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Induced spawning of African catfish (*Clarias gariepinus* Burchell, 1822) after pre-spawning prophylactic disinfection; the breeding performance and tissue histopathological alterations are under scope

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Abstract

Adult African catfish (*Clarias gariepinus*) were used to investigate the effects of pre-spawning prophylactic disinfection during a seven days quarantine period on the breeding performance and the possible histopathological influences on *C. gariepinus* brooders. Based on broodfish sex; a total of 36 females were stocked into 6 tanks (6 females tank^{-1}) and 18 males were stoked into other 6 tanks (3 males/tank) signifying three treated groups (T_1: T_3) of two replicates (6 females+3 males/replicate). T_1 was assigned as a control non-treated broodfish, whereas T_2 broodfish was disinfected with formalin (FA) (15 ppm) for 6 h and T_3 broodfish were disinfected with malachite green (MG) (1 ppm) for 45 minutes. Brooders treated with MG (T_3) delivered lower egg numbers, lower hatching percent and high percentage of larval deformity. Additionally, pronounced histopathological lesions were observed in the liver, testis and ovaries of both the FA and MG- treated catfish that is to give a further explanation for the declined breeding performance of both groups. Herein, the pre-spawning disinfection of *C. gariepinus* during the quarantine period is not necessary especially when proper management, transportation and handling is to be followed. Nevertheless, if pre-spawning disinfection to be applied; the MG should not be prescribed for *C. gariepinus* broodfish unless no other cheap and much safer disinfectants rather than FA existed. But still, a future research is required to explore its possible negative effects on both reproductive efficiency and the prospected histopathological changes of broodfish being recovered from FA application.

Keywords: Induced spawning, *Clarias gariepinus*, Pre-spawning quarantine, Prophylactic disinfection, Spawning performance, Histopathology

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Introduction

Egypt has assembled the biggest aquaculture industry in Africa, providing over 90 million Egyptian citizens with an inexpensive protein. Among many fish and crustacean species cultured in Egypt, the African catfish, *Clarias gariepinus* has received little attention accounting only for 2.5% of the total fish production (GAFRD, 2010), and is being used mainly to control the over-breeding of tilapia in fish ponds. Recently, fish farmers have realized that catfish has many advantages such as high growth rate, acceptable taste, low feed requirements, good market price and resistance to diseases. Of course, this will change the image of catfish from just an undesirable species in tilapia ponds to an important and potential species for Egyptian aquaculture. However, obtaining enormous number of superior hatch is a major quest by fish farmers which is hindered by the scarcity of natural spawning in captivity, diseases (that might affect the different stages of the production cycle) and numerous other factors (Mylonas *et al.,* 2010; Duncan *et al.,* 2013; Mylonas *et al.*, 2016). Spawning induction of *C. gariepinus* has gained a significant worldwide attention by many authors (Kouřil *et al.,* 1992; Inyang and Hettiarchichi, 1994; Brzuska *et al.*, 1999; Brzuska, 2004) and recently in Egypt by (Shourbela, 2013; El-Hawarry *et al.*, 2016).

On the same context, the fungal infection of different aquaculture species including catfish eggs particularly, Saprolegnia and parasitic infestation (which cause egg mortality and reduces hatchability of viable fertilized fish eggs, leading to a huge loss to aquaculture industry) have also been extensively investigated. Therefore, several chemotherapeutics have been examined and used as curative agents for external ciliated protozoan parasites in food fishes (Athanassopoulou *et al.,* 2009; Picón-Camacho *et al.,* 2012) Formalin (FA), malachite green (MG) and sodium chloride are very good examples of the therapeutants applied in aquaculture.

Formalin is an extensively used therapeutant for controlling fungal infection in aquaculture; both in hatcheries and in ponds. It is also highly efficacious in controlling the hatcheries fungal infection of fish and fish eggs (Gieseker *et al.,* 2006; Pedersen *et al.,* 2010; Yisa *et al.,* 2014) both as a prophylaxis as well as a treatment. Similarly, due to its anti-parasitic and anti-microbial properties malachite green (MG) has been used as early as the 1930s to combat ectoparasites and to control fungal infection of fish eggs, juveniles and adult fish (Zahn and Braunbeck, 1995; Diggles, 2001; Jokinen, 2015; Sharifpour *et al.,* 2016).

