ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com
Journal DOI: 10.21474/IJAR01

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

AGROCHEMICALS INDUCED GENE EXPRESSION ALTERATIONS IN O.MOSSAMBICUS.

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Manuscript Info

Manuscript History:

Received: 12 April 2016
Final Accepted: 19 May 2016
Published Online: June 2016

Key words:
Agrochemicals, Fish, Adverse Effects, HPG axis.

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Abstract

The effect of agrochemicals in general and pesticides in particular on non-target organisms has been a source of worldwide attention and concern for decades. Pesticide residues in water are a major concern as they pose a serious threat to biological communities including humans. The present study deals with the sub-lethal effect of four agrochemicals namely insecticide (Imidaclorprid-IMI), Herbicide (Pyraonsulfuron Ethyl-PE), Fungicide (Curzate-CZ) and plant nutrient (Mixture of Trace metal ions-MN) on the alteration in gene expression pattern of various target genes of hypothalamus pituitary gonadal axis (HPG) in *Oreochromis mossambicus*. IMI exposure resulted in an up regulation of the kisspeptin 2 and GnRH-I mRNA level in hypothalamic region, gonadotropin receptors (GtH-Ir and IIr) and estrogen receptor (ERI) in ovary. PE and CZ exposure altered the GtHIIr and Estrogen receptor II (ERII) mRNA levels in brain and ovary. Micronutrient mixture (MN) exposure altered the kiss 2 and ER-I transcript in hypothalamic region of brain and GtH-IIr and ER-I receptor in ovary. The result of the present study implies that agrochemicals has modified HPG axis which ultimately lead to the reduced reproductive fecundity of the fish.

Introduction:-

The endocrine system is comprised of numerous organs throughout the body, which work in tandem with the central nervous system (CNS) to regulate biological processes. Reproduction in fish is controlled by the highly conserved hypothalamus pituitary gonadal (HPG) axis . At its apex are the GnRH neurons in the hypothalamic-preoptic area of the brain that ultimately control the reproduction by integrating information from social and environmental signals with internal information such as nutritional and hormonal state (Maruska and Fernald 2011). The hypothalamic neuron stimulates the anterior and posterior pituitary to regulate the entire system by delivering its hormone to target organ**.** However, due to lack of hypophyseal portal system in teleost, neurohormones show their action by the use of nerve fibers from the preoptic region to the pituitary (Levavi-Sivan B et al., 2010).

The world population is expanding rapidly and is likely to be 8 billion by the year 2025. Limited availability of additional arable land and water resources and the declining trend in crop yields globally will make food security a major challenge in the 21st century (Sadekarpawar and Parikh 2013). According to the projections, food production on presently used land must be doubled in the next two decades to meet the food demand of the growing world population. To achieve the required huge increase in food production, greater emphasis in application of fertilizers and improvements of soil fertility are indispensable (Parikh et al., 2010). Gujarat state, In India is one of the important agro-economic states; hence use of pesticides for the better yield of crops is the routine practice. Due to pesticides toxic properties there is an obvious risk that non target organisms are affected, either at the application site or due to unintentional spreading, at nearby or even distant areas (Akerblom 2004).Chemicals Originating from agricultural activities can enter the nearby aquatic environment through agricultural runoff and disturbs the whole aquatic life through bio-accumulation , one of the non-targeted species which is affected the most are the fishes (Ullah and Zorriehzahra 2014).

Recently, advancement in the field of toxicology is to develop and evaluate new molecular and cellular methods to supplement traditional methods of toxicity testing and risk assessment (Jang et al., 2014).In this context, various studies have been carried out; such as proteomic studies in zebra fish (Biales et al., 2011) exposed to fungicide prochloraz have reported an altered expression of proteins in brain region (Biales et al., 2011); endocrine disrupting activity of endosulphan is been well demonstrated in *Cichlasoma dimerus* on gonadotropin-releasing hormone (GnRH) I, II, and III and β follicle-stimulating hormone (βFSH) activity by Piazza et al., (2011); genetic profiling has also been studied by Weber et al (2013) in zebra fish on exposure of atrazine. Multiple studies have been conducted to assess the effect of pesticides on the HPG axis of fish (Palais et al., 2012; Shenoy 2012; Freeman et al., 2014; Wirbisky et al., 2016). As proposed by Mennigen et al., (2008) the inhibitory effects of agrochemicals on reproduction may be mediated through changes in regulation at the any level of the HPG axis.

