



## Study of effect of heavy metals CuSO<sub>4</sub> & ZnSO<sub>4</sub> on GSI of fresh water teleost through HPG axis

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### ABSTRACT

This histopathological study evaluated the effect of CuSO<sub>4</sub> & ZnSO<sub>4</sub> on Gonadosomatic Index (GSI) through hypothalamic-pituitary-gonadal (HPG) axis of fresh water teleost female *Anabas testudineus*. The hypothalamus is projected ventrally as a round infundibulum, which is connected with the hypophysis (pituitary gland) in the brain by an indistinct pituitary stalk; this concept is called as hypothalamo-neurohypophysial system or hypothalamic-pituitary-gonadal (HPG) axis.

For the study experiment were carried out by exposing the female *Anabas testudineus* to the test solutions containing various concentrations of heavy metals CuSO<sub>4</sub> & ZnSO<sub>4</sub> (copper & zinc sulphate) and 50% mortality was recorded at 96 hours exposure period. The LC<sub>50</sub> value calculated was 2.5 mg/l for CuSO<sub>4</sub> & 3 mg/l for ZnSO<sub>4</sub>. On the basis of lethal doses test animals were exposed to the sub-lethal doses for a long period of 30 days then histopathologically and quantitatively observed the effect of heavy metals on neurohypophysial complex of fish. Results indicated that the neurohypophysial complex of fresh water fish *Anabas testudineus* is sensitive to the heavy metal pollutants and affect the spawning of fish. Effect of Copper & Zinc sulphate on GSI was found statistically significant ( $P<0.05$ ) after 30 days exposure between control and pollutant group.

**Key Words:** Neurohypophysial complex, hypothalamic-pituitary-gonadal (HPG) axis, heavy metal Pollutant (HMP), Infundibulum, Histopathological.

### INTRODUCTION

Fishes exhibit huge aquatic diversity in shape, size, biology and in the habitats they occupy. They are the most primitive vertebrate from which all other vertebrates including man has evolved. They are important not only because of their economic value, but also because of sensitivity to ecological changes. So, fish assemblages have widely been used as biological indicators to estimate and evaluate the level of degradation and health of rivers and streams<sup>1</sup>.

Analysis of most of the natural water bodies revealed a critical loss of biodiversity. The level of contamination with heavy metals has been increasing in the last decades, partially because of increase in the use of such metals in a variety of agricultural and industrial processes; this has stimulated studies to identify the potential toxicity induced by such agents. In this regard, it has been reported that heavy metal like copper and zinc can exert toxicity at specific experimental conditions, which may also depend on the evaluated organism and the associated environmental factors<sup>2,3</sup>.

Though some heavy metals like Cu and Zn are essential to both plants and animals and they serve some useful biological functions in their body but their excessive intake can be highly toxic due to their ability to be absorbed quickly in biological system. After being absorbed in the cell they affect their normal functions, reproductive cycles and breeding they also affect structural proteins, enzymes & nucleic acid. Zinc at a excessive intake cause death due to hypoxia. because of rapid drop of blood oxygen and pH<sup>4</sup>. A range of responses have also been reported from copper exposure of which elevation of the hormone cortisol and an increase in adrenergic response, such as release of catecholamine's, can explain many of the observed effects such as on behavior and reproduction. Chromic effect of copper adversely affects the growth and reproduction in fish<sup>5</sup>.

It was found that the toxic effect of metal mixtures are the sum of the toxicities of each metal (additive effect), corresponding to the bio-available form of the metals<sup>6,7</sup>. These toxic substances may knock down immune system, reproductive system and endocrine system in animal and these effects can be at organ, tissue and cell level<sup>8</sup>. Metals and organic contaminants affect the spawning behavior and spawning duration of fish<sup>9</sup>.