The earthen pond system, on the other hand, is the dominating culture system in Egypt. It favors the fungal and parasite growth due to overcrowding and unfavorable water quality parameters. Nevertheless, most hatcheries depend on broodfish captured from ponds as the main source of breeders, a practice that might play a

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significant role in infestation of the hatchery. Additionally, the situation will be exaggerated with the increasing possibility of having bruised or injured broodfish with decreased immune status as a direct result of the stress imposed by transportation and handling (Ellsaesser and clem, 1986; Barton, 2011; Shourbela *et al.,* 2016). Bruised broodstock are much liable to bacterial, fungal and parasitic infestation which in turn negatively affect their reproductive performance (Adebayo, 2006).

Accordingly, a wide variety of pre-spawning disinfection schemes was recommended with a sequence of medicated baths including; formalin and/or malachite green and furanic antibiotics not only to protect the broodstock but also to prevent vertical transmission of diseases (Nagaraj *et al.,* 2000; Aftabuddin *et al.,* 2009). The recommended prophylactic doses are quite variable and to be determined by the type of the therapeutants used, time of exposure, fish species, life stage and water temperature (Aftabuddin *et al.,* 2009). Similarly, such practice of prophylactic disinfection was routinely applied for marine fish broodstock in pre-spawning quarantine tanks; a combination of 100-ppm MG and 100-ppm FA was used to disinfect Mud crab *(Scylla serrate)* against the bacteria and fungi by bathing them overnight in an aerated seawater bath (Davis *et al.,* 2004). Likewise, Gopakumar *et al* (2013) appreciated the application of 200 ppm formalin and 0.2 ppm malachite green mixture for 1-2 minutes as a prophylactic disinfection of marine fish broodstock.

However, very few reports were published on the impact of these chemical compounds on the breeding response of the African catfish and their consequent effects on the produced larvae. Earlier studies used FA or MG and even other disinfectants on only certain stages of the catfish production cycle, and despite that Adeyemo *et al.* (2011, 2012), tested the detrimental effects of FA and MG as disinfectants for *C. gariepinus* broodfish, they did not, however, address a clear report about their effects on the reproductive performance of the experimental broodfish and its relation to the histopathological lesions obtained in their studies. Nevertheless, the malachite-green application was banned in most European countries (from 1 October 2001); it was incorporated in this investigation owing to its efficacy and traditional use as a bath treatment against ectoparasitic ciliates and fungal infections. Also, despite being banned, it is still widely applied for routine disinfection of pre-spawning and quarantined broodstock fish both in Africa and Asia.

Therefore; the current research work was assigned to speculate the effects of pre-spawning prophylactic disinfection during a seven days quarantine period on the breeding performance and the possible histopathological influences on *C. gariepinus* brooders.

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Materials and methods

Broodstock selection

Healthy adult African catfish (*C. gariepinus*) were obtained from a private fish farm during the breeding season (May-August, 2015), and transferred to the hatchery unit in our laboratory. For proper selection of the female broodfish, a catheter was used to get a small egg sample from their ovaries. The egg diameter of more than 90 % of the ripe ovaries were bigger than 900 µm when examined under a calibrated ocular micrometer.

Broodstock management

The selected broodfish were allocated to 12 circular plastic tanks (200 L tank^{-1}). Based on their sex; a total of 36 females were stocked into 6 tanks (6 females tank^{-1}) and 18 males were stocked into the other 6 tanks (3 males tank^{-1}) signifying three treated groups (T_1: T_3; 12 females + 6 males treatment^{-1}) of two replicates (6 females + 3 males replicate^{-1}). T_1 was assigned as the control non-treated broodfish and stocked directly in their broodstock tanks, whereas T_2 broodfish was disinfected with formaldehyde (15 ppm) for 6 h (Janssen, 1987) and T_3 broodfish were disinfected with malachite green (1 ppm) for 45 minutes (Aftabuddin *et al.,* 2009), before being stocked into their corresponding tanks. Experimental broodfish were then disinfected every other day for another couple of times. Tanks were supplied with aerated water, and those tanks containing female brooders were not fed for five days as an acclimatization period. The water quality of the experimental tanks was maintained through a complete daily water exchange (temp; 27±0.52 °C, pH; 8.07±0.36, and DO; 6.04±0.16 mg L^{-1}) under natural photoperiod.