Earlier findings have proved the toxicity of IMI (Desai et al., 2014); CZ (Parikh et al., 2015); PE (Upadhyay et al., 2014); and MN (Sadekarpawar et al., 2015 a,b,c), however there is a gap in our understanding of the adverse effect of these chemicals on HPG axis. We hypothesize that, in addition to the toxicity of these agrochemicals, they may have endocrine disrupting property. Hence the objective of the study is to have an insight into the effect of agrochemicals on hypothalamic pituitary gonadal axis (HPG) of O.mossambicus.

Materials and Method:-

Fish:-

Healthy male and female adult O.mossambicus was procured from the pure brooders of length 12±3cm and weight 25±3g. Fishes (5 males and 5 females) were kept in a clean glass aquarium for an acclimation period of 12-15 days in de-chlorinated water at 27 ± 4^0C, pH 7.4 ± 0.05, dissolved oxygen 8 ± 0.3 mg/L, total hardness 188 mg/L $CaCO_3$ with a 12:12 light:dark photoperiod. They were fed with the commercial available healthy food during the period of study. 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 the fishes were divided into 5 groups having 5 females and 5 males (n=10) and 4 replicates were performed for each group. 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 pyrazonsulfuron Ethyl-PE (50 mg/L i.e. $1/10^{th}$ of LC_{50}), Group IV Exposure of fungicide Curzate-CZ (4.9mg/L i.e. $1/10^{th}$ of $LC_{50)}$,Group V Exposure of micronutrient mixture-MN (500mg/L i.e. $1/10^{th}$ of LC_{50}). The exposure was for the period of 14 days and on the 15th day fishes were sacrificed via rapid cervical transection. Tissues from brain (including hypothalamus and pituitary), ovary were sampled and preserved in TRIzol reagent for subsequent RNA isolation.

Total RNA extraction and cDNA synthesis:-

Total RNA was isolated by Trizol method (Invitrogen**)** (Peterson and Freeman 2009) and concentration was measured spectroscopically by Perkin elmer. cDNA was reverse transcribed from 50ng total isolated RNA using Thermo Verso cDNA synthesis kit (AB-1453/B) and PCR for the candidate genes was performed with their specific primers and with standardized condition i.e. denaturation was performed at 95^0C for 1 min, annealing was carried out for different primer according to their respective T_m for 30 sec and extension was carried out at 72^0C for 7 mins with 18srRNA as the reference gene (Table 1).A total of 35 cycles were carried out for all the genes and finally the amplicon obtained were checked on 2% agarose gel and images were taken using ABI gel documentation system. Relative quantification analysis of the PCR products was done using Image J software.

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 was used 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.

Western Blot study:-

30 micrograms of total protein 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 (kisspeptin 1 and 2, procured as a gift from Prof.Parhar, 1:1000dilution),

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).

Estradiol (E_2) Hormonal Assay:-

At the 15^{th} day, blood was collected using caudal peduncle with the help of heparinized syringe. Plasma was separated and circulating steroid levels were measured by Cayman ELISA kit (Cat #582251). Each sample was assayed in triplicates were 100 ul of ELISA buffer was added to all the wells, followed by addition of 50 ul of estradiol standard to each well. Estradiol standard was made using serial dilution from the stock solution (400ng/ml) in 8 tubes.50ul of sample was added to each triplicate trailed by 50ul AChE tracer in each well except for blank and TA (total activity). Finally 50ul 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:

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

B/B0 (Sample or Standard Bound/Maximum Bound)
B0- Maximum binding.
B-Sample or Standard Bound.

