the following formula:

$$\text{logit}(B/B0) = \ln [B/B0/(1 - B/B0)]$$

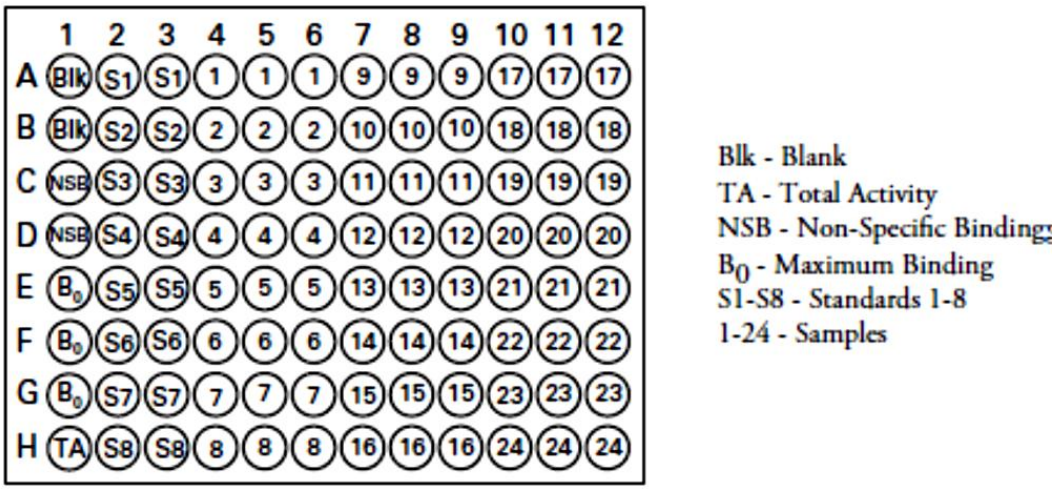

Figure XI: Schematic representation of experiment carried out on ELISA plate for 11-KT

B/B0 (Sample or Standard Bound/Maximum Bound)

B0- Maximum binding

B-Sample or Standard Bound

Cortisol Hormonal Assay:

Principle:

The assay is based on the competition between cortisol and cortisol acetylcholinesterase (AChE) conjugate (cortisol tracer) for a limited number of cortisol-specific mouse monoclonal antibody binding sites. Because the concentration of the cortisol tracer is held constant while the concentration of cortisol varies, the amount of cortisol tracer that is able to bind to the Cortisol monoclonal antibody will be inversely proportional to the concentration of cortisol in the well. This antibody-cortisol (either free or tracer) complex binds to the goat polyclonal anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of cortisol tracer bound to the well, which is inversely proportional to the amount of free cortisol present in the well during the incubation.

$$\text{Absorbance} \propto [\text{Bound Cortisol Tracer}] \propto 1/[\text{Cortisol}]$$

Procedure:

Plasma was separated from the blood and circulating steroid levels were measured by Cayman ELISA kit (Cat # 500360*)*. Each sample was assayed in triplicates were 100 µl of ELISA buffer was added to all the wells, followed by addition of 50 µl of cortisol standard to each well. Cortisol standard was made using serial dilution from the stock solution (400ng/ml) in 8 tubes.50µl of sample was added to each triplicate trailed by 50µl AChE tracer in each well except for blank and TA (total activity). Finally 50µl of cortisol antiserum was added to each well expect for the well of TA and NSB (Non-Specific binding). The plate was incubated at room temperature on orbital shaker for 1 hr, and was developed using Ellman's reagent. The standard curve and sample concentration was determined using the following formula:

$$\text{logit}(B/B0) = \ln [B/B0/(1 - B/B0)]$$

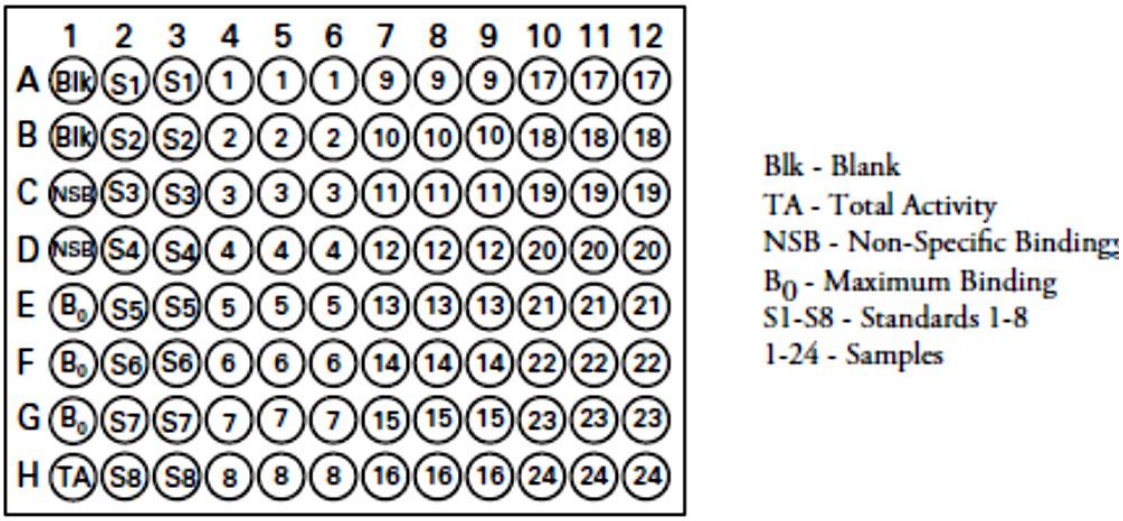

Figure XII: Schematic representation of experiment carried out on ELISA plate for Cortisol