It was shown that accumulation of heavy metals results in inhibition of the secretion of noradrenalin and stimulation of the secretion of dopamine in the hypothalamus and the brain is also known to be a site of stress hormone synthesis<sup>10</sup>. These processes results in a disturbance of hormonal equilibrium of the HPG axis, which can unfavorably influence the efficiency of spawning in fish. It should be noted that the HPG axis functions as a dynamic system throughout each life-stages of an organism, early in development through gonad development and finally into adult life-stage<sup>11, 12</sup>. The primary tissues involved in this hormonal cascade are the hypothalamus, pituitary gland, and gonads. The hypothalamus located at the base of the brain is sensitive to signals from sensory receptors and releases hormones in response to environmental cues, principal among these hormones are gonadotropic releasing hormones (GnRH), which travel from the hypothalamus to the pituitary gland. Certain cells of the pituitary receive GnRH and release it into the bloodstream. The pituitary is responsible for a wide variety of functions including growth and reproduction. The gonadotropic hormones travel to the gonads which synthesize steroids responsible for final maturation of the gametes<sup>13</sup>.

The hypothalamus of teleost fishes comprises the Gomori-positive nucleus preopticus (NPO) and the Gomori-negative nucleus lateralis tuberis (NLT)<sup>14</sup>. The NPO, which is composed of the pars magnocellularis and the pars parvocellularis, is situated on both sides of the preoptic recess. The axons (Type A peptidergic fibres containing 100-200 nm granules) originating from the cells of the NPO form the preopticohypophysial tract which penetrates the pituitary, ramifies and terminates in the neurointermediate lobe<sup>15-17</sup>. These axonal fibres originated from the NPO cells forms tractus preopticohypophysis, which travels on the floor of hypothalamus and terminates in the neurohypophysis of the pituitary gland. The neurosecretory material is transported from NPO to the pituitary gland through this tract so tractus preopticohypophysis and neurohypophysis are important components of neurohypophysial complex<sup>18-20</sup>. The gonadotrophs are also directly innervated by these fibres originating in the NPO. This implicates both, the NPO and the NLT in the regulation of gonadotropic functions of the pituitary. The presence or absence of NLT varies in different teleost species. In *Anabas testudineus* NLT is absent<sup>21</sup>.

## MATERIAL AND METHODS

### COLLECTION OF SPECIMENS

Collections from February to August were mainly used to fix the maturity of spawning period of test fish *Anabas testudineus* (weight: 45 grams and above).

### EXPERIMENTS

For the control and effect of heavy metal pollutants on the neurohypophysial complex and gonads, in the laboratory experiments were conducted in three phases.

### PHASE I EXPERIMENT

In the first phase of experiment 20 female test fish as control were acclimatized in normal water fish tank for copper & zink sulphate respectively in natural condition for one month (May-June). Dry prawn powder as fish food was provided and medium was changed daily. After acclimatization dissect out the animal in laboratory and following measurements were recorded during experimentation.

- ✓ Total length of each fish.
- ✓ Body weight of each fish.
- ✓ Gonad weight of each fish.
- ✓ The volume of gonad, measured by liquid displacement method.

### PHASE II EXPERIMENT

In the second phase short term tests of acute toxicity were performed over a period of 96 hours using heavy metal pollutant copper & zink sulphate.

For CuSO<sub>4</sub> preliminary experiments were conducted to choose the concentration that resulted in the 50% mortality at 96 hours exposure period. The LC<sub>50</sub> value calculated was 2.5mg/l. The dose in which fishes were survived for a long period of a month in the fish tank is 2mg/l, is taken as sub-lethal dose.

For ZnSO<sub>4</sub> preliminary experiments were conducted to choose the concentration that resulted in the 50% mortality at 96 hours exposure period. The LC<sub>50</sub> value calculated was 3mg/l. The dose in which fishes were survived for a long period of a month in the fish tank is 2.5 mg/l, is taken as sub-lethal dose.

### PHASE III EXPERIMENT

In the third phase of experiment 20 test fishes were acclimatized in separate fish tank, in the sub-lethal concentration of heavy metals CuSO<sub>4</sub> (2 mg/l) & ZnSO<sub>4</sub> (2.5 mg/l) for 30 days in spawning period. The solution of the pollutants was changed daily and fishes were aerated and feed properly. After 30 days sacrificed animals one by one and dissect out the fish for brain & ovary after recording the necessary measurements (as recorded in ex. no.1).

