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Monitoring the Effect of Garlic (*Allium sativum*) and Black Seed (*Nigella sativa*) on *Fusarium moniliform* Infection in Fish with Emphasis on Fecundity

Nashwa S. Elias and Nadia A. Abd El Ghany

Department of Fish Diseases, Animal Health Institute, Dokki, Giza, Egypt

Abstract: The effects of *Fusarium moniliform* infection on *Clarias gariepinus* fecundity were investigated. Drastic effects were detected in 100% of females and 70% of males of the artificially injected fish with 0.1 ml. of spores suspension contained *Fusarium moniliform* spores with concentration of 1×10^4 and the percentage of mortality was 20%. The external clinical signs appeared as severe hemorrhagic patches of the skin, redness around the mouth, erosion of fins and tail, erythematic and ulceration of muscles, ulceration around the site of inoculation, circular black wounds were found on the ventral side with complete loss of coloration. Fungal re-isolations were performed from all females and 70% of males 48 h post injection. Males revealed vacuolation and necrosis of seminferous tubules with high percent of dead sperms, while females copied highly severe drop in the number of matured ova accompanied by atretic follicles. External examinations of some infected ovaries revealed either hemorrhagic patches and asymmetry. Fish fecundity was obviously dropped through gonadal weight (W_G), gonadosomatic (I_G), in addition to relative and absolute fecundity. Moreover, the total protein and liver enzymes of both sexes were markedly decreased. 30% of the injected males showed no changes either externally or internally. *Nigella sativa* oil at concentration of 0.3ml. / 100 g fish body weight exerted no significant improvement in infected fish fecundity of both sexes. In the same time, *Fusarium moniliform* was reisolated from a few percent (about 10%) of treated males. On the other hand, fecundity of *Allium sativum* powder treated males and females at concentration of 30 g / Kg. diet, obviously improved fecundity, but it is still low than that of the control group. It was recommended to use garlic powder in fish diet for prophylaxis rather than for medication against *Fusarium* infection.

Key words: *Fusarium moniliform* · Fecundity · Gonads · *Nigella sativa* · *Allium sativum*

INTRODUCTION

Fungi are a group of organisms called heterotrophs that required living or dead matter for growth and reproduction. They are present everywhere in the environment of fresh or salt water fish and in cool or warm temperatures. In most cases, fungi serve a valuable ecological function by processing dead organic debris.However, under certain condition, they might become a problem, especially if fish are stressed by either disease, or poor environmental conditions. Malnutrition, injuries or spawning are stressful conditions which enable the fungus to attack fish.

Fungi cause serious diseases in economically important species of freshwater fish and crustaceans [1,2]. *Fusarium* species, well- known plant pathogen in terrestrial habitat being a serious opportunistic pathogen causing serious diseases to freshwater fish [1,3]. *F. culmo*rum was found on the skin of carp and was responsible for the mortality in a new earthen pond [4]. In Tilapia species and Nile catfish *fusarium* caused skin lesions [5,6]. *F.solani* is common in the aquatic environment and it has been recovered from wound lesions in several species of juvenile marine turtles [7] and from intestinal ulceration of a captive sea lions [8]. Also, *Fusarium* species caused ulceration of skin and gills of *Tilapia mossambica* [9]. *F.oxysporum* had been world wide distributed in *Macrobrachium rosenbergii* were they had also been associated with exoskeleton, branchial and ocular lesions in several species of Penaeid shrimp [10]. *F.tabacinum* was documented as a gill disease in freshwater Cray fish [11] and *F.solani* was the cause of black gill disease in Hermit crabs [12]. Furthermore, *F. oxysporum*,*F.moniliform*, *F.solani* and *F. incarnatum* were described in *Penaeus aztecus* and *Penaeus monodon* [13,14].

Corresponding Author: Dr. Nadia A. Abd El Ghany, Department of Fish Diseases, Animal Health Institute, Dokki, Giza, Egypt
E-mail: noniaelghany@yahoo.com

It had been proved that *fusarium* exert hyperestrogenic syndromes and reproductive disorders in both fish sexes [15].

