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Medroxyprogesterone Acetate Drug Induced Reproductive Changes in Female Mosquito Fish *Gambusia Affinis*

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Abstract:
Medroxyprogesterone acetate (MPA) is an hormone analogue of progesterone that is usually used in combination with estrogen content in restarting the menstrual cycles that has been abruptly stopped, this will be done as a part of hormone displacement therapy. The presence of this compound have evidently been found in the aquatic ecosystems. The effect of this compound was found out on the freshwater mosquito fish viz *Gambusia affinis.* The commercially available pill containing MPA is Modus-10 which was used to test upon the fish. Fresh stock of the test fish were procured from the ponds available in Karnataka State Akkamahadevi Women University and as well as from the Fisheries Research and Information Centre, Bhutnal, Vijayapura. The fishes were reared in laboratory conditions for acclimatization before starting the experiment, the fishes were maintained in 3 groups of 6 individuals each (n=6), 3 males and 3 females. The fishes were maintained in static-renewal toxicity system. One group served as a control while other two groups were treated with two different doses of the drug 6mg and 12mg per aquarium per day. They were fed with commercial fish feed at 0.5gm per day. The female fishes were dissected on 29th day and it was found that the drug treatment had affected their ovaries morphologically and physiologically. It was found out in the treated group of fishes that the eggs had reduced yolk granules, reduced number of eggs, larval mortality, over-ripen eggs, and the rate of fertilization was also affected. Further the histopathological studies of the treated fish ovaries revealed remarkable changes in the structure of the ova when compared with the structure of the ovary from the control group. The pre-vitellogenic follicles and vitellogenic oocytes were also malformed. The stress caused due to the drug exposure might be attributed to the changes in the ovaries. The ovaries from higher dosage exposure group(12mg) were contrarily found to be less affected when compared to lower dose, this can be related to the findings by Schreck *et. al* 2010, that the stressor molecules when present in higher concentration makes the fish to show coping type of responses so the histological details show less affect of the drug to the ovary but the ova which were found structurally appeared normal but no fertilized eggs were found, so the reproductive aspects are somehow affected. So the results found here substantiates the economic use of these pills and responsible disposure of the left outs so that the non-target animals aren't affected by this.
Keywords: *Gambusia affinis*, Medroxyprogesterone acetate, histopathology, reproduction.

Introduction:

Medroxyprogesterone is used to treat amenorrhea, abnormal uterine bleeding and it is also used to prevent endometrial hyperplasia (thickening of the lining of the uterus or womb) in women, who are in taking conjugated oestrogen. Progesterone is a hormone that is important for the regulations of ovulation and mensuration, progesterone is also produced in high amounts in female by the ovary, and it is also produced in smaller amounts by the adrenal gland both in males and females. Changes of developmental and reproductive parameters can be a major drivers of alternations in population growth (Gleason and Nacci, 2001; Newman, 2001; Grist et al., 2003; Gurney, 2006).The presence of natural and synthetic steroid hormones in the environment and their adverse effect in wild animals, especially the disruption of sex hormone signalling pathways and reproduction in fish and amphibians (Christian et al., 2010).

Growth, gonadal development and reproductive in fish are influenced by many factors such as feed composition, quality and quantity are among the most important (Sampath and Pandian1984; James et al., 1993; Jobling 1998). Reproductive peculiarities that make fish particularly vulnerable to toxic, impact on oviparity and external

fertilization. Vitellogenesis, a process associated with oviparity, has been found to be highly responsive to toxicants (Fair brother et al., 1999).

In addition, there is a growing concern in hormones and compounds with some similar activities as special oestrogen, progesterone and glucocorticoids could also possess risk to many aquatic organism. Effect of assessment of environmental steroid hormones in vertebrates is limited to the hypothalamic-pituitary-gonadal access, related to specific endocrine end points such as fecundity and hormone levels (Sumter and Johnson.2005). In aquatic organisms, there is a decrease in fecundity and also results in adverse effect on reproduction, steroid hormone imbalance, feminization or muscularizations, altered sexual behaviour, and also changes in gonadal histology and alteration of sex development have been reported upon various environmental steroid hormone exposure (Jobling et al., 2013).