#### Histopathological analysis

First Control fish then the fish exposed to sub-lethal doses of CuSO<sub>4</sub> & ZnSO<sub>4</sub> for 30 days, in the spawning period were sacrificed. Their brains and ovaries were dissected out and fixed in aqueous bouin and hollande's modified, dehydrated in graded ethanol series, cleared in xylene and embedded in paraffin. Finally the paraffin blocks were prepared and slices were cut at 5μ to 6μ (micrometer thick) by means of rotary microtome (Leica RM 2145). Slides of these slices were dehydrated and stained with Aldehyde Fuchsin (AF) and Chrome Alum Haematoxylin Phloxine (CAHP) for brain sections, Mallories Triple Enrich's Acid Alum Haematoxylin stains were used for gonads. The sections were examined and photographed using an Olympus BX 51 microscope.

#### Statistical analysis

One of the most common measures to analyse the effects of pollutants is gonadosomatic index (GSI). The GSI as a percentage weight of ovary to the body weight has been used as a maturity index of fish. The GSI for both groups (Control & HMP- CuSO<sub>4</sub> & ZnSO<sub>4</sub> treated groups) were calculated according to the formula:

$$\text{Gonosomatic index} = \frac{\text{Weight of gonad}}{\text{Weight of fish}} \times 100$$

Where, GW = Gonad weight of fish in milligram (Mg).

BW = Body weight of fish in gram (Gm).

Means ± standard deviation (SD) were calculated for both groups. Statistical analysis (Two Way ANOVA) was done using the software Graph pad Prism 5, at a 5% significance level. The results of the experimental groups (HMP-CuSO<sub>4</sub> & ZnSO<sub>4</sub> treated groups) were compared with the control group to quantify the effect of the CuSO<sub>4</sub> & ZnSO<sub>4</sub> on the test animals.

## RESULTS AND DISCUSSION

### HISTOPATHOLOGICAL RESULT OF NEUROHYPOPHYSIAL COMPLEX

The activity of the NPO have correlated with the annual gonadal cycle, the NPO is active during the spawning periods and inactive in the sexually quiescent period. Similarly, seasonal fluctuations in the quantity of neurosecretory material in NPO have also been correlated with the gonadal activity<sup>22, 23</sup>.

In *Anabas testudineus* NPO of the control fish are spherical in shape. The cytoplasm is granular and deeply stained. The nucleus is situated near the periphery and contains one or two nucleoli, with

chromatin material. The neurosecretory cells are more concentrated (granulation) and uniformly distributed with the cytoplasm. The staining intensity is also deep (figure 1). Tractus preoptico hypophysis and neurohypophysis also shows similar conditions (figure 2, 3) in control fish. The histological change in the NPO shows its involvement during reproductive cycle. The NPO cells of copper sulphate treated fish are highly affected. The NPO cells of zinc sulphate treated fish are also affected but less than that of copper sulphate. Most of the NPO cells are smaller in size with depleted neurosecretory materials (figure 4). The nucleus is also smaller in size and the nucleolus is rarely visible. Some of the NPO cells are hypertrophied and some are hypotrophic condition. Tractus preopticohypophysis and neurohypophysis also shows further decrease in the quantity and intensity of neurosecretory material (figure 5, 6) due to which the spawning of fish is severely affected in both pollutants. Thus it can be inferred that greater the effect of heavy metal pollutants to the secretion of HPG axis neurohormones (FSH & LH), greater the effect to the spawning period & lesser the process of oogenesis<sup>24</sup>.

## HISTOPATHOLOGICAL RESULTS OF OVARY

Ovary in fish represents the most dynamic organ having a high cell turnover during the reproductive period. Fully mature ovaries of control fish shows advance stages of oogenesis and mature ovum (figure 7). Pollutant Copper and Zinc sulphate treated fishes cause greater loss to advance stages of oogenesis. partial or total atresia of oocyte, marked damage in germinal epithelium, stromal hemorrhage, vacuolization of oocytes and general inflammation<sup>25, 26</sup>.