Hormones and chemicals

The following materials were used in this experiment;

- Carp pituitary extract (CPE); 4 mg carp pituitary in one ml physiological saline, prepared as described by Brzuska (2004).
- Formalin; El-Gomhouria Company for Trading Chemicals and Medical Appliances, Alex., Egypt.
- Malachite green; Tiba comp for hatchery facilities, Kafr El Sheikh, Egypt.

Hormone injection

For accurate calculation of the proper hormonal dose, the experimental broodfishes were weighted before hormonal injection. Each broodfish (629.58±129.84) received intramuscular injection of prepared hormone (4 mg kg^{-1} b.w.) above the lateral line in the evening between 7 pm and 7.30 pm (Nwokoye *et al.,* 2007) and returned to its respective aerated tank for further investigations.

Ovulation and fertilization

Female broodfishes induced to spawn were checked for ovulation 10 h after injection (Brzuska, 2004). Gentle hand stripping and squeezing of the abdomen towards the genital opening was

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necessary to check for successful ovulation (Richter *et al.,* 1987). Successfully ovulated broodfish was considered ovulated upon yielding an adequate amount of green brown eggs. Three subsamples of eggs (one gram each) were taken from each female, and fixed in 5 % formalin for subsequent calculation of egg numbers (Phelps *et al.,* 2007). The eggs from each broodfish were collected into a clean and dry plastic bowl which was labeled according to its respective treated group.

Testes of the sacrificed male broodfishes were removed immediately before female stripping and squashed in physiological saline solution (one g testis per 5ml 0.9 % NaCl solution). The milt suspension was coarsely filtered and kept under 5^{o}C (Phelps *et al.,* 2007). The milt taken from three males of each treated group was added and mixed with the egg masses of the corresponding treated female group. The eggs and sperm mixture was stirred gently with a feather while clean freshwater was added in small amounts in order to activate sperms and fertilization of eggs. The mixture was then allowed to stand for two minutes for complete fertilization and rehydration of eggs. After that the eggs were washed and rinsed with freshwater several times for complete removal of residuals before transferring them to the incubators.

Incubation of eggs

The fertilized eggs were spread over a single layer of a horizontal 1 mm mesh size mosquito net, suspended in a well aerated plastic trough (10 cm water depth) (Haylor, 1993). A random sample of 100 eggs was obtained from each female hatching trough (24 h post incubation) in order to check the fertilization rate under a binocular microscope (Brzuska, 2004). Once the hatching of larva occurs, the new hatchlings descend to the bottom of the hatching troughs, while the egg shells, dead eggs, and deformed larva remain adhered to the mesh net. The deformed larvae were then simply counted, as the unfertilized eggs and egg shells were immediately removed to avoid fungal infection. After complete hatching, the hatchability percentage and the percentage of deformed larvae were calculated for each female broodfish (Haniffa and Sridhar, 2002).

Histopathological assessment

Following the third exposure of the broodfish to the prophylactic doses of the tested disinfectants, two females and one male from each replicate were sacrificed, and their gonads and liver were removed and prepared for histopathological studies according to Culling (1983).

Statistical analysis

The recorded data were statistically analyzed using SAS (2008) to perform the requirements of the statistical model.

$X_{ij} = \mu + t_i + r_j + e_{ijk}$

X_{ij} : Egg and larval quality parameters.

μ: Population mean.

t_i: Treatment effect.

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r_j: Replicate effects.
e_{ijk}: experimental error.