B/B0 (Sample or Standard Bound/Maximum Bound)

B0- Maximum binding

B-Sample or Standard Bound

Thyroid Stimulating Hormone Assay (Biodetect kit # 1003)

Principle:

Sample, antibody and enzyme labeled were incubated in a microwell for 60 minutes. After obtaining equilibrium, the excess reagents were washed off. A chromogen substrate (TMB-H_2O_2) was added. After 15 min of incubation, the reaction was stopped by the addition of strong acid. The colour development is inversely proportional to the concentration of unlabelled TSH in calibrators, control and patient samples. A graph was plotted using calibrators TSH concentration on x-axis and relative absorbance on y-axis, thus by plotting the absorbance the concentration for unknown samples can be interpolated.

Procedure:

50µl of fish plasma sample was dispensed into the designated well. 100µl of given enzyme reagent (TSH-conjugate) was added per well. After shaking the microplate around 10 seconds, it was incubated at room temperature for 60 minutes. Further, the content was decanted and microplate was washed three to four times with wash buffer and then substrate (TMB-H_2O_2) was

added to each well. The plate was kept in dark and incubated for 15 minutes at RT. The reaction was stopped by adding stop solution (0.5M H_2SO_4) and absorbance was read at 450nm.

Triidothyronine (T_3-Biodetect kit # 1001)

Principle:

As the kit was procured from biodetect systems, it had same principle and procedure as that of TSH. Sample, antibody and enzyme labeled were incubated in a microwave for 60 minutes. After obtaining equilibrium, the excess reagents were washed off. A chromogen substrate (TMB-H_2O_2) was added. After 15 min of incubation, the reaction was stopped by the addition of strong acid. The colour development is inversely proportional to the concentration of unlabelled T_3 in calibrators, control and patient samples. A graph was plotted using calibrators TSH concentration on x-axis and relative absorbance on y-axis, thus by plotting the absorbance the concentration for unknown samples can be interpolated.

Procedure:

50µl of fish plasma sample was dispensed into the designated well. 100µl of given enzyme reagent (T_3-conjugate) was added per well. After shaking the microplate around 10 seconds, it was incubated at room temperature for 60 minutes. Further, the content was decanted and was washed three to four times with wash buffer and then substrate (TMB-H_2O_2) was added to each well. The plate was kept in dark and incubated for 15 minutes at RT. The reaction was stopped by adding stop solution (0.5M H_2SO_4) and absorbance was read at 450nm.

Thyroxine (T_4-Biodetect kit # 1002)

Principle:

As the kit was procured from biodetect systems, it had same principle and procedure as that of TSH. Sample, antibody and enzyme labeled are incubated in a microwave for 60 minutes. After obtaining equilibrium, the excess reagents were washed off. A chromogen substrate (TMB-H_2O_2) was added. After 15 min of incubation, the reaction was stopped by the addition of strong acid. The colour development is inversely proportional to the concentration of unlabelled T_3 in calibrators, control and patient samples. A graph was plotted using calibrators TSH concentration on x-axis and relative absorbance on y-axis, thus by plotting the absorbance the concentration for unknown samples can be interpolated.

Procedure:

50µl of fish plasma sample was dispensed into the designated well. 100µl of given enzyme reagent (T_4-conjugate) was added per well. After shaking the microplate around 10 seconds, it was incubated at room temperature for 60 minutes. Further, the content was decanted and was washed three to four times with wash buffer and then substrate (TMB-H_2O_2) was added to each well. The plate was kept in dark and incubated for 15 minutes at RT. The reaction was stopped by adding stop solution (0.5M H_2SO_4) and absorbance was read at 450nm.

Pathway representation:

The amplification of target gene resulted in multiple action site of the agrochemicals. Thus, bioinformatic tools were used for representation of pathway and to investigate which pathways were being affected by the tested agrochemicals. Individual candidate gene network maps were generated using Pathway Common and visualized in the open source software platform Cytoscape.

Statistical Analysis:

The difference between the control and the exposed fishes was determined by One-Way ANOVA using Graph pad Prism software version 6. If there was any significant difference, post Hoc test was carried out where Dunnett multiple comparison test were employed to recognize difference in the alterations found in between the control and the exposed groups. The significant level of the tests was set at 5% ($p<0.05$).The data was also exposed to regression analysis only for candidate genes of HPG axis per agrochemical exposed and significance was tested at $\alpha=0.05$ and noted $*p<0.05$ using Graph pad Prism software version 6.