The main side effect of progesterone is unusual vaginal bleeding and causes pain or burning when urinate in women, breast lump, vision problems, headache, diarrhoea, sleep problem are some common side effects. Although the result of core contract studies and pre-clinical animal test has suggested several foetal effect attributed to medroxyprogesterone acetate (Mines, D; Tennis*, et al.,* 2014). A previous study reported that Gambusia fishes should comfortable response with model during GABA depleted analysis on many different fertility drugs including medroxyprogesterone (Runnells, *et al.,* 2013).

Fig 1: A pair of male and female *Gambusia affinis*

Materials and Methods:

Fish Collection:

Healthy and active *G. affinis* adults were procured from the Fisheries research and Information Centre, Bhutnal and also collected from KSAWU Campus Vijayapura, the fish were brought to the laboratory in large plastic covers. They were acclimatized to the laboratory condition for 14 days prior to the commencement of experiment. Fishes were kept in fresh water large plastic tub (volume -20L), and there was continuous supply of oxygen through the aerator to the plastic tubs, there was continuously supply of aerator for all plastic tubs, and on daily basis the water was renewed. Changing of water for aquarium is necessary, the goal of changing water is to keep the fish healthy. Changing of fish water regularly is important because even if your tank water looks clear particles of food and waste are still present. Not only has it caused a physical build-up, accumulation of pollutants such as nitrate and phosphate. Aeration is the processing of adding oxygen to the water. Fishes were fed with Commercial dry pellets (Optimum food for aquarium fish). Pellets were of small size as fish can feed on it very easily, food was given on daily basis morning and evening time, but feeding was stopped 1 day prior to expose the fishes for the experiment.

Fig 2: Fish collection at Fisheries Research Centre, Bhutnal, Vijayapura

Fig 3: Fish Collection site in KSAWUV campus

Dosage preparation:

Before starting the experiment one day prior to exposure dosage was prepared. Medroxyprogesterone Acetate tablet of 10 mg was grinded with mortar and pester up to small crystals of the drug. And crystals were weighed with the help of weighing machine. The weighed drug was packed in paper by naming one as 6mg and other as 12 mg. And the doses were prepared for 28 days.

Progesterone treatment:

The study stet up included three groups, n = 6 [9 Male & 9 female fishes totally in three groups] in each tubs. Each group had 3 male and 3 female fishes. The first group of fishes were served as an initial control. The 6 fishes were kept in 8L water containing plastic tubs (tub - A) and these tub of fishes were exposed to 6mg concentration of Medroxyprogesterone acetate (micro labs) drug where another 6 fishes were kept in 8Lwater containing plastic tub (tub-B), fishes were exposed to 12mg concentration of MPA drug/day for a for a period of 28 days. The powdered drug was directly put in the rearing tubs. The fishes in all the three tubs were fed with commercial fish feed pellets and a constant amount of feed 0.50g per day was supplied. And there was continuous supply of air with aerator to all the plastic tubs. On daily basis the water was changed regularly for 28 days, to avoid the accumulation of pollutants and physical build-up.

Dissection:

The fishes exposed to MPA were sacrificed on 29th day. Before dissection, the treated fishes of both concentration 6mg, 12mg and control group fishes were sedated by chloroform to make them unconscious. The control and MPA drug treated group of fishes were compared to see the changes as a result of the exposure. The number of eggs seen in the ovary, morphology of the eggs or embryo if any were considered for observation.

Further to study the changes occurring at cellular or tissue level the histopathology studies related to the ovaries were undertaken. The fishes were sacrificed on 29th day of the exposure by making them sedated with chloroform to study and compare the changes among MPA treated and control group of fishes. Ovary was separated from the fish & was collected in small Eppendorf tubes filled with formalin (10%) and were outsourced to Dr. Karigoudar Diagnostic Laboratory, Vijayapura for sectioning and staining (with Haematoxylin and Eosin) from control and MPA treated fish group.