Statistical Analysis:-

The computed data was analyzed using PRISM 6 Software. One and two way ANOVA followed by DUNNETs multiple comparison were used to the test for significant differences among the individual treatment combinations. Statistical significance was accepted at $*p \leq 0.05$ for all tests.

Results:-

There was an up regulation of GnRH-I in all the agrochemicals expect in case of herbicide (PE), which showed significant ($*p<0.05$) down-regulation (Fig –1). Kiss2 mRNA expression was found to be was up-regulated maximally (Fig-3) in case of micronutrient mixture (MN) followed by insecticide (IMI) and fungicide (CZ) which was establish to be statistically significant ($*p<0.05$).

On the other hand, the expression pattern of kiss-I revealed down-regulation of its transcript (Fig-2) in all the groups, however the significance ($*p<0.05$) was noted in case of insecticide (IMI), micronutrient mixture (MN) and Herbicide (PE).

GtH-Ir: The expression pattern of receptors were also studied in ovary, among which GtH-Ir was up regulated in all case (Fig-5), but was significantly ($*p<0.05$) elevated in case of IMI and CZ. Analogous results were obtained for GtH-IIr, that showed up regulation among the different class of agrochemicals (Fig-4), but the significant change ($*p<0.05$) was noted under the exposure of MN followed by IMI.

ER-I & ER-II: The expression of estrogen receptor (ER-I & ER-II) was studied in brain and ovary both to witness the fold change in the tissue level gene expression (Fig-6-9).ER-I showed a significant change ($*p<0.05$) of its expression under the exposure of IMI and MN in both tissues. However, there was a down regulation of it when exposed to PE in both the tissues, which was found to be non-significant. However, this pattern of expression was not true for ER-II, which showed significant over expression under the influence of CZ, IMI, PE and CZ, PE in brain and ovary respectively. Other agrochemical exposure in both the tissues showed the alteration, but it was non-influential. Further, western blotting was performed for Kiss 1 and Kiss 2 (Fig- 10, 11, & 15). There was significant down regulation of Kiss 1 expression in all the groups. Correspondingly, Kiss 2 expression was also attributed to be up regulated under the exposure of MN, IMI and CZ ($*p<0.05$).

Accession No.	Gene Name	Sequence	Tm	Species	Amplicon Size (bp)
NM_001113489	Kiss1	FP:CTCAGGGGAACAGACACTCG	59^0C	*Daneo rerio*	400
		RP:GCAAATACCTCAGAGAGGACCA			
NM_001279468.1	Kiss 2	FP:5':GGATCCCAGCCTCTGCTTTT3'	60^0C	*O.niloticus*	214
		RP:5':TCAGGTGGGTACCTCCAGTT3			
AB104861.1	GnRH1	FP:5'CGCCATTTCCTCTCCAGCTTA3'	60.1^0C	*O.niloticus*	180
		RP:5'CGCTACTCCAACAGAGGTCG3'			
AB042422,	GTH Ir	FP:5'ACCTGCTGGAGAGTATCGGT3'	60^0C	*O.mossambicus*	276
		RP:5' AGGCGGTGGAATGGATCTTG3'			
AB042423,	GTH Iir	FP: 5'AAATGCTCCCCAAAGCCAGA3'	60^0C	*O.mossambicus*	214
		RP:5'GCCAGTCTGTGGCTGATTGT3'			
NM_001279770.1	ER-α (Type I)	FP:5'GGAGGTATGCGTAAGGACCG3'	53^0C	*O.niloticus*	85
		RP: 5'GCAGGTCTTTGGCTGGTTTG3'			
NM_001279774.1	ER-β (Type II)	FP:5'CAATGTCATGCATGGGTTGTCT3'	52^0C	*O.niloticus*	198
		RP:5'TCCATGTTGGGGTTGCATCA3'			
AF497908	18srRNA	FP:5′-TATTGTGCCGCTAGAGGTGAA-3′	51^0C	*O.mossambicus*	102
		RP:5′-CCTCCGACTTTCGTTCTTGA-3′			