Immunestimulants, the modern promising tool in aquaculture were capable of enhancing cultured fish resistance against bacterial or fungal diseases and all stressors[16].

Garlic, *Allium sativum* had been agreed upon as an antibiotic for fungal – associated diseases, [17,18]. Its ability to inhibit growth of all tested fungi was confirmed by [17].

Black seeds was known as an antifungal due to the fungicidal effect of its' crude or essential oils and other compounds, thus used in medicinal applications [16].

The in hand research aimed to highlight the drastic effect of *F. moniliform* on male and female fecundity of *Clarias gariepinus* fish with special interest to two treatment trials to monitor the efficiency of garlic and black seeds.

MATERIALS AND METHODS

1-Fish for isolation of fungi and experimental infection: A total number of 200 males and females *Clarias gariepinus* with average body weight of 300±50 g. 40 males and females for isolation of fungi and 160 *Clarias gariepinus* for experimental infection were transferred from a private farm to Fish Diseases Department at the Animal Health Research Institute and was allowed to acclimatize in glass aquaria with re-circulating water at 25±0.5°C for one week with daily adjustment of oxygen and ammonia.

2-Fish Examination: Fish were examined externally to exclude fish with any external lesions or abnormalities. Also, samples from livers, gonads and muscles were examined to assure the absence of any pathogens.

3-Preparation of fungi

- Fungus isolation: Fish to be examined were firstly killed by striking the cranial top above the eyes with large scissors. Surface sterilization was done before opening the fish by absolute alcohol and flamed spatula to redness. A sterile inoculating loop was inserted through the sterilized area of liver, gonads and muscles, the obtained inocula were inserted into plate containing Sabourauds dextrose agar media with 500 units penicillin and 2 mg. streptomycin per ml^{-1} to prevent bacterial growth [19]. Cultures were kept at 25° C for 7 days. Pure cultures were established using single spore isolation method as follows: a small part of fungal mycelium from the 7 days culture was transferred using sterile medical needle and inoculated on PDA (potato dextrose agar)[20]. Cultures were inoculated at 25° C for 3 – 7 days. 3 and 7 days post inoculation of the spores were stained with lactophenol cotton blue and examined microscopically.

-Fungal Identification: A morphometric identification of the fungal isolates was held out based on cultural characteristics: colony colour, type of mycelium, shape and septation of conidia (micro- and macroconidia), [20].

-Preparation of Spores Suspension: Fungal strains of *F. moniliform* cultured on PDA at 25°C for 7 days, then conidial mass was harvested by adding 20 ml. sterile distilled water into each culture plate, followed by collection of the suspension in 30 ml. sterile autoclave tubes. Suspensions were filtered through two layers of sterile medical gauze to ensure that filtrate contain fungal conidia, concentrations of which were calculated using an erythrocyte counting chamber and adjusted to 1×10^4 conidia ml^{-1} in sterile distilled water [21].

4 - Experimental design: A total number of 160 *Clarias gariepinus* males and females for experimental fish of each sex were divided into 4 groups of 20 fish each:

1st group served as the control and it was injected I/ M with 0.1 ml. saline.

Remaining 3 groups were all injected I/ M with 0.1 ml. of concentrations 1×10^4 of the *F. moniliform* conidial suspension.

2nd group was examined soon after appearance of external lesions and mortality. In males 2nd group divided to 2 A and B according to clinical signs appearance.

3rd and 4th groups were treated with *Nigella sativa* oil 0.3ml. / 100 g. fish body weight [22] and *Allium sativum* (garlic) powder 30 g / Kg. diet [18] soon after appearance of external lesions, respectively.

5-Growth measurements and morpho-anatomical parameters: These parameters were calculated for each fish from each group [23].

6-Fungal Reisolation: Specimens from liver, gonads and muscle at injection site from each living or dead fish were microscopically examined and inoculated into PDA plates to assure reisolation and identification of fungus.

7-Total protein and liver enzymes (ALT and AST): Total protein and alanine aminotransferase (ALT)&aspartate aminotransferase (AST) were assayed in serum separated from blood obtained from caudal vein using diagnostic Kits (Diamond Company).

