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ORIGINAL ARTICLE

An Evaluation of Combined Impact of Water pH and Cypermethrin on Ovarian Reproductive Physiology of Olive Barb (*Puntius sarana sarana*) Hamilton, 1822

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ABSTRACT
Pesticides like cypermethrin are very much resistant to biological degradation and consequently stored in the food chain. Fish health can be impacted by abiotic factors in a number of ways. Temperature, pH, water column length, turbidity, salinity, and alkalinity are examples of abiotic factors that have been proven to have a major influence on fish reproduction. In this work, we observed the detrimental effects of pH and cypermethrin together on ovarian volume and oocyte stage in Puntius sarana. Cyper 25 was administered at a sublethal dosage to each treatment group. In all treatment groups stage I was the most prevalent oocyte stage. Stage IV oocyte was found highest in number among all types of oocyte in control group. For stage I and stage V oocyte the percentage of oocyte increased significantly with the increase of acidity of the medium (pH 6 and 5). In basic medium (pH 8.5 and 9.5) the percentage of oocytes increased compared to control group. The percentage of stage II oocyte showed a significant decrease ($p<0.05$) in acidic medium (pH 6 and 5) and in pH 9.5 + toxicant group. Both stage III and stage IV oocyte showed similar pattern in all experimental groups. However, in pH 8.5 + toxicant group the percentage of both stage III and stage IV oocyte retained almost control values. During all spawning period a significant ($p<0.05$) gradual decrease in the volume of ovary was recorded in pH 6 + toxicant, pH 5 + toxicant group and pH 9.5 + toxicant group. In pH 8.5 + toxicant group volume of ovary almost retained the volume as control without any significant change.
Key Words: *alkalinity, cypermethrin, oocyte, salinity, spawning, toxicant, turbidity*

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INTRODUCTION

Many pesticides have an effect on animals' endocrine systems. Pesticides like cypermethrin are very much resistant to biological degradation and consequently stored in the food chain. Endocrine disrupting chemicals (EDCs) can induce physiological and reproductive problems by attaching to hormone receptors and mediating their function. Pesticides found their way into water bodies after being applied, causing harm to aquatic animals particularly to fish. Because of hydrophobic nature of most pesticides they can readily be mixed into the soil and then wash off into water bodies such as rivers, lakes, and ponds, resulting in aquatic contamination [1, 2]. When they accumulate in aquatic organisms, they may infiltrate the food chain [3].

Gonads and liver play an important role in the quantity of plasma sex steroid hormone either through secretion from ovary and testis or by the rate of deactivation of hepatic tissue and excretion. Many histopathological changes were detected in gonads due to exposure to pesticides. Vacuolation with vitollogenic fluid in the parenchyma of ovary and primary oocyte necrosis, degenerative and necrotic alterations in the seminiferous tubules, and atretic oocytes are examples of these abnormalities [4-7]. The

oocyte atresia indicates decrease of estradiol hormone. Low plasma concentration of vitellogenin explained vitellogenic oocytes and necrosis of hepatopancreas [8].
Study of the stages of oocyte is very important to know the proper functioning of oogenesis in fish which ultimately reflect reproductive health of fish. It had been observed that vitellogenic and atretic oocytes are the most important stages to determine the development of oocyte, as both the stages are linked to the fish oogenesis capability [9]. The larger number of atretic cells indicates either inappropriate ovarian growth, in which the oocytes were unable to mature due to endocrine disruptor toxicity or decreased supply of gonadotropin from the hypophysis, or direct effect of pollution on the intra-ovarian scene [10]. Similar results were demonstrated where malathion inhibited acetyl cholinesterase activity in brain of *Clarius batrachus* in a dose-dependent manner, resulting in ovarian cycle stage I and II egg loss [11]. Several studies showed that ovary volume is also disrupted due to stress [12].
The pH of the water is critical for fish survival and reproduction. All of a fish's bodily activities are performed in the water. Proper understanding the physical and chemical properties of water is the crucial point for effective aquaculture because fish are completely reliant on water for respiration, feeding, development, excretion, maintenance of salt balance, and breeding. Abiotic variables can impact fish health in a variety of ways. Abiotic variables that affect fish reproduction, such as temperature, pH, water column length, turbidity, salinity, and alkalinity, are found to have a significant impact [13, 14]. Of all these abiotic variables, pH is thought to be one of the most significant ones affecting aquatic life. Fish physiology is significantly impacted by variations in water pH. Fish die at pH 4, which is an acidic state, according to [15]. At very low pH values (less than pH 3), mucus coagulation on surface of gill and consequent oxygen deficiency may be the predominant reason of death [16]. In this study we documented the adverse combined impact of Cypermethrin and acidic and basic pH in ovary function of Puntius sarana.