Results:

Acute toxicity of medroxyprogesterone acetate on the *G affinis* showed reproductive and developmental deformities in mosquito fish (*G affinis*). From many studies we came to know that Progesterone is highly toxic to fish. Mortality was also observed during the experimental period. During this study conducted the results are as follows.

Reproductive and developmental changes in treated fish:

First study was done to assess the reproductive and developmental changes of fish with two concentrations of medroxyprogesterone drug. The result of all the article which were studied reveals that, an acute toxicity of drug MPA showed effect on reproduction and development in mosquito fish *(Gambusia affinis).*

In the present study the control fish behaved normally. There was no variation in the controlled fish; therefore, the results of these non-exposure series were taken as standards for whole test periods. MPA is involved in the regulation of reproduction in the mosquito fish (G. affinis). The treatment of MPA for 28 days has been shown to suppress reproduction and developmental behaviour at concentration 6 mg and 12mg / lit in the aquarium.

Reproductive and developmental deformities:

1. Vitellogenesis
2. larval mortality
3. Reduced number of eggs
4. Over ripe eggs
5. Low fertilization rate
6. Reduced hatchability

Reduced vitellogenesis:

As we know oestrogen induces the vitellogenesis in fishes. Vitellogenesis is the process through which maturing oocytes in the ovary accumulate egg yolk. VTG is synthesized in the female liver as the precursor of egg yolk. In the present study the Vitellogenesis process in the MPA drug exposed fishes was shown to be affected by the drug exposure when compared with the control group of fish. It was observed in the exposed group of fishes that the yolk granules in the egg cytoplasm were considerably reduced compared to the control fishes' eggs which had heavily yolk laden cytoplasm. The results are as shown in Figure 4. This further shows the effect of the drug on the hepatocytes which plays an important role in vitellogenesis and the same should be further investigated (Carl B. Schreck *et. al* 2010).

Larval malformation*:*

Progesterone is also involved in the production of higher number of eggs but, due to the effect of drug the eggs were undeveloped (Shashikant Sitre *et. al* 2014). Only 50% of eggs were developed when compared with control group fishes' as shown in Figure 5

Reduced number of eggs:

Normally the mosquito fish produces 20-25 eggs in a month. While dissecting, we observed that MPA treated fishes were containing only 10-12 eggs. The progesterone has affected the number of eggs normally produced. As founded out by Carl B. Schreck *et., al* 2010, higher concentrations of the progesterone results in reduction in number of eggs, the results found here shows relevancy with the earlier findings.

Over-ripen eggs:

Over-ripening of ovulated eggs takes place inside the lumen of the ovary and has been recognized as a possible cause of poor egg quality in fishes. If spawning or spontaneous release of the eggs does not take place, they may become over-ripe and hard and in many cases they may also remain in the ovary. These over-ripe eggs lost for reproduction and also blocks the spawning (Chrysoula Roufidou *et. al* 2016). In the treated group of fishes it was observed that eggs were retained in the ovary and weren't ovulated, the same wasn't observed in the control group fish's ovary where either the eggs were in follicular phase or if developed completely were ovulated. The result is as shown Figure 6.