Table 1: Depicts the forward and reverse primer sequence of candidate genes with its accession number, T_m and amplicon size

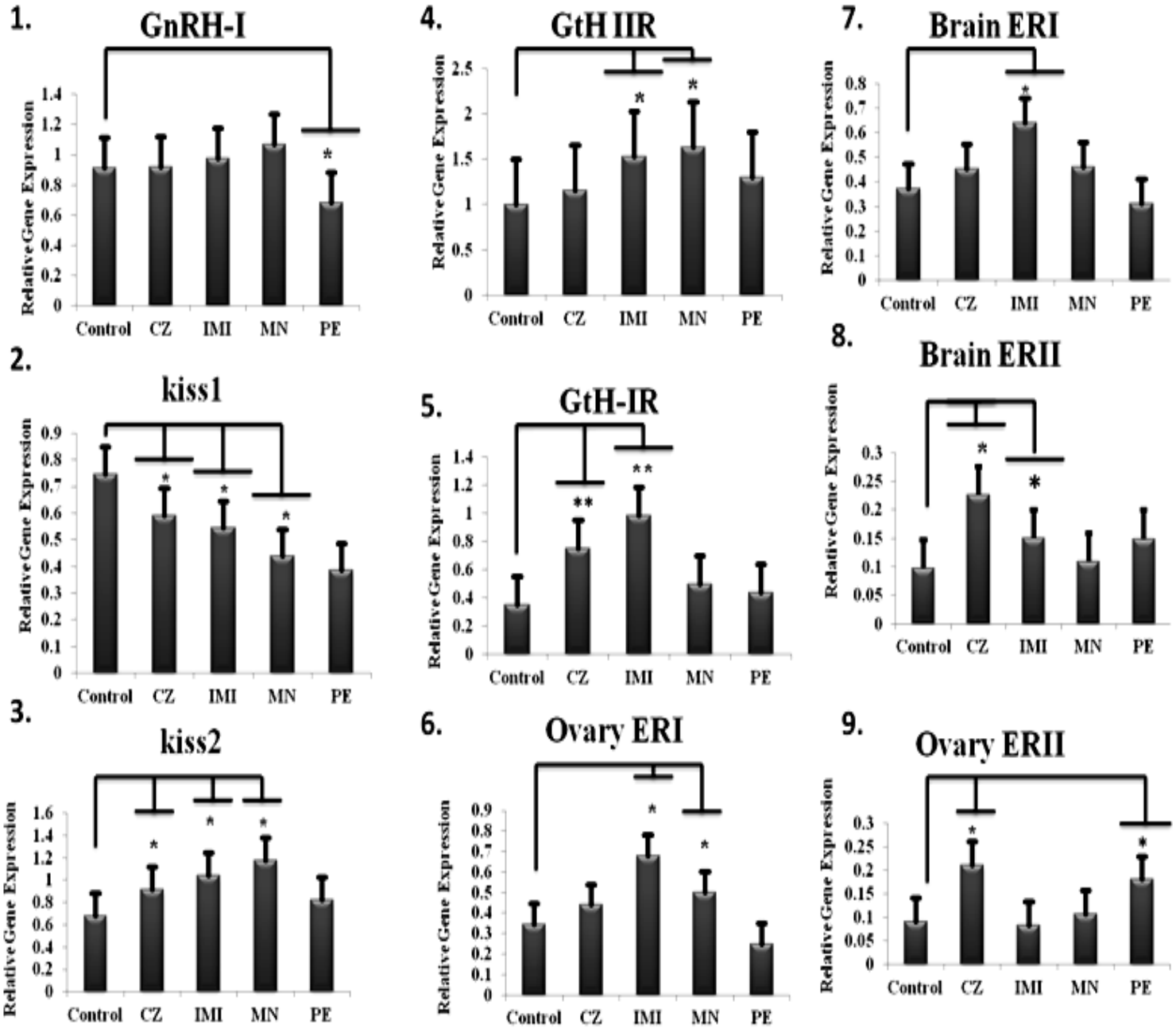

Fig 1-9: Relative gene expression pattern of GnRH-I,Kiss1,Kiss2 in brain hypothalamic region GtH-Ir, GtH-IIr in ovary and ERI , ERII in brain and ovary. (*) denotes level of significance at *p<0.05,p<0.01,***p<0.001.**