MATERIAL AND METHODS

Experimental Fish:

A local fish pond called Sasan, located near Taki Road in Barasat, West Bengal, India, provided the healthy freshwater *P. Sarana* (average length 17-19 cm and width 4-6 cm) (Figure 1). Fish were exposed to a potassium permanganate solution (0.5% w/v) for one minute in order to remove any skin adhesion. For duration of two weeks, the fish were acclimated to room temperature (32 ± 1.0°C) in a 500-liter rectangular glass aquarium filled with dechlorinated aerated tap water. Fish were regularly fed 3% of their body weight in commercial meal pellets during this time. Dead fish were removed as soon as possible to prevent polluting the water. Prior to the experiment, the water quality parameters were assessed using an Aquaponics water quality test kit. (Table 1)

Figure 1: *Puntius sarana* (Experiential fish)

Parameters	Range
Temperature	29-31°C
pH	7.3-7.6
Dissolved O_2	6.1- 6.7 mg/L (average)
Nitrate	0.18 mg/L (average)
Free CO_2	4.5 mg/L
Alkalinity	118-132 mg/L (as $CaCO_3$)
Total Hardness	108-116 mg/L (as $CaCO_3$)

Table 1: Water quality parameters.

Toxicant and Experimental design:
Cyper 25 was used in present study (Figure 2). Before exposure of fishes to pesticides, the water quality was examined in accordance with the APHA guidelines. LC_{50} dose of Cyper 25 for *puntius sarana* was considered as 3.65µg/L. For sub lethal test in different pH the fishes were assigned to six groups of ten specimens for each group (Table 2). For sublethal tests one tenth concentration of 96 h LC_{50} value was considered. The pH of water was regulated with the application of hydrochloric acid and sodium hydroxide. The water in the aquaria was changed every 24 hours, and the pH and various cypermethrin concentrations were brought back to normal as previously mentioned. Since the pyrethroids used were soluble in acetone, the control tank was kept at an equivalent acetone volume.

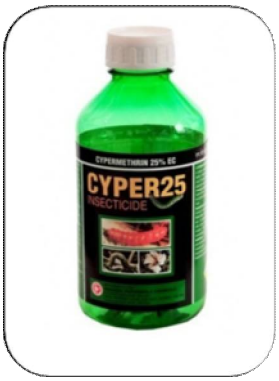

Figure 2: Toxicant used in present investigation (Cyper 25)

Table 2: Sub lethal experimental groups

	Group	Parameters
10 fishes/ group	1 (Control)	pH 7 with no Cypermethrin
	2	pH 7 + Sublethal concentration Cypermethrin
Exposure period: 15 days	3	pH 5 + Sublethal concentration Cypermethrin
	4	pH 6 + Sublethal concentration Cypermethrin
	5	pH 8.5 + Sublethal concentration Cypermethrin
	6	pH 9.5 + Sublethal concentration Cypermethrin

Oocyte staging:
After the histological preparation of ovary different stages of reproduction were calculated using earlier research with a few tweaks [17, 18]. The stages of oocytes were classified into stage I, as previtellogenic oocyte stage II, as early Vitellogenic oocyte, stage III, as mid vitellogenic oocyte, stage IV, as mature oocyte and stage V, as atretic oocyte. Each selected fish's oocyte stages were documented, and an average value of count was reported. Formula below was used to calculate each group's oocyte staging.
Number of oocytes of each stage × 100/ Total number of oocytes

Ovary volume:
To study the toxic effects of cypermethrin and variable pH on ovaries, the experiments were performed throughout the year in glass aquaria of 40 L capacity, containing tap water of pH 7 (control) and tap water with sub lethal dose of cypermethrin 25% EC in different pH (6, 5, 8.5 and 9.5). In each aquarium, a batch of five fish was housed. After the exposure period, all of the fish in the aquarium (control and treated) were netted out, dissected for the removal of ovaries, and the volume of ovaries were calculated using the water displacement method using the formula.
Initial volume of water in cylinder = A ml
Final volume of water in cylinder with ovary = B ml
Volume of ovary = B – A = C ml

Statistical analysis
Data of all experiments were represented as mean ± standard error. Apart from descriptive statistics of the outcome variables, appropriate multivariate statistic such as ONE WAY ANOVA, LSD, t-TEST was done with Minitab, version 17 Software to understand the causal relationship between parameters.