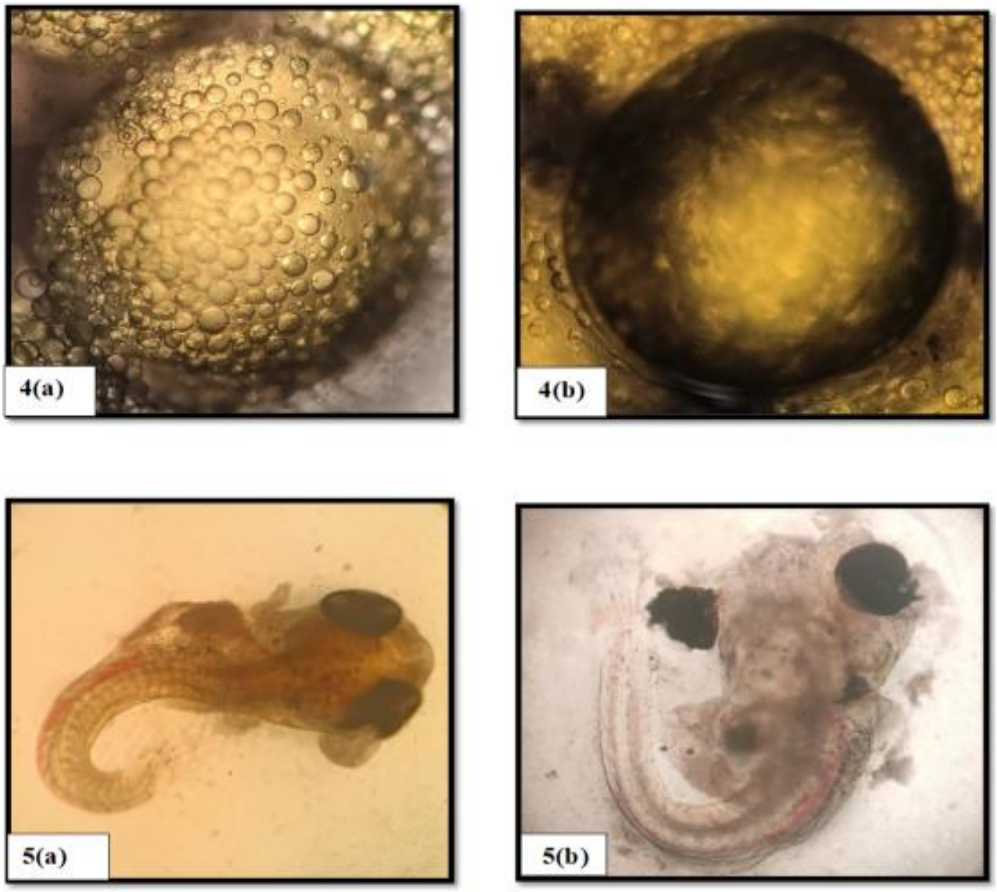

Figure (4-5): Showing Reproductive and developmental deformities in G. affinis exposed to MPA drug.

Fig 4 : (a) showing normal vitellogenesis; yolk granules and (b) showing r educed vitellogenesis (12mg concentration of MPA drug).

Fig 5: (a) showing normal larvae and (b) showing larval malformation (6mg concentration of MPA drug).

Low fertilization rate:

During the experiment, we observed the low- fertilization rates in treated fishes when compared with control group of fishes. Generally mosquito fish fertilization is internal. As low fertilization is caused due to lack of nutrition, feeding, physical and chemical parameters of the water, genetics, and age of the brood stock and characteristics of the gametes, especially related to aging (over-ripeness) associated with the period of residence in the reproductive tract or the period between spawning. And also have an impact on the survivability of embryos or larvae (Ioanna Katsiadaki *et.,al* 2016) (Monika Schmitz *et.,al* 2016). The number of fertilized eggs or the number of young ones given birth were around 15-18 in control group and were reduced to less than 10 in the treated groups.

Reduced hatchability:

Hatching is the condition which larvae emerges from the fertilized egg, female Gambusia fish produces eggs that hatch within their bodies. On average mosquito fish will produce 120 eggs and young one are released in broods of 25-30 at a time in a four year span. Most common reason for the reduced hatchability are early embryonic death, egg rots, broken yolk and hatchery malfunctions. Poor results in hatching are commonly caused by the poor or improper potential nutrition, improper control of temperature or humidity (Arshi Mustafa *et. al* 2017). When the temperature or humidity is too high or too low for a longer period of time, the normal growth and development

of the embryo is affected. Due to the effect of MPA drug it also resulted in reduced number of hatches in progesterone treated fishes. as seen in Figure 7.