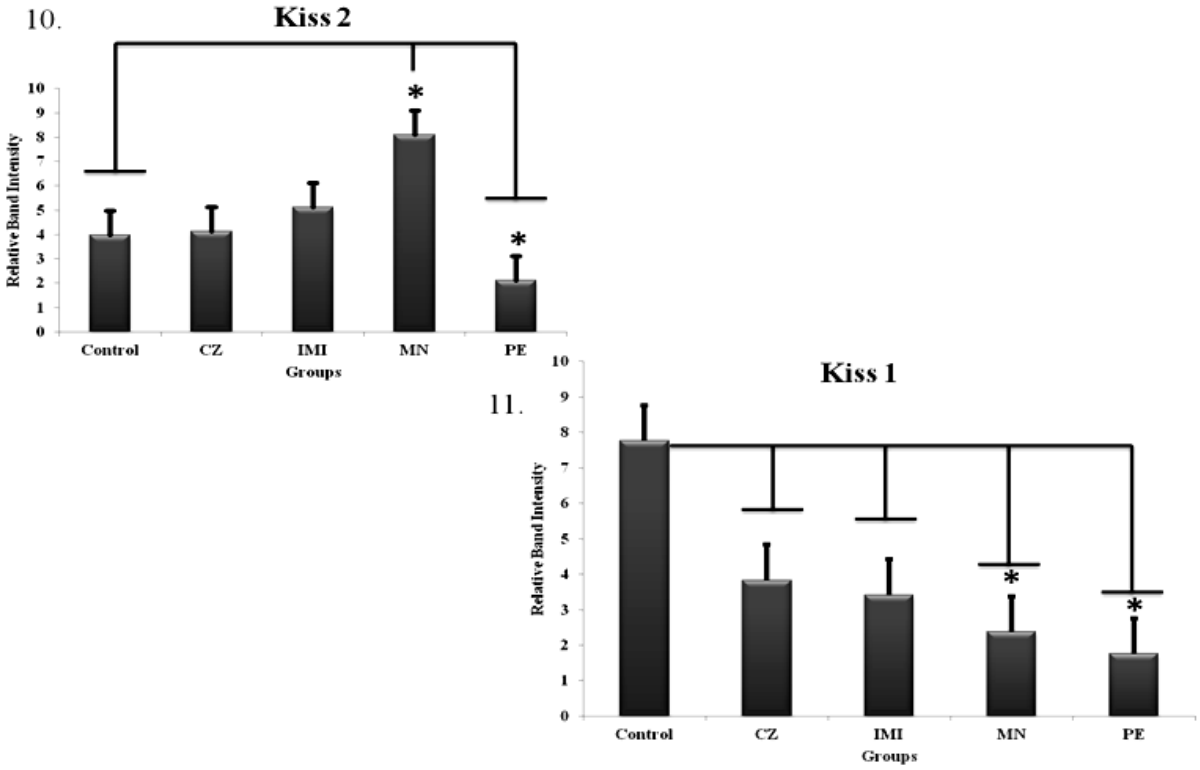

Fig 10 & 11: Relative band density of Kiss 1 and Kiss 2 of 4 groups compared to control. (*) denotes level of significance at *p<0.05, **P<0.01, ***P<0.001