RESULT AND DISCUSSION

Oocyte staging
In the pH 5 + toxicant, pH 6 + toxicant, pH 7 + toxicant, pH 8.5 + toxicant, and pH 9.5 + toxicant treatment groups, stage I was the most prevalent oocyte stage. Stage IV oocyte was found highest in number among

all types of oocyte in control group (Table 3, Figure 3). When the fishes were subjected to sublethal dose of cypermethrin 25% EC stage I and stage V oocytes increased significantly ($p<0.05$) where as a significant ($p<0.05$) decrease in number was recorded for stage II, III and IV (Table 3, Figure 3).

For stage I oocyte the percentage of oocyte increased significantly with the increase of acidity of the medium (pH 6 + toxicant and pH 5 + toxicant). Highest value was found in pH 5 + toxicant group (Table 3). In basic medium (pH 8.5 + toxicant and pH 9.5+ toxicant) the percentage of oocytes increased compared to control group.

The percentage of stage II oocyte was found to be the minimum among the experimental groups. The percentage of stage II oocyte showed a significant decrease ($p<0.05$) in acidic medium (pH 6 + toxicant and pH 5 + toxicant) and in pH 9.5 + toxicant group. Stage II oocytes % were highest in pH 8.5 + toxicant group (10.45 ± 0.40) where a significant ($p<0.05$) increase is noticed compared to the control fish (Table 3).

Both stage III and stage IV oocyte showed similar pattern in all experimental groups. The values were decreased significantly when the fishes were subjected to the sub lethal dose of Cypermethrin 25% EC. In acidic medium and pH 9.5 + toxicant groups the values showed gradual significant decrease ($p<0.05$). However, in pH 8.5 + toxicant group the percentage of both stage III and stage IV oocytes % increased significantly ($p<0.05$) retaining almost control values (15.51 ± 0.68, 34.43 ± 0.53) respectively (Table 3, Figure 3).

In case of stage V oocyte, a gradual significant increase was noticed with the increase of acidity of the medium (pH 6 + toxicant and pH 5 + toxicant). Maximum percentage of oocyte is found in pH 5 + toxicant treatment groups. In pH 8.5 + toxicant group the oocyte percentage was recorded as (21.52 ± 0.23) that is very near to the control value.

ANOVA showed a significant ($p<0.001$) difference in oocyte percentage in different experimental groups; with an F value of 589.38 for stage I, 80.64 for stage II, 55.98 for stage II, 906.30 for stage IV and 108.11 for stage V oocyte (Table 4). In stage II, III and V oocyte percentage the mean difference with the control value was recorded least in pH 8.5 + toxicant treatment group (Table 5).

In treated fish, the percentage of stage I oocytes was found to be higher in the toxicant group as well as different pH groups as compared to the control group (Table 3) while other type of oocytes decreased, except atretic ones (stage V). Like other researchers, in present investigation these oocytes (stage V) demonstrated an upward percentage (from 23.360 ± 0.99 to 65.211 ± 0.12) [19, 20 & 21]. The vitellogenic and atretic oocytes were the most significant phases to analyse in oocyte development because both of these stages indicate the female fish's potential to reproduce [22]. The higher percentage of atretic oocytes found in this study could be due to either improper ovarian growth and development, resulting in decreased oocyte growth and inability to mature due to cypermethrin and pH toxicity, decreased supply of gonadotropin from hypophysis, or direct impact of pollution on the intra-ovarian scene [23].