8-Fish fecundity: Absolute and relative fecundity and gonads examination were evaluated referring to [24].

9-Histopathological Examination: Livers were histopathological processed and stained with Haematoxylin and Eosin. Gonads placed in Bouin's for 48 hours before staining according to [25]. All figures examined at X 660 and X 1200.

10-Statistical Analysis: Data obtained were statistically analyzed according to [26].

RESULTS

Clinical signs:

External lesions: The general clinical signs in infected *Clarias gariepinus* appeared as severe hemorrhagic patches on the skin (Fig. 1), redness around the mouth, erosion of fins and cleavage of tail, ulceration of muscles (Fig. 2), ulceration around the site of inoculation, circular black wound were found on the ventral side of fish (Fig. 3) with complete loss of coloration.

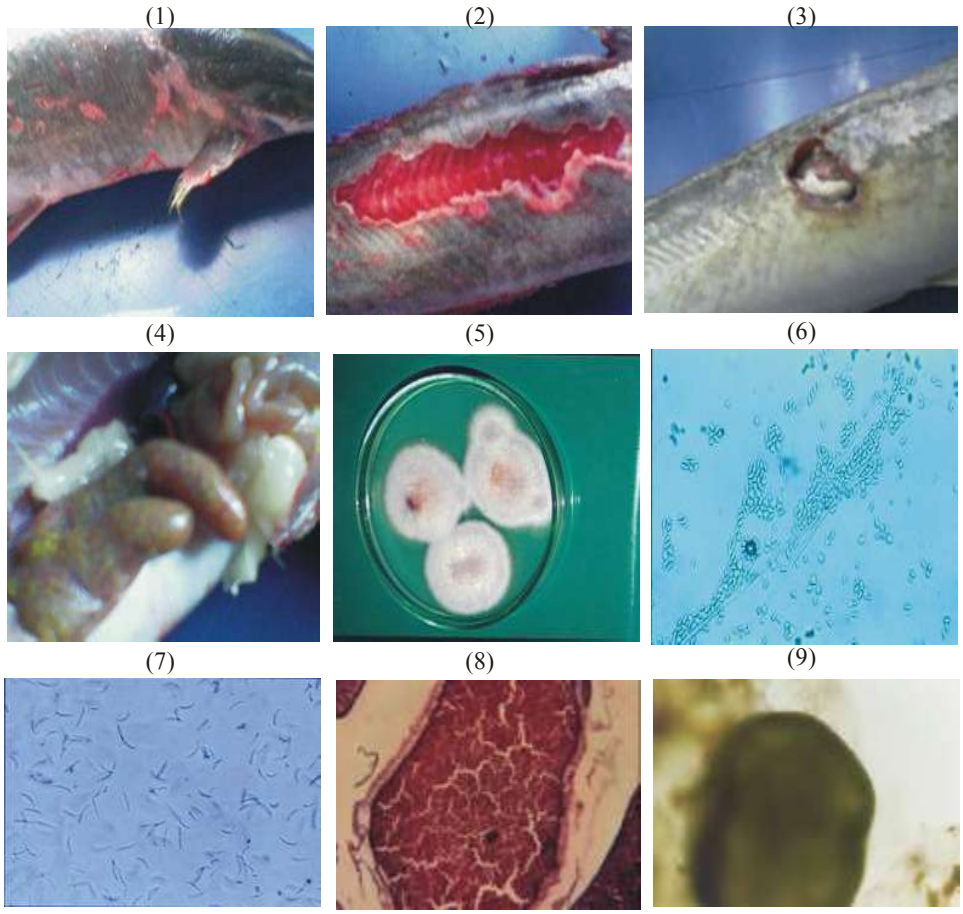

Fig. 1: *C. gariepinus* infected by *fusarium moniliform* showing hemorrhagic patches on skin
Fig. 2: *C. gariepinus* infected by *fusarium moniliform* showing erythematous muscle
Fig. 3: *C. gariepinus* infected by *fusarium moniliform* showing circular black wound on ventral side
Fig. 4: *C. gariepinus* of affected ovary showing asymmetrical lobes and large in size
Fig. 5: *Fusarium moniliform* culture on PDA showing dense aerial white to dark violet mycelium
Fig. 6: *Fusarium moniliform* culture showing microconidia were formed in chains, fusiform to clavate cells (x10)
Fig. 7: *Fusarium moniliform* culture showing macroconidia long, slender, straight and thin walled
Fig. 8 & 9: *C. gariepinus* normal female ovary containing large sized ova completely filled with vitellogenin