Results

Table 1 illustrates the spawning performance parameters and larval quality of *C. gariepinus* following the FA and MG treatment. The latency period data ranged from 12 to 13 h and demonstrated non-significant differences (p>0.05) between the ovulated experimental groups. Similarly, results of the relative fecundity were not significantly different (p>0.05) among the treated broodfish groups. The control (non-treated) group and the FA-treated group gave the highest percentage of ovulation (75% for both) whereas, the MG-treated group revealed a significantly lower ovulation percentage (50%). The broodfish of the control group showed the highest significant (p<0.05) egg weight (66.18 g) and egg number (46325 eggs) followed by the FA- treated broodfish (egg weight; 53.73g and egg number; 37611 eggs).

On the contrary, the MG-treated broodfish delivered significantly lower (p<0.05) egg weight (41.17 g) and egg number (28821 eggs) compared to the control group and FA-treated group. On the other hand, the control broodfish showed a significantly (p<0.05) higher fertilization rate (84%) and hatchability percentage (84.72%) whereas; the lowest records were observed in the MG-treated group. Meanwhile, a significantly higher larval deformity percentage (7.08%) was recorded for the MG-treated broodfishes compared to the values recorded for the control brood fish (4.28%) and FA-treated group (5.56%).

The liver of the control (non-treated) *C. gariepinus* broodfishes (Fig. 1a) displayed hepatocytes (H) arranged in a definite chord like pattern with a prominent lumen (L) and sinusoidal layer (SL). The nucleolus of each hepatocyte was either spherical or slightly ovoid with regular surfaces scattered chromatin granules and one or more nucleoli. On the other hand, the liver of FA-treated broodfish showed congestion of blood vessels and sinusoids (Fig. 1b). Moreover, there were pyknotic nuclei with multifocal hepatic necrosis (Fig. 1c). Whereas, the liver of MG-treated broodfish, showed congestion of the blood vessels and focal hepatic necrosis together with inflammatory cell infiltrations (Fig. 1d and e).

The testis from a control catfish was covered by collagenous connective tissue capsule (tunica albuginea) which gave many septa dividing the testis into several lobules. The histological sections showed marked asynchronous development in the spermatogenesis within the same testis.

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Table 1: Spawning performance parameters and larval quality (means±SD) of *Clarias gariepinus* following the formalin (FA) and malachite green (MG) pre-spawning disinfection (*n*=8).

Performance parameters	Treatments		
	T_1 (Control)	T_2 (Formalin; 15 ppm)	T_3 (Malachite green; 1 ppm)
Fish weight (g)	690.50±79.12[a]	632.38±82.47[a]	611.88±77.54[a]
Latency period (h)	12.00±0.63[a]	12.33±1.03[a]	13.00±1.16[a]
Ovulation rate (%)	75	75	50
Egg weight (g)	66.18±13.00[a]	53.73±15.78[ab]	41.17±16.81[b]
Egg No	46325±9103[a]	37611±11048[ab]	28821±11766[b]
Relative fecundity	67.80±13.60[a]	59.22±17.57[a]	48.66±20.43[a]
Fertilization rate (%)	84.00±7.73[a]	75.78±9.55[b]	66.33±12.23[c]
Hatchability rate (%)	84.72±7.09[a]	75.44±7.94[b]	75.92±8.69[b]
Deformed larvae (%)	4.28±1.90[b]	5.56±2.43[ab]	7.08±3.34[a]

Means in the same row with different superscript are significantly different (p<0.05).