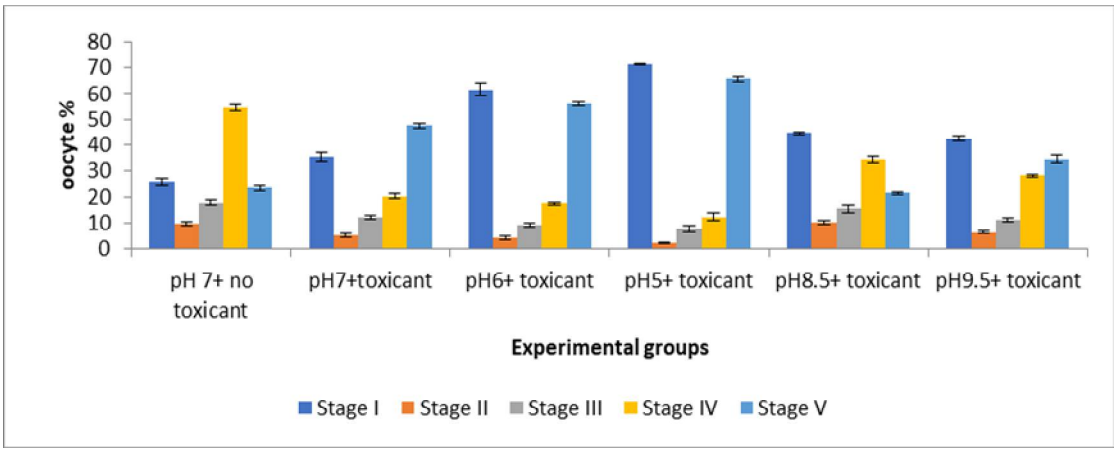

Figure 3: Percentage of different stages of oocytes of *Puntius sarana* (female) exposed to sublethal dose of cypermethrin 25% EC in different pH. Data represented as Mean ± SE.

Malathion induced a dose-dependent reduction of activity of acetyl cholinesterase in the brain of *Clarius batrachus*, resulting in a decrease in stage I and II oocytes in the reproductive cycle, according to Das & Sengupta [11]. The combined influence of the pesticide and pH was observed to have impeded development of ovary and increase in size and weight of the ovaries. In comparison to the norm, the volume and frequency % of all types of oocytes were on the decline. However, in all the situation the percentage of oocyte in pH 8.5 + toxicant group retained the normal situation as control indicating that in this pH fishes were somehow able to overcome the toxic effect of cypermethrin.

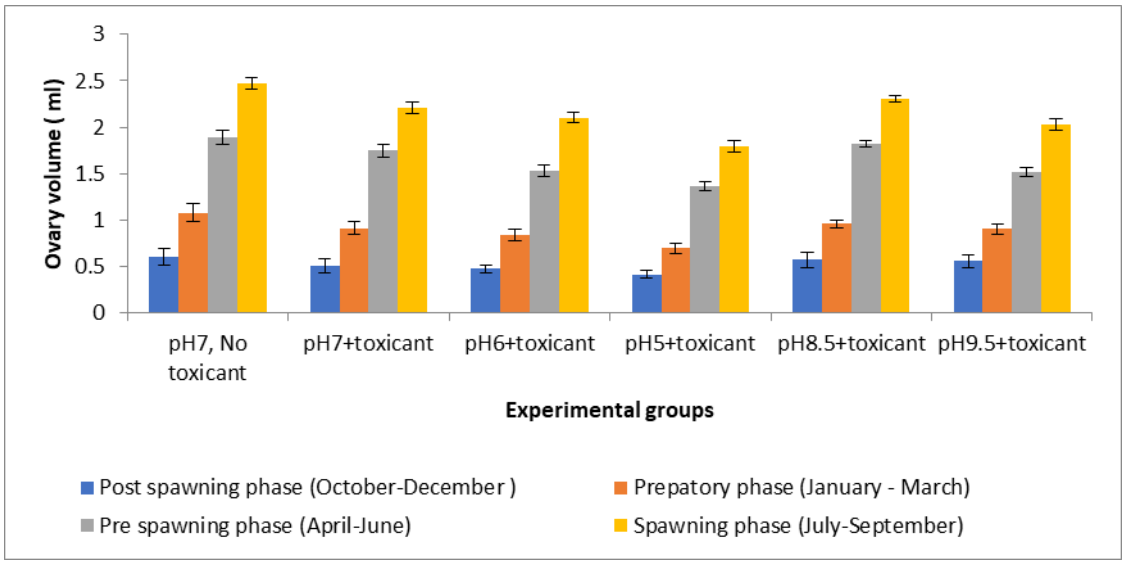

Figure 4: Volume of ovary of *Puntius sarana* (female) subjected to sublethal dose of cypermethrin 25% EC in different pH. Data represented as Mean ± SE

Table 3: Percentage of different stages of oocytes of *Puntius sarana* (female) exposed to sublethal dose of cypermethrin 25% EC in different pH, * indicates P<0.05. Each value represented as Mean ± SE.