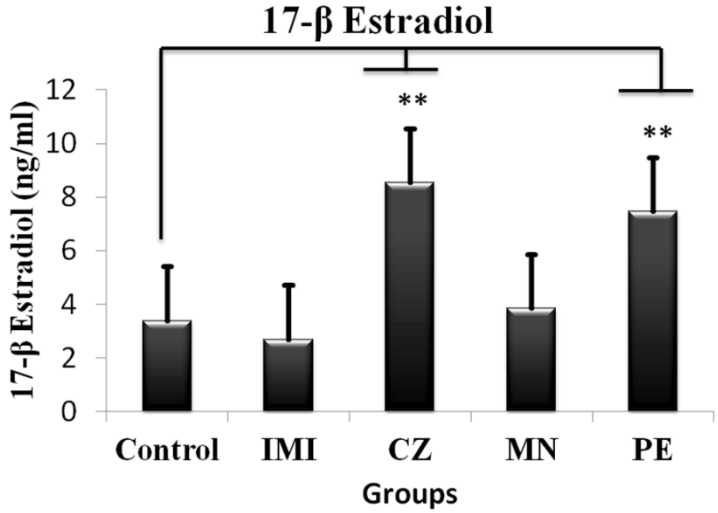

Fig 12: Graph showing the Plasma estradiol level (ng/ml) of *O.mossambicus* . (*) denotes significance at *p<0.05, **p<0.01, ***p<0.001

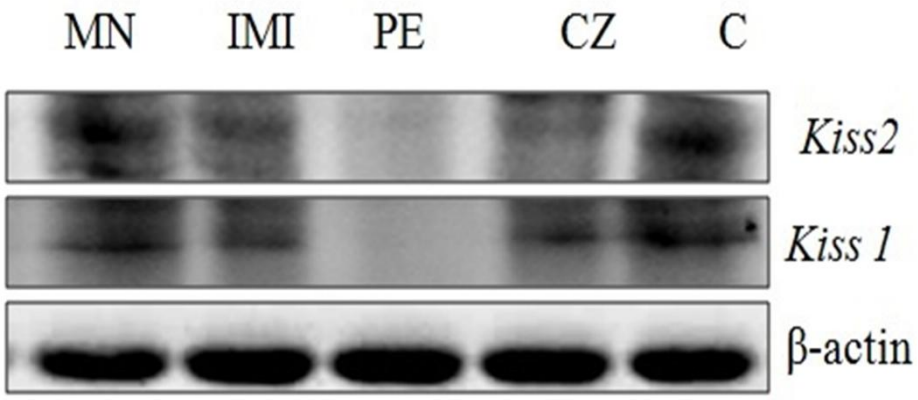

Fig15: Western blot analysis of kisspeptin 1 and kisspeptin 2. B-actin was taken as internal control for densitometric analysis