The spawning periodicity is extremely affected and confirms the histological changes during reproductive cycle. The Pollutant CuSO<sub>4</sub> treated fish indicates a sharp downward trend in the activity of ovary in which later stages of oocytes became atretic and get finally absorbed (figure 8). This metal cause greater loss to advance stages of oogenesis than zinc sulphate.

Pollutant ZnSO<sub>4</sub> treated fish also indicates a downward trend in the activity of ovary in which later stages of oocytes shows lysis, swelling, atresia. Severe necrosis, haemorrhage and nuclear pyknosis (figure 9).

Exposure dependent histological alterations in the ovary were seen. The zinc and copper toxicity causes significant decrease in survival, growth and reproduction over the complete life cycle of the fish. Progressively the chronic exposure of both pollutant affects reproduction, later stages of oocytes undergo atresia and extensive damage to advance stages of oogenesis (figure 8, 9). In the present study the effect of zinc sulphate and copper sulphate is found to be maximum at the 30<sup>th</sup> day of exposure on the ovary, thus it is concluded that the heavy metal pollutants have been found distractive in nature<sup>27, 28</sup>.

## STATISTICAL RESULT

According to the results obtained from the seasonal spawning phases of *Anabas testudineus* the size of the oocyte increases with the temperature of the environment. Increase or decrease in ovary weight and volume correspond with the increase or decrease in water temperature. In April, May and June the highest ovary weight and ovary volume was observed and higher the GSI of control group i.e. 8.49, 9.05 and 9.07 respectively. Thus the GSI increases with the maturation of fish and reaches to its maximum at

the peak period of maturity that is why the GSI has been considered as reliable estimate for gonadal maturity and spawning of any species. Its abrupt decrease indicates beginning of spawning. Our study confirms the observations of earlier workers<sup>29-33</sup>.

The reduction of GSI of test fish subjected to HMP-ZnSO<sub>4</sub> is 5.70, 5.21, 4.92 & HMP-CuSO<sub>4</sub> is 5.01, 4.51, 4.15 (figure 10,11) these results also reflect the reduction of the gonad mass and gonad volume as compare to the control group in the month of April, May and June. Similar findings were reported by earlier workers<sup>34, 35</sup>.

Increase in GSI signified active spawning state in the ovaries of control group, but these values are decreasing in pollutant copper & zinc sulphate treated groups. When compared the GSI of control group and pollutant groups, the GSI of pollutant groups were found to have decreased more than 50% ( $p<0.05$ ) after 30 days of exposure but copper sulphate cause more reduction of GSI than that of zinc sulphate (figure 12).

The Spawning phase between Control group and Pollutant copper sulphate group shows statistically significant difference ( $8.87 \pm 0.32$ ,  $4.45 \pm 0.43$ ,  $p<0.05$ ) in between them (Table 1) as well as Pollutant zinc sulphate group also shows statistically significant difference ( $8.87 \pm 0.32$ ,  $5.27 \pm 0.39$ ,  $p<0.05$ ) in between them (Table 2).

**Table 1 - GSI of Ovary- Control group verses HMP group CuSO<sub>4</sub>**

Sr. no.	Phases	Control group			Polluted group			P value
		N	Mean	S.D.	N	Mean	S.D.	
1	preparatory phase	03	0.77	0.33	03	0.65	0.25	0.64
2	Pre-spawning phase	03	3.72	2.04	03	2.51	1.35	0.43
3	spawning phase	03	8.87	0.32	03	4.45	0.43	0.0002