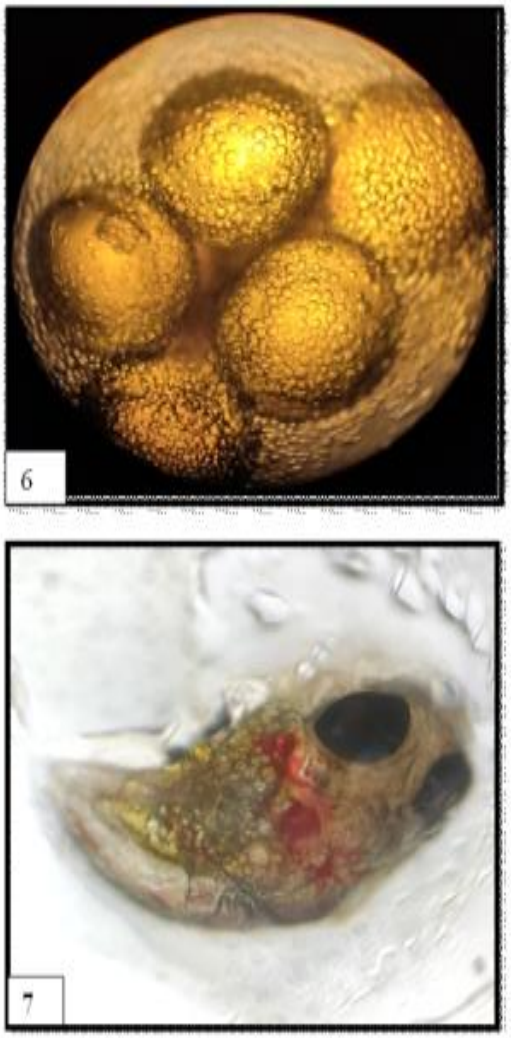

Figure (6 -7): Showing Reproductive and developmental deformities in G. affinis exposed to MPA drug

Fig 6 - showing over ripen eggs (12mg concentration of MPA drug).

Fig 7 - showing reduced hatchability(12mg concentration of MPA drug).

Histopathology of ovary:

Control fish ovary:

- *G affinis* reveals that it is surrounded by an ovarian wall which is differentiated in to an outer thin peritoneum, a thicker tunica albuginea made up of connective tissue, muscle fibre and blood capillaries the inner most layer is the germinal epithelium which joins with the albuginea at several places and projects in to the central lumen, the ovacoel, in the form of finger like projection called ovigerous lamellae.
- Histological sections of ovaries were visualized microscopically and analysed to classify the stages of oogenesis (Jackson et al., 2019). The stages of oogenesis in female mosquito fish was categorise as primary

oocyte, maturing cortical and /or early vitellogenic oocytes , or mature ovum based on the classification by Leusch et al., (2006). All ovaries were scored non-affected. (Plate I-C)

Treated Fish:

Treated Fish ovary (6mg concentration of MPA drug):

During our study, fishes were exposed to 28 days with the concentrations of 6mg & 12mg MPA drug, to observe the reproductive and developmental deformities. We observed the morphological changes in the ovary and abnormal shape of ova. And also this resulted in elongation of ovarian follicles. Clumping of cytoplasm and degeneration in the follicular cells was also observed. And also resulted in restraint of oocyte maturation. The result is said on the basis of the findings by Carl B. Schreck *et. al* 2010.

And also fish's social environment can also affect the reproduction through interactions with the endocrine stress response. However the degenerative effect of liver can be predicted as the possible reason behind atresia of vitellogenic follicles of ovary. Low levels of stress may have a positive effect on reproductive processes while greater stress has negative effects on fish reproduction (Sana Ullah *et., al* 2014) (Plate I-A).