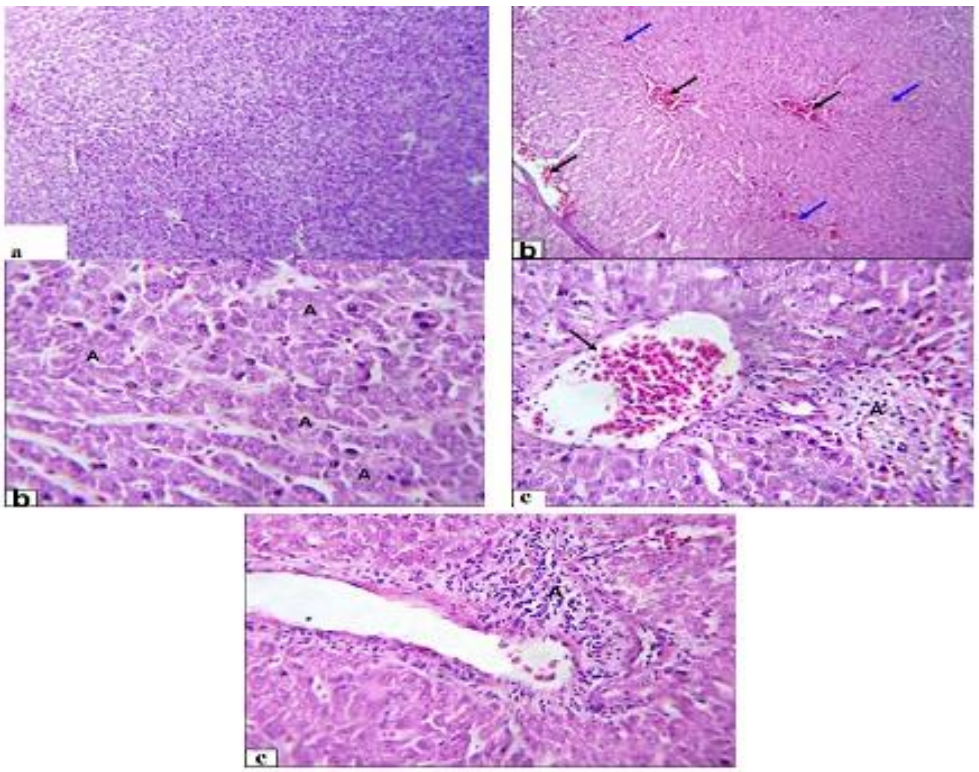


Figure 1: (a) Photomicrograph of catfish liver of control group showing normal histological structure (H&E, X160). (b) Photomicrograph of catfish liver exposed to formalin (15 ppm) for 6 h showing congestion of blood vessels (black arrows), sinusoids (blue arrows) (X160, H&E), and pyknotic nuclei with multifocal hepatic necrosis (A (H&E, X200). (c) Photomicrograph of catfish liver exposed to malachite green (1 ppm) for 45 minutes showing congestion of blood vessels (arrow) and Focal hepatic necrosis with inflammatory cells infiltrations (A) (H&E, X200).

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The sections revealed irregularly shaped lobules of varying dimensions, all development stages could be observed inside these lobules and in the interior portion of the lobules, spermatozoa were packed, basophilic and filled the lobular lumen (Fig. 2a). The seminiferous lobules of FA-treated catfish were filled with a moderate density of spermatozoa with or without germinal cells (Fig. 2b). Testicular sections of the MG-treated broodfish had thick interstitium and germinal cells in the seminiferous lobules which were either partially fully distended or nearly empty of spermatozoa, besides the congested interstitial blood vessels (Fig. 2c).

The histological sections of the ovaries obtained from the control broodfish displayed different normal developmental stages generations of ova. The primary oocyte revealed increased nuclear size together with an increased nucleoli number. Additionally, Balbiani bodies were noticed all over the cytoplasm of the late stages of the primary oocytes. The mature follicle had increased size, zona radiata and completely yolk-filled structures (Fig. 3a). The ovarian sections of the FA-treated catfish showed a moderate to severe pyknosis and shrinkage of the primary oocyte beside separation of zona radiate by edema represented by a pale eosinophilic substance (Fig. 3b). The microscopic findings of the ovarian sections of MG-treated catfish resemble the FA-treated one but with less severity (Fig. 3c).

Discussion

This study described the effects of pre-spawning prophylactic disinfection during a seven days quarantine period on the breeding performance of *C. gariepinus* broodfish whereas most of the previously research works were focused on other production phases in different fish species including; catfish (Adeyemo *et al.*, 2011, 2012), Nile tilapia (*Oreochromis niloticus*) (Mert *et al.*, 2015), milkfish (*Chanos chanos*) (Cruz and Pitogo, 1989) and trout (Meyer and Jorgenson, 1983).