Oocyte staging %	pH 7+ no toxicant	pH7+ toxicant	pH6+ toxicant	pH5+ toxicant	pH8.5+ toxicant	pH9.5+ toxicant
Stage I	25.8 ± 0.68	35.39 ± 0.80*	61.64 ± 1.17*	71.12 ± 0.11*	44.51 ± 0.34*	42.37 ± 0.52*
Stage II	9.76 ± 0.39	5.43 ± 0.44*	4.47 ± 0.37*	2.35 ± 0.15*	10.45 ± 0.40	6.56 ± 0.24*
Stage III	17.73 ± 0.47	12.43 ± 0.40*	9.39 ± 0.38*	7.61 ± 0.62*	15.51 ± 0.68*	11.40 ± 0.40*
Stage IV	54.42 ± 0.58	20.33 ± 0.41*	17.51 ± 0.20*	12.49 ± 0.79*	34.43 ± 0.53*	28.42 ± 0.28*
Stage V	23.36 ± 0.49	47.45 ± 0.58*	56.09 ± 0.38*	65.65 ± 0.56*	21.52 ± 0.23*	34.60 ± 0.83*

Table 4: ONE WA ANOVA table for different stages of oocytes of *Puntius sarana* (female) exposed to sublethal dose of cypermethrin 25% EC in different pH.

ANOVA						
		Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
Stage I	**Between Groups**	5631.101	5	1126.220	589.379	0.000
	Within Groups	34.395	18	1.911		
	Total	5665.497	23			
Stage II	**Between Groups**	194.840	5	38.968	80.640	0.000
	Within Groups	8.698	18	0.483		
	Total	203.538	23			
Stage III	**Between Groups**	284.305	5	56.861	55.978	0.000
	Within Groups	18.284	18	1.016		
	Total	302.590	23			
Stage IV	**Between Groups**	4594.836	5	918.967	906.298	0.000
	Within Groups	18.252	18	1.014		
	Total	4613.088	23			
Stage V	**Between Groups**	6429.068	5	1285.814	108.113	0.000
	Within Groups	21.414	18	1.190		
	Total	6450.482	23			

Table 5: Post hoc Test (LSD) Multiple Comparison table for Percentage of different stages of oocytes in *Puntius sarana* (female) exposed to sublethal dose of cypermethrin 25% EC in different pH. *, indicates P<0.05.

Dependent Variable	(I)	(J)	Mean Difference (I-J)
Stage I	Control	pH7+tox	-9.58*
		PH6+ tox	-35.83*
		pH5+tox	-45.32*
		pH8.5+tox	-18.71*
		pH9.5+ tox	-16.57*
Stage II	Control	pH7+tox	4.33*
		PH6+ tox	5.28*
		pH5+tox	7.40*
		pH8.5+tox	-0.60
		pH9.5+ tox	3.19*
Stage III	Control	pH7+tox	5.29*
		PH6+ tox	8.34*
		pH5+tox	10.11*
		pH8.5+tox	2.21*
		pH9.5+ tox	6.33*
Stage IV	Control	pH7+tox	34.09*
		PH6+ tox	36.90*
		pH5+tox	41.92*
		pH8.5+tox	19.98*
		pH9.5+ tox	25.99*
Stage V	Control	pH7+tox	-24.09*
		PH6+ tox	-32.72*
		pH5+tox	-42.29*
		pH8.5+tox	1.84*
		pH9.5+ tox	-11.23*

Table 6: Volume of ovary of *Puntius sarana* (female) subjected to sublethal dose of cypermethrin 25% EC in different pH. *, indicates P<0.05. Each value represented as Mean ± SE.

Ovarian phase	pH 7 + no toxicant	pH 7 + toxicant	pH 6 + toxicant	pH 5 + toxicant	pH 8.5 + toxicant	pH 9.5 + toxicant
Post spawning phase **(October-December)**	0.59 ± 0.04	0.50 ± 0.03*	0.46 ±0.02*	0.41 ±0.02*	0.56 ± 0.04	0.55 ± 0.03
Preparatory phase **(January- March)**	1.07 ± 0.04	0.91 ± 0.03*	0.83 ±0.03*	0.69±0.03*	0.95±0.02*	0.90 ± 0.03*
Pre spawning phase **(April-June)**	1.89 ± 0.03	1.75 ± 0.03*	1.53 ±0.03*	1.36 ±0.02*	1.82 ± 0.02	1.52 ± 0.02*
Spawning phase **(July-September)**	2.47 ± 0.03	2.21 ± 0.03*	2.10 ±0.03*	1.79 ±0.03*	2.30 ±0.02*	2.03 ± 0.03*

Table 7: ONE WAY AVOVA TABLE for ovary volume of *Puntius sarana* (female) subjected to sublethal dose of cypermethrin 25% EC in different pH.