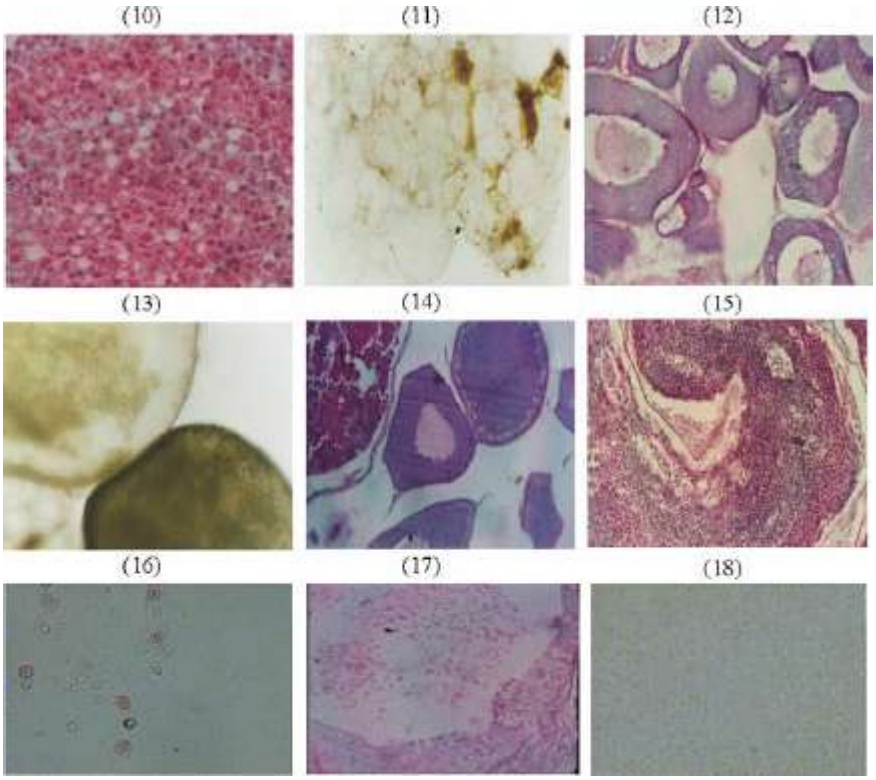

Fig. 10: *C. gariepinus* infected by *f. moniliform* showing liver with hemorrhages, cells rapture, necrotic hepatocytes and multifocal granulomatous hepatitis H&E.X 660

Fig. 11: *C. gariepinus* infected *f. moniliform* showing ovary with empty large sized ova H&E.X 1200

Fig. 12: *C. gariepinus* infected by *f. moniliform* showing ovary with oedema and many atretic Follicles H&E.X 1200

Fig. 13 & 14: *C. gariepinus* females treated with *Nigella sativa* showing ovary with many empty large sized ova H&E.X 1200

Fig. 15: *C. gariepinus* normal uninfected male showing testes filled with spermatids, spermatozoa and well differentiated sperm cells. H&E.X 1200

Fig. 16: *C. gariepinus* normal uninfected male showing testes filled with high % of living sperms. H&E.X 660

Fig. 17: *C. gariepinus* infected male showing testis with necrosed nuclei while lumen of seminferous tubules contained very few sperms. H&E.X 660

Fig. 18: *C. gariepinus* of male treated with *Nigella sativa* showing testis with high % of dead sperms. H&E.X 660

Internal lesions: Livers were pale in color with several dark red areas and enlarge gall bladder. Ovaries revealed asymmetrical lobes (Fig. 4).