Treated fish ovary (12mg concentration of MPA drug):

We observed the degeneration of pre-vitellogenic and vitellogenic oocytes. It also gave clarity about oocyte atresia. Many research outcomes shows that fishes that undergoes long time exposure to drug becomes more stressed, as stress response consists of a physiological cascade of events that is initiated by perception of a stressor and communicated to the body via the central nervous system acting both neuronally and hormonally. It was surprisingly to see that due to stress the affected ovary of fish had recovered as stress affects primarily on hormones of the hypothalamic–hypophyseal–internal axis and possible interaction with those of the hypothalamic–hypophyseal–gonadal axis. Due to stress the affected ovary of fish had recovered in this group, we observed coping-types of responses in the fish that can resist the stressor for some period of time and have energy available, called allostatic overload, stress can affect reproduction in various ways, depending on when in the life cycle it is experienced and the severity and duration of the stressor (Carl B. Schreck *et. al* 2010). It can accelerate ovulation or inhibit reproduction. Constraints involving mate choice can also result in greater number of gametes to compensate for poor-quality of gametes (Gowaty et al., 2007). So it was observed that the fishes exposed to high doses of the drug for longer period of time had coped up the stress and recovered in a good way but compensation to this there were no fertilized eggs in the reproductive tract of the fishes. It can be stated from this that the fishes while coping up with the stress had managed to recover or retain their gamate structure and development but it had severely affected their rate of fertilization and hence a delayed effect of the stress by the drug exposure has been observed. (Plate I-B). Some drugs are known as Endocrine disrupting chemicals (EDC) which can interfere with normal functioning of endocrine system in fish. Adverse effects of MPA on the Hypothalamus-pituitary-gonad axis results in reproductive failures in fish.

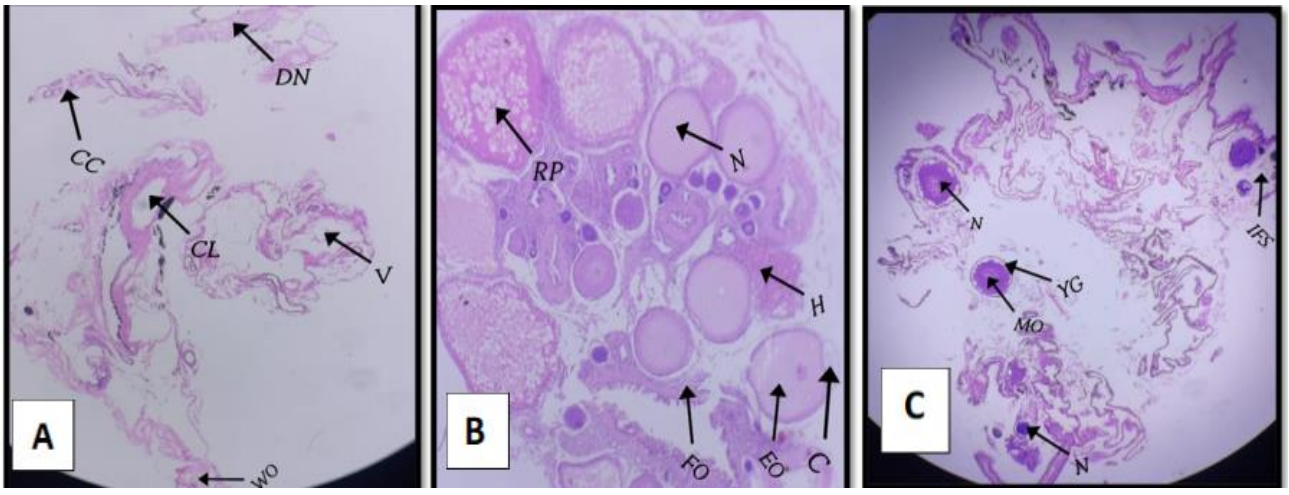

Plate I. A tissue is of 6mg MPA drug exposed tissue; B tissue is of 12mg MPA drug exposed tissue; C represent the control tissue of ovary with normal structure.