Using CPE as a spawning agent has successfully induced *C. gariepinus* broodfish to spawn. The ovulation percentage recorded for the control (non-treated) and the FA-treated groups was greater than the MG-treated broodfish, whereas the latency periods were almost the same. Such successful spawning induction in *C. gariepinus* by CPE was reported in several previous studies (Hossain *et al.*, 2013; Sahoo *et al.*, 2014; El-Hawarry *et al.*, 2016).

Meanwhile, the usage of FA or MG as pre-spawning disinfectants has resulted in lower egg weight, egg number, and fertilization rate and hatchability percentage.

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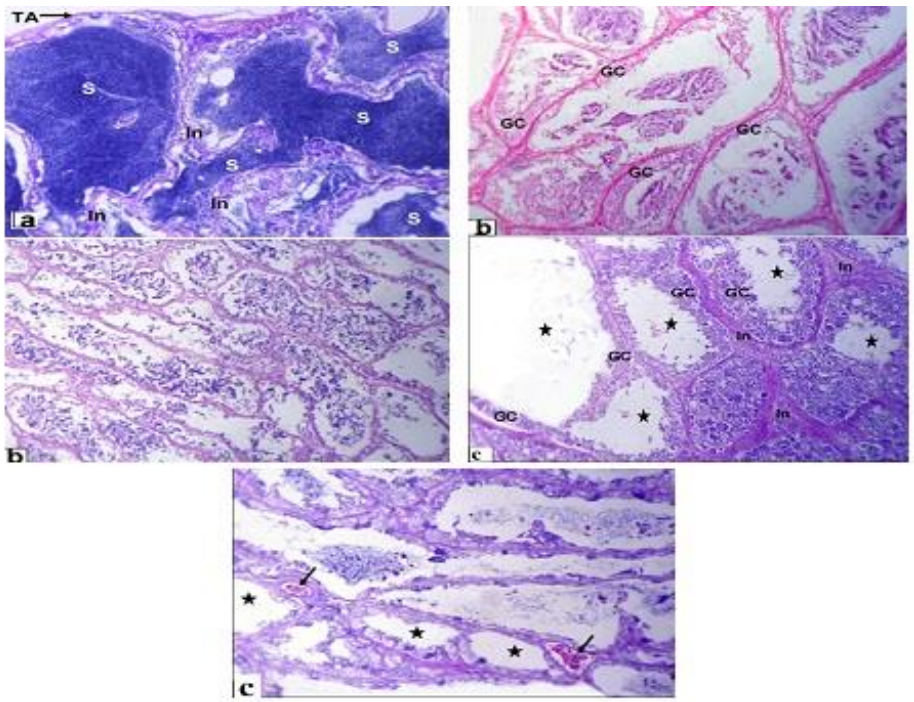


Figure 2: (a) Photomicrograph of catfish testis of control group showing normal histoarchitecture. The seminiferous lobules are filled with spermatozoa (S); tunica albuginea (TA) and interstitium (In) (H&E, X160). (b) Photomicrograph of catfish testis exposed to formalin (15 ppm) for 6 h and stained with H&E, 160x showing the seminiferous lobules filled with a moderate density of spermatozoa with presence and absence of germinal cell (GC). (c) Photomicrograph of catfish testis exposed to malachite green (1 ppm) for 45 minutes showing the seminiferous lobules had germinal cell (GC), thick interstitium (In) and the lumina empty from spermatozoa (stars) or the seminiferous lobules either fully distended partially or nearly empty lobules (stars) by spermatozoa beside congestion of interstitial blood vessels (arrows), 200x.

Additionally, the quality of larvae obtained from the control (non-treated) African catfish females was better (after 24 h of incubation) than the larvae produced by the FA or MG-treated broodfish. These results might be attributed to the differences in the egg quality obtained from the differently treated brood fish group. Adeyemo *et al.* (2012) reported that the eggs obtained from FA-treated *C. gariepinus* broodfish after a three days treatment before spawning induction were clustered eggs, but still clumped and with irregular edges. Similarly, Adeyemo *et al.* (2011) also described the eggs obtained from MG-treated African catfish females as irregularly shaped with focal necrotic lesions. However, it is worth stressing that, the current study conveyed the possibility of fertilization and hatching of eggs obtained from FA or MG-treated broodfish, contradicting Adeyemo *et al.* (2011, 2012), who concluded that the fertilized eggs of the FA and MG-treated groups did not hatch and did not develop as normally as those eggs collected from the non-treated broodfish.