Morphometric criteria of the isolated fungi: The recovered fungi were isolated on Sabourauds dextrose agar media and preliminarily identified on PDA, morphometrically as members of the genus *Fusarium*, namely *F. moniliform* colonies grow rapidly on PDA with dense aerial white to dark violet mycelium which reverse to brownish colour,showing average growth rate per day at 25°C are which were delicately floccose to felted (Fig. 5). Microconidia were formed in chains, fusiform to clavate with a slightly flattened base produced from long phialides. They occasionally become one septate and chlamydoconidia was not produced (Fig. 6). Macroconidia usually long and slender, almost straight, thin walled but often appeared as sharply curved apical cell and pedicellat basal cell (Fig. 7).

Mortality, morbidity and reisolation: Clinical signs appeared severely among 100% females. In only about 70% males it took a moderate picture while 30% showed no clinical sings. On the contrary, mortality % was more among males (about 25%) than in females was nearly 10%.

Table 1: Comparative fecundity in normal, experimentally infected, treated with Black seed and Garlic female Clarias gariepinus

Item		Control	Infected	Treated with Nigella s.	Treated with Garlic
Growth Measurements	B.L (cm.)	35.7±0.8	36±2	41±1A^{+++}	42±0.2 A^{+++}
	B.W (gm.)	374±2	298±3 ***	379±1A^{+++}	385±3 A^{+++} B^{+++}
	W_G (gm)	18±1.4	5.9±1.3 ***	8.6±1.2A^{+++}	8.7±1.4 A^{+++}
	W_H (gm)	7.3±0.03	3.5±0.4 ***	4.4±1.2 A^{+}	6.4±0.7 A^{+++}
Morpho – anatomical Parameters	I_G	7.2±0.8	1.4±0.04***	5.6±0.4A^{+++}	5.1±0.2 A^{+++} B^{+}
	I_H	4.9±0	1.2±0.03	1.8±0.2	2.9±0.2A^{+++} B^{++}
	K	0.6±0.01	0.6±0.02	0.6±0.03	0.6±0
Relative Fecundity	F.B.L	3928±3	3987±6 ***	5174±3A^{+++}	5446±5A^{+++} B^{+++}
	F.B.W	2202±2	1823±6 ***	2229±6A^{+++}	2256±3 A^{+++} B^{+++}
	F.O.W	3868±5	1584±4 ***	2134±5A^{+++}	2155±2 A^{+++} B^{+++}
Absolute Fecundity	Dep. Eq.	160±2	136±2 ***	214±1A^{+++}	180±3 A^{+++} B^{+++}
	T. Egg No.	4891±3	96±1 ***	1206±3A^{+++}	3374±2 A^{+++} B^{+++}
S.Total protein	T.P. gm/dl	12.7±0.3	3.1±0.8 ***	3.6±0.3	8.7±0 A^{+++} B^{+++}
Liver enzymes	ALT μ / ml	19.6±0.7	13.2±0.8	14.4±1	18.2±1 A^{+++} B^{+++}
	AST μ / ml	20.8±2	6.2±1.4***	15±1.4A^{+++}	17.4±0.4A^{+++} B^{+++}

body length (B.L.), body weight (B.W.), gonadal weight (WG) and liver weight (WH), gonadosomatic (IG) and hepatosomatic (IH) indices, condition factor (K) body length (B.L.) – body weight (B.W.) – body depth (B.D.) – ovarian weight (WG) and liver weight (WH) – gonadosomatic index (I G) – hepatosomatic index (I H) – condition factor (K) – relative fecundity to body length (F.B.L.) - relative fecundity to body weight (F.B.W.) - relative fecundity to gonad weight (F. O.W) – dependence equation according to biomass (Dep. Eq.) – total egg number (T Egg No.). Mean ± S.E. N = 10

*: compared to Control

A: Nigella or Garlic compared to infected 2B: Garlic gp. compared to Nigella group