Exposed Tissue A
Morphological changes of oocyte[c]
Elongated ovarian follicle [EO]
Fragmented ova [FO]
Degenerated nucleus [N]
Oocyte rupture [RP]

Control C
Control tissue normal nucleus[N]
Normal yolk granules [YG]
Normal mature oocyte [MO]
Inter follicle space [IFS]
Cytoplasm [N]

Exposed Tissue B
Degeneration of nucleus [DN]
Cytoplasmic clumping [CC]
Cytoplasmic liquefaction [CL]
Vacuolation [V]
Wrinkled oocyte [WO]

Discussion:
In the present study, the Medroxyprogesterone Acetate drug shows the acute effects on fish reproduction and development. During the study group of fishes were exposed to MPA for three weeks and this showed the acute effects. The changes include embryo deformities, exposed fishes resulted in abnormal development of embryo such as only head part was developed while compared with control fishes. Vitellogenesis was reduced in exposed fishes when compared with control fishes. And also there was larval malformation by comparing with control fishes we come to know that, physical structure of fish was not in proper way. Due to the effect of drug, the eggs were over-ripened and in control fish eggs were ripened. And also resulted in reduced hatchability in exposed fishes compared to control group. This is evident from the present study that there are reproductive and developmental deformities in fish exposed to concentrations of Medroxyprogesterone Acetate drug.
Histological abnormalities in ovaries may be caused by several factors, VIZ.., ionizing radiations, electric current, parasitic infections, mechanical injuries and by a variety of drugs and aquatic pollutants. Almost similar histopathological findings were reported in the ovaries of *Gambusia affinis*. After the exposure of fishes to 6mg concentration of Medroxyprogesterone Acetate drug, it resulted in the disruption of ovary, abnormal shape of ova. Due to the effect of drug ovary of cytoplasm was clumped where in control ovary cytoplasm was normal. And also there was elongation of ovarian follicles.
And also observed the cells assimilation of yolk granules during the exposure and also resulted in the arrest of vitellogenesis and this caused the reduction in size of oocytes. Fishes exposed to higher concentration of Medroxyprogesterone acetate drug i.e. 12mg this also resulted in the histopathological changes. There was degeneration of pre-vitellogenic and vitellogenic oocytes in the ovary of exposed fish. These fishes were under the stress and showed coping type of responses ad showed recovery in their ovaries. Endocrine disrupting chemicals (EDC) such as Medroxyprogesterone Acetate this may interfere with normal functioning of endocrine system in fish. Adverse effects of MPA in the Hypothalamus-pituitary- gonad axis also resulted in the reproductive failures in fish.

Conclusion:
From the experiment, the result here shows that Medroxyprogesterone Acetate is toxic to Mosquito fish and it affects reproductive and developmental deformities in *Gambusia affinis*. This work shows that fertility drugs of Modus-10 are capable of blocking the action of hormones in fish and causing reproductive dysfunction and abnormal development.
As Mosquito fish (Gambusia affinis) are indispensable to modern mosquito control programs and are provided free of charge. These primarily feeds upon aquatic insects and prefers mosquito larvae. These fishes are referred as natural predators in the environment. Hence, on dumping medroxyprogesterone acetate to water bodies it affects the health of aquatic animals as Mosquito fish. These fishes feed on mosquito larva and controls the growth of mosquito population and also avoids the spread of many diseases such as Malaria, Dengue, Yellow fever etc. If the fishes intake medroxyprogesterone acetate, the fish undergo several reproductive changes as well as developmental deformities, from this there will be tremendously increase in mosquito population and also results

in spreading of many diseases. We conclude that, their fecundity and reproduction is potential and this will be affected by the stress the fishes face under such exposures apart from the natural stress. Such kinds of studies are useful for protecting the biota in natural environment.

Conflict of interest:
None

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Nil

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