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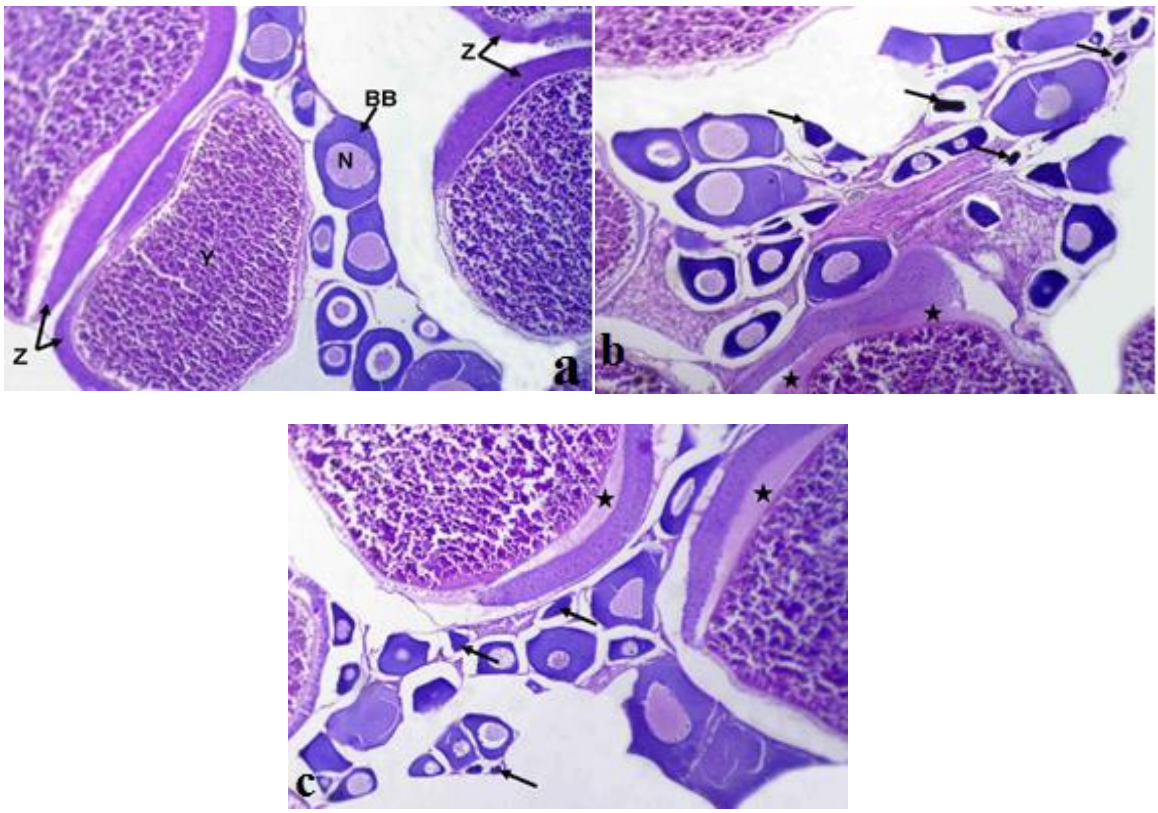


Figure 3: (a) Photomicrograph of catfish ovary of the control group showing normal histoarchitecture. BB: Balbiani bodies; N: nucleus; Y: yolk; Z: zona radiate (H&E, X160). (b) Photomicrograph of catfish ovary exposed to formalin (15 ppm) for 6 h showing pyknotic and shrinkage of the primary oocyte (arrows) and separation of zona radiate by edema (stars) that was represented by faint eosinophilic material (H&E, X160). (c) photomicrograph of catfish ovary exposed to malachite green (1 ppm) for 45 minutes and stained with H&E showing pyknosis and shrinkage of the primary oocyte (arrows) and separation of zona radiate by edema (stars) that was represented by faint eosinophilic material (H&E, X160).