2A group with external lesions 2B group with no external lesions

* / A^{+} / B^{+}: P < 0.01 **/ A^{++} / B^{++}: P < 0.005 *** / A^{+++} / B^{+++}: P < 0.001

Table 2: Comparative fecundity in normal, experimentally infected (with / without signs), treated with Black seed and Garlic male Clarias gariepinus

Item		Control	Group 2 A (70%)	Group 2 B (30%)	Treated with Nigella s.	Treated with Garlic
Growth Measurements	B.L (cm.)	44.6±0.4	38±0.4 ***	37±0.6 ***	37.4±0.4 A $^{+++}$	42.6 ±0.4 A^{+++} B^{+++}
	B.W (gm.)	400±1	298±1 ***	361±6 ***	342±1 A^{+++}	419±1 A^{+++} B^{+++}
	W_G (gm)	3.6±1	0.9±0.2***	0.5±0.1***	2.7±0.3	3.3±0.3 A^{+++} B^{++}
	W_H (gm)	4.2±0.06	2.4±0.3 **	2.2±0.4	4.8±0.6 A^{+++}	4 ± 0.3 A^{+++}
Morpho – anatomical Parameters	I_G	3.7±0.3	0.2±0.02 ***	0.3±0.02***	0.8±0.1	2.6±0 A^{+++}
	I_H	0.9±0.2	0.6±0.01 **	0.3±0.03	1.4±0.2	1.1±0.1
	K	0.7±0	0.7±0	0.5±0.01*	0.7±0	0.5±0
Relative Fecundity	F.B.L	6143±3	4447±2 ***	3347±0.4***	4307±3A^{+++}	5554±6 A^{+++} B^{+++}
	F.B.W	2328±2	2225±1***	2138±4***	2046±4A^{+++}	2422±4 B^{+++}
	F.O.W	1114±1	576±2 ***	482 ± 6 ***	932 ± 4 A^{+++}	1032± 6 A^{+++} B^{+++}
Absolute Fecundity	Sperm density	1416±3	186±4 ***	580±5 ***	793±1 A^{+++}	999±2 A^{+++} B^{+++}
	% live	90±0.1	23±3***	66±0.8 *	69±3 A^{+++}	86±1 A^{+++} B^{+++}
	% dead	10±0.02	77±3 ***	34±0.9 *	31±2 A^{+++}	16±1 A^{+++} B^{+++}
S. Total protein	T.P. gm/dl	9.8±0.2	3.4±0.3***	3.9±0.6***	5.3±1 A^{+++}	6.7±0.2 A^{+++}
Liver enzymes	ALT μ / ml	18.3±1	13.2±0.3 ***	14.2±0.1***	19±1.1 A^{+++}	7.8±0.4 A^{+++} B^{+++}
	AST μ / ml	21.8±0.4	12.4±2 ***	9.6±1 **	20±1.2 A^{+++}	11.5±0 A^{+++} B^{+++}

body length (B.L.), body weight (B.W.), gonadal weight (WG) and liver weight (WH), gonadosomatic (IG) and hepatosomatic (IH) indices, condition factor (K) body length (B.L.) – body weight (B.W.) – body depth (B.D.) – ovarian weight (WG) and liver weight (WH) – gonadosomatic index (I G) – hepatosomatic index (I H) – condition factor (K) – relative fecundity to body length (F.B.L.) - relative fecundity to body weight (F.B.W.) - relative fecundity to gonad weight (F. O.W) – dependence equation according to biomass (Dep. Eq.) – total egg number (T Egg No.). Mean ± S.E. N = 10

*: compared to Control

A: Nigella or Garlic compared to infected 2B: Garlic gp. compared to Nigella group

2A group with external lesions 2B group with no external lesions

* / A^{+} / B^{+}: P < 0.01 **/ A^{++} / B^{++}: P < 0.005 *** / A^{+++} / B^{+++}: P < 0.001

Fungal reisolation was positive from all infected fish livers, testis & ovaries.

Treatments effects:

- Both treatment trials exerted zero mortality and morbidity rates in both sexes.
- No fungus was reisolated from males and females after treatment with garlic and isolated from a slight % (not more than 10%) of males after treatment with black seed.