ANOVA						
		Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
Post spawning phase	**Between Groups**	0.122	5	0.024	4.933	0.003
	Within Groups	0.119	24	0.005		
	Total	0.241	29			
Preparatory phase	**Between Groups**	0.402	5	0.080	17.807	0.000
	Within Groups	0.108	24	0.005		
	Total	0.511	29			
Pre spawning phase	**Between Groups**	1.063	5	0.213	63.513	0.000
	Within Groups	0.080	24	0.003		
	Total	1.144	29			
Spawning phase	**Between Groups**	1.378	5	0.276	81.549	0.000
	Within Groups	0.081	24	0.003		
	Total	1.459	29			

Table 8: Post hoc Test (LSD) Multiple Comparison table for volume of ovary in different phases of spawning of female *Puntius sarana* exposed to Sublethal dose of cypermethrin 25% EC in different pH. *, indicates P<0.05.

Dependent Variable	(I)	(J)	Mean Difference (I-J)
Post spawning phase	Control	pH7+tox	0.09*
		PH6+ tox	0.13*
		pH5+tox	0.18*
		pH8.5+tox	0.03
		pH9.5+ tox	0.04
Preparatory phase	Control	pH7+tox	0.16*
		PH6+ tox	0.23*
		pH5+tox	0.38*
		pH8.5+tox	0.12*
		pH9.5+ tox	0.17*
Pre spawning phase	Control	pH7+tox	0.14*
		PH6+ tox	0.36*
		pH5+tox	0.53*
		pH8.5+tox	0.06
		pH9.5+ tox	0.36*
Spawning phase	Control	pH7+tox	0.26*
		PH6+ tox	0.37*
		pH5+tox	0.68*
		pH8.5+tox	0.17*
		pH9.5+ tox	0.44*

Ovary volume

From October to September, i.e. during the post-spawning to spawning period, the volume of the ovary in the control group grew dramatically (0.590 ± 0.04 ml to 2.470 ± 0.03 ml) (Table 20). When the fishes were subjected to the sublethal dose of toxicant a significant ($p<0.05$) decrease of ovary volume was seen in all the four phases from post spawning to spawning (Table 6, Figure 4).

During post-spawning period (October-December) the volume of the ovaries decreased progressively with the increase of acidity of the medium. Lowest ovary volume was found in pH 5 + toxicant experiment group, however in basic medium (pH 8.5 and 9.5) no significant difference was noticed with the control value. (Table 6, Figure 4).

During preparatory period (January-March) the volume of the ovaries remained between 1.07 ± 0.04 ml to 0.69 ± 0.03 ml range. A gradual significant ($p<0.05$) decrease in the volume of ovary was recorded with the increase of acidity in the medium. Ovary volume decreased also in basic medium (Table 6, Figure 4).

In pre spawning period (April-June) ovary volume ranged between 1.89 ± 0.03 ml to 1.36 ± 0.02 ml. The lowest volume was recorded in pH 5 + toxicant medium. A significant ($p< 0.05$) gradual decrease in the volume of ovary was recorded in pH 6 + toxicant, pH 5 + toxicant group and pH 9.5 + toxicant group. In pH 8.5 + toxicant group volume of ovary almost retained the volume as control without any significant change (Table 6, Figure 4).

During spawning period (July- September) ovary volume ranged between 2.47 ± 0.03 ml - 1.79 ± 0.03 ml. The lowest volume was recorded in pH 5 + toxicant medium. A gradual significant decrease in the volume of ovary was also recorded in pH 6, pH 5 and pH 9.5 groups in spawning phase. In pH 8.5 + toxicant groups the difference with the control was found to be significant but it was very closer to the control value (Table 6, Figure 4).

One way ANOVA demonstrated a significant ($p<0.003$ in post spawning phase and $p<0.001$ in other three phases) difference in ovary volume in different experimental groups in each of the four ovarian phases (Table 7). LSD showed the mean difference in the values of ovary volume was least in pH 8.5 + toxicant group compared to the control in all the four phases (Table 8).

CONCLUSION

Decrease of volume of ovary due to toxicity stress was found to be very common in fishes. In our investigation it was evident that the toxicity stress adversely damages the histoarchitecture of fish ovary leading to the necrosis of cells. This may be one of the causes of ovary volume decrease as evident in the present investigation. Decrease in reproductive hormone level may also be a cause of decrease in ovary

volume. Decrease of ovary volume due to stress was reported by several workers. So the findings of our study are in good agreement with these previous findings.

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