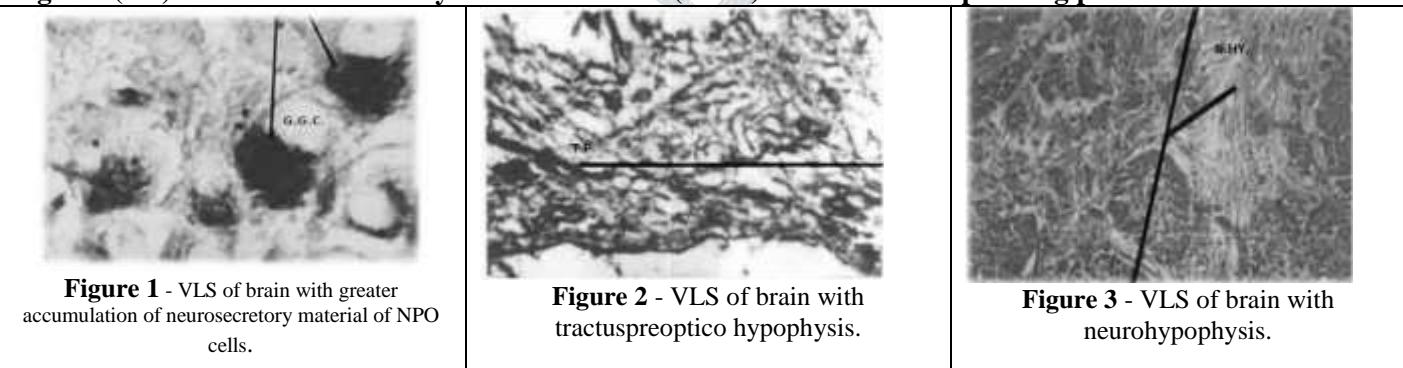
In the Spawning period the GSI of Ovary between Control and HMP treated CuSO<sub>4</sub> groups shows statistical significant difference in between them ( $8.87 \pm 0.32$ ,  $4.45 \pm 0.43$ ,  $p<0.05$ ).

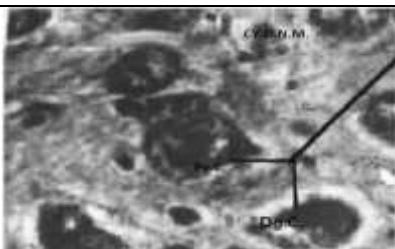
**Table 2 - GSI of Ovary- Control group verses HMP group ZnSO<sub>4</sub>**

Sr. no.	Phases	Control group			Polluted group			P value
		N	Mean	S.D.	N	Mean	S.D.	
1	preparatory phase	03	0.77	0.33	03	0.72	0.31	0.85
2	Pre-spawning phase	03	3.72	2.04	03	2.97	1.21	0.641
3	spawning phase	03	8.87	0.32	03	5.27	0.39	0.0002

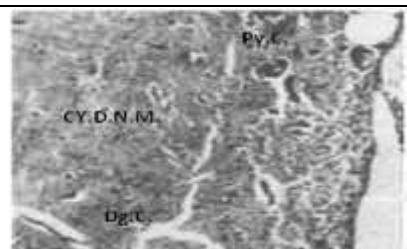
In the Spawning period the GSI of Ovary between Control and HMP treated ZnSO<sub>4</sub> groups shows statistical significant difference in between them ( $8.87 \pm 0.32$ ,  $5.27 \pm 0.39$ ,  $p<0.05$ ).

#### Figures (1-8) of Control and Heavy Metal Pollutants (HMP) treated fish in spawning period

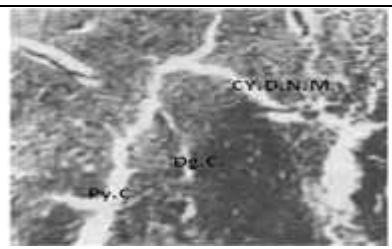




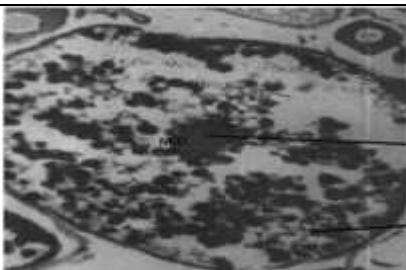
**Figure 4** - VLS of HMP treated brain with depleted neurosecretory material of NPO cells.