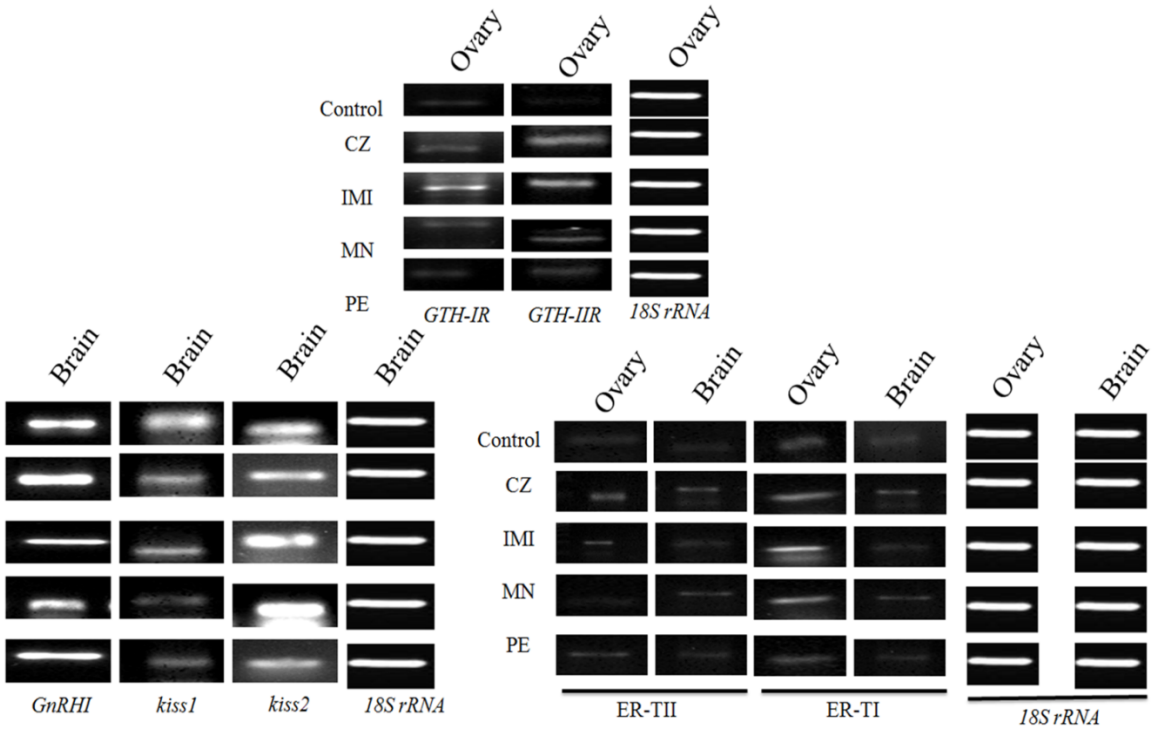

Fig 14:Gene expression profile on agarose (2%) of Kisspeptin 1 & 2, GnRH I-Gonadotropin Releasing Hormone, GtHIr & GtHIIr-Gonadotropins Hormone Receptor, ERI& ERII Estrogen receptor.

Discussion:-

The increase in the spray of agrochemicals has resulted in elevation of toxicity in the environment that has end result in bioaccumulation from one trophic level to the other, ultimately affecting the humans (Ribeiroa et al., 2005; Guoa et al., 2008; Singh and Singh 2008). Cichlids are one of the remarkable models of study, ranging from developmental to toxicological studies (Fujimura and Okada 2007). In the present study, *O.mossambicus* was taken as the model organism with the aim to find the endocrine disrupting properties of the agrochemicals by studying the key genes responsible for the altered physiology on exposure of the widely used agrochemicals in the state of Gujarat.

In the present study, of all the agrochemicals exposed, the expression of GnRH-I was found to be up regulated except in case of PE, where significant down regulation was found. Similarly, Kiss2 mRNA expression was also found to be significantly up regulated under the exposure of MN, IMI and CZ, implying that it is exerting its effect by up-regulating GnRH-I and Kiss2 neurons. Teleost particularly *O.mossambicus* are known to have three isoforms of GnRH (GnRH-I ,GnRH-II ,GnRH-III) that are distributed in various tissues, till now GnRH-I is known to regulate HPG **(**Sempere et al., 2012**)**. As reported by Oakley et al ., (2009) GnRH I neurons are known to be regulated by kiss 2 neurons of discrete nuclei of hypothalamus in some teleost, thus intimately regulating each other (Parhar et al., 2004; Clarkson et al .,2010). In the present study MN exhibited the maximum alteration in the Kiss2 gene expression pattern , this may be because it is an amalgamation of trace metal ions (Zn^{2+},Fe^{2+},Cu^{2+},B^{+},Mn^{+}) suggesting the synergistic or individual action of metal ions (Mebane et al., 2012; Sadekarpawar et al., 2015). . IMI belongs to neonicotinoid group, the increase in GnRH-I and Kiss2 on IMI exposure proves an additional role of IMI apart from its usual mode of action (Gibbons et al., 2015; Crosbya et al., 2015; Desai and Parikh 2013).

Kisspeptins are a group of peptides that stimulate GnRH release and are required for puberty and maintenance of normal reproductive function. Studies in teleosts have revealed the presence of multiple kisspeptin forms (Kiss1, Kiss2) in the brain. It has been suggested that there is a double site of Kisspeptin action in the brain, either in the hypothalamic-hypophyseal region or in the median eminence, an area located outside the blood brain barrier (Nocillado et al., 2008). The important role of Kiss 1 has also proved to regulate gonadotropin secretion, proving the pivotal role in regulation of reproduction (Akazome et al., 2008) .In the present study, mRNA expression and western blot analysis has confirmed the expression of kiss 1 in *O.mossambicus*, and mRNA expression of Kiss 1 was found to be significantly downregulated under PE exposure, suggesting its pleotropic role (unpublished data) in other than regulating the HPG axis. However, the exact mechanism by which it happens is still illusive. Immune-histochemical studies can shed more light on the same.