On the same manner, Meyer and Jorgenson (1983) reported an increased percentage of trout eggs that reached the eyed eggs stage following MG treatment compared to the controls (probably due to reduced fungal infestation of the MG-treated eggs). He also added that this increase did not compensate for the loss of larvae due to malformations (head and jaws malformation, spine deformity and/or missing fins) caused by MG treatment.

Furthermore, the histopathological findings of the ovaries and testis taken from the experimental broodfish may support the former reproductive performance recorded in the present study. The observed pyknotic and shrunken primary oocyte of the ovaries are in agreement with the findings described by Adeyemo *et al.* (2011, 2012). Additionally, the low density of spermatozoa detected in the seminiferous tubules might be a direct consequence of the germ cells necrosis or cessation of spermatogenesis that appeared in the testis of the FA-treated broodfish males and the MG-treated one respectively, which may consequently give a further explanation

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to the declined fertilization rate and poor hatchability percentages recorded in those treated broodfish groups. Similarly, necrotic disrupted and depleted seminiferous tubules were recognized in the histological sections of testis obtained from the FA or MG-treated males of *C. gariepinus* (Adeyemo *et al.*, 2011, 2012).

The liver of FA and MG-treated catfish showed congestion of vasculature and multifocal hepatic necrosis. These reported liver damages in the FA and MG-treated groups may also give a supportive explanation to the declined reproductive performance recorded in these broodfish groups. Such effects might be attributed to the fact that, liver tissue plays a major role in the process of egg formation (Lubzens *et al.*, 2010).

Similarly, a reported liver damage was reported in several fish species following the exposure to FA treatment including; multifocal necrosis of the hepatocytes of catfish after exposure to 37 mg L^{-1} FA for three repetitive days for 30 minutes (Adeyemo *et al.*, 2012), tissue damage and genotoxicity in *Oreochromis niloticus* with as low as 15 mg L^{-1} FA concentration (Mert *et al.*, 2015) and cloudy swelling, hemorrhage, deposition of pigments, and necrosis in liver parenchyma of milkfish, yet partial recovery of tissues was observed in fish after 10 days in FA-free sea water (Cruz and Pitogo, 1989). Contradictory to our results, Chinabut *et al.* (1988) revealed that the liver of common carp fry showed no histopathological lesions following FA treatment (8 weeks at equal doses).

Moreover, focal liver necrosis, sinusoidal congestion, mitochondrial damage and nuclear alterations were also seen in the liver of rainbow trout exposed to MG treatments on seven consecutive occasions (Gerundo *et al.*, 1991). Additionally, histopathological examination of MG-treated carp revealed dystrophic changes in the parenchymatous tissues (Svobodova *et al.*, 1997).Consequently, it can be inferred that the present study comprehensively demonstrated the possible detrimental effects of using FA and MG as a prophylactic therapeutants for *C. gariepinus* broodstock before being admitted to the spawning induction. The protective effects obtained by both therapeutants did not reimburse for the declined fertilization rate, hatchability percentage and loss of larvae due to malformations. Nevertheless, in this study the *C. gariepinus* control (non-treated) broodfish did not show any signs of fungal and parasitic disease and its reproductive efficiency was within the known normal levels. Moreover, the FA-treated broodfish showed nosignificant differences in the ovulation percentage, egg weight, egg number and larval deformity compared to the *C. gariepinus* control (non-treated) broodfish. Herein, the pre-spawning disinfection of *C. gariepinus* during the quarantine period is not necessary especially when proper management and transportation handling is to be followed. Nevertheless, if pre-spawning

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disinfection to be applied; the MG should not be prescribed for *C. gariepinus* broodfish unless no other cheap and much safer disinfectants rather than FA existed. But still a future research is required to explore its possible negative effects on both reproductive efficiency and the prospected histopathological changes of broodfish after recovery from the FA treatment.

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