**Figure 5** - VLS of HMP treated brain with tractus preoptico hypophysis



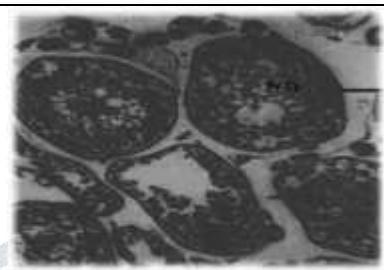
**Figure 6** – VLS of HMP treated brain with neurohypophysis



**Figure 7** - T.S. of ovary showing advance stages of oogenesis- mature ovum.

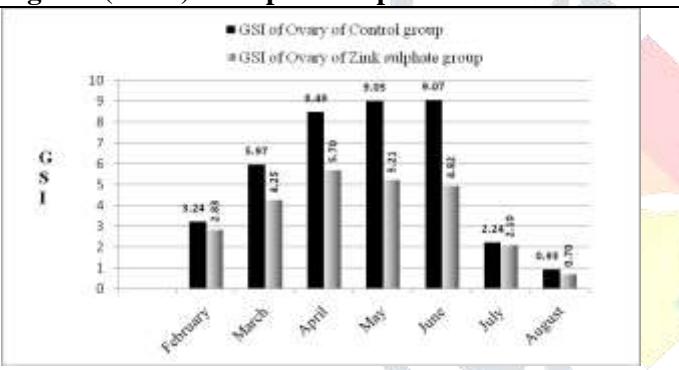


**Figure 8** - T.S. of HMP CuSO<sub>4</sub> treated ovary showing nuclear pyknosis & pyknotic cytoplasm

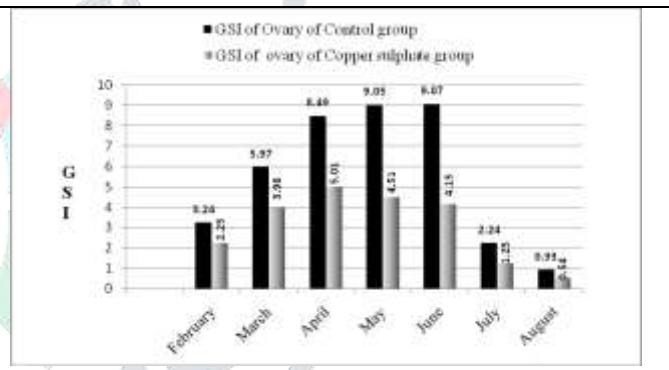


**Figure 9** - T.S. of HMP ZnSO<sub>4</sub> treated ovary showing Nuclear pyknosis & pyknotic cytoplasm

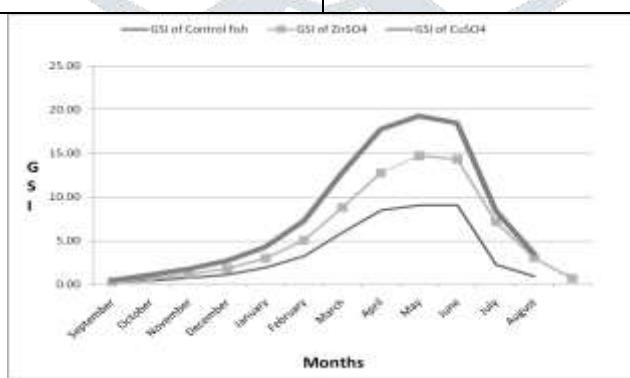
#### Figures (10-12) - Graphical representations



**Figure 10** - Showing the monthly comparison of GSI between Control group and ZnSO<sub>4</sub> treated group



**Figure 11** -Showing the monthly comparison of GSI between Control group and CuSO<sub>4</sub> treated group



**Figure 12** - Copper sulphate treated fishes cause greater loss to advance stages of oogenesis than zinc sulphate

## CONCLUSION

The survival and existence of any species is ultimately determined by the ability to reproduce successfully in a fluctuating environment and show the diversity aspect. Maturation and spawning are very important biological processes for existence. Variation in these processes due to alteration in geographical locations and the environment are common, but heavy metal toxicity in natural water is the major source of contamination which have adverse effects on the HPG relationship of fish and disturbs the aquatic biodiversity which is responsible for maintaining and

supporting overall environmental health. An understanding of these factors is essential for the existence, preservation, development and conservation of the species to maintain the Global biodiversity because fish are irreplaceable bio-indicators of the degree of damage to the water environment in terms of ecological aspect.

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