GnRH-I upon activation acts on anterior cells of pituitary, thus initiates the release of GtH-I (LH like) and GtH-II (FSH like) peptides. This in turn binds to its receptor (GtH-Ir, GtH-IIr) present either on testes or ovary (Yaron et al., 2003; Chen and Fernald 2008). It has been demonstrated that sex reversal is influenced in a large number of teleost species exposed to agrochemicals that have estrogenic effect (Cheshenko et al., 2008; Scholz and Kluver 2009;Hachfi et al., 2012;Frye et al., 2012). IMI and MN exposure resulted in impairment of gonadal activity and the outcome was that on 15^{th} day the dissected fish showed only the presence of ovary, suggesting the possibility of sex reversal phenomena which was corroborated by earlier reported reduction in Gonadosomatic index (GSI) (Sadepawar and Parikh 2013; Desai 2013). Receptor profile of GtH-Ir was found to be up regulated under all agrochemicals exposed, but the significance was reported only in case of IMI and CZ, while the GtH IIr expression was significantly higher in IMI, MN exposed groups. Our results are in accordance with earlier reported sex reversal in various teleost species (Jobling et al., 2002; Orlando et al., 2004; Kortenkamp 2007; Brown et al., 2016).

ER-I and ER-IIr which are analogous to mammalian estrogen receptors α and β (Nelson and Habibi 2013; Nagler et al., 2007) were also studied in ovary and brain as they illustrate negative feedback directly or indirectly (Guiguen et al., 2010;). Among all the agrochemicals exposed, there was significant up regulation of ER-I in IMI and MN in ovary and brain, possibly governing the action by some downstream signaling mechanism (Selin et al., 2009), confirming the endocrine disrupting action of these chemicals. ER-II did not show same pattern of regulation as CZ and PE exposure resulted in a significant up regulation of ER-II mRNA in both brain and ovary, while IMI accredited the higher expression of ER-II only in brain tissue. CZ being the mixture of cymoxanil and mancozeb, has resulted into constitutive receptor activation leading to its up regulation (Coumailleau et al., 2015), which may be due to its mimicking action as that of estrogen. Apart from the conventional studies done on various groups of herbicide (Parikh et al., 2014), very few studies are accounted for the negative effects of PE on any organism. PE

which belongs to the group of sulfonylurea too expressed the parallel effect as that of CZ, suggesting its mimicking role to that of estrogen which was well supported by an increase in plasma level of 17- β estradiol. Our result are parallel to the result of Kim et al.,2014; Servili et al., 2011.

Conclusion:-

Among all the agrochemicals examined, in brain IMI, MN exerts its effect by up-regulating Kiss-2 and GnRH-I transcripts, altering the HPG axis. While PE acts via acting on Kiss-1 neurons in hypothalamic region of brain. Ovary, receptor profile was well being altered by IMI, CZ and MN, which illustrated higher GtHIr and GtH-IIr mRNA levels respectively. Increased expression of ER-I by IMI ,MN and that of ER-II by PE, CZ suggests its mimicking role of estradiol (E_2). Hence, from the results of the current investigation, it can be concluded that all agrochemicals could be potential endocrine disrupting chemicals. The exact mode of action needs to be delineated by further investigations. The results obtained can be considered as a preliminary hint about these chemicals on HPG axis and encourages in-depth analysis of the mechanisms involved therein.

Acknowledgement:-

The authors are highly thankful to Head, Department of Zoology for providing instrument facilities for the present work and are also thankful to Prof. Ishwar Parhar, Monash University Malaysia for providing the kisspeptin antibodies.

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