Fecundity estimation: Results (Table 1 and 2) show that the injected females and males (Group 2 A and B) exerted highly significant drop in growth measurements, morpho- anatomical parameters, relative and absolute fecundity as well as T.P. and liver enzymes values as compared to the control group. These parameters improved after as compared to before treatment.

Gonadal histopathological examinations:

- Figures 8 and 9 reveal the control ovary where all ova were completely filled with vitellogenin.
- Infected livers showed hemorrhages and cells rapture accommodated by the necrotic hepatocytes and multifocal granulomatous hepatitis (Fig. 10).
- Infected ovaries showed large number empty (Fig. 11) or oedematous with atretic follicles (Fig. 12)
- Treated ovaries with *Nigella sativa* showed many large sized empty ova free of vitellogenin (Fig. 13 and14)
- Control testis showed enlarged seminferous tubules full of spermatids, spermatozoa and well differentiated sperm cells (Fig. 15), whereas its milt contained large % of living sperms (Fig. 16). Infected testis of gp. 2A and B characterized by germinal epithelial of testicular ducts with necrosed nuclei while lumen of seminferous tubules contained vacuolar degeneration of spermatocytes (Fig. 17)
- Treated testis with *Nigella sativa* showed thickening of seminferous tubules with large % of dead sperms (Fig. 18)

DISCUSSION

Fusarium species are of cosmopolitans' distribution and well known important filed plant pathogen. Many species survive in soil and dead vegetation.

In this work, *F. moniliform* were present among the fungi isolated from skin and internal organs (liver and gonads) of examined fish. Several cases of infection related to *fusarium* species have been reported in skin of freshwater fish such as *mirror carp* and *grass carp* [27,28]. Following experimental infection with *F. moniliform* in *Clarias gariepinus* by I/M route, obvious histopathological changes occurred which agree with [29]. The supernriouty of I/M route of infection met with in case of *F. moniliform* is attributed to hypotheses postulated by [30] who stated that hyaluronic acid enzyme secreted in excess by this fungus acting as a virulence factor facilitating the pathological process ending it in the benefit of the fungus.The conclusions was confirmed by [29] who recorded that the autolysis of fish muscles is duo to the hydrolyze of the mucopolysaccarides and the chitin of muscles.

Fusarium has been proved to be a growth inhibitor as well as an immunosuppressant agent [31]. In addition, [32] had added reducing infected fish productivity to its' disadvantages. In the present research *Fusarium* exerted severe external lesions whose severity appeared more in females. Fins were deteriorated, red patches were scattered allover the body followed by muscles' erosion which then went deeply till invading internal organs.

Fish B.W. and F.B.W. of both sexes were markedly dropped, which may be due to loss of fish appetite.

Livers, in both sexes, appeared dark brown friable where as their dysfunction was confirmed by the marked decrease in W_H, T.P. and liver enzymes (ALT & AST). These findings coincide with the occurrence of hemorrhages and cells rapture in infected fish accommodated by the necrotic hepatocytes and multifocal granulomatous hepatitis proved by [33].

In females, external lesions and fungal reisolation from livers and ovaries were clear in 100% of experimentally infected fish. This explained the highly significant drop in females' fecundity. Ovaries revealed obvious drop in weight beside presence of hemorrhages and asymmetry. Also infected fish' ovaries copied either immaturity or oedematous which all stand for the highly significant drop in absolute fecundity. These findings agreed with the disturbances in the ovarian maturity and follicular development processes together with ovarian atrophy presented by [34, 35].

Concerning males, infection with *fusarium* caused mortality rate (about 25%) which was higher than that in females. In this research infected males were divided into about 70% (group 2 A) which showed external lesions and the fungus was reisolated from livers and testes. The remaining 30% (group 2 B) externally appeared apparently healthy without any of clinical signs although sequenced by fungal reisolation from testes. Still both groups (2 A&B) showed highly significant drop in W_G,W_H, I_G, I_H and F.O.W. Consequently, these followed obtaining

faint white watery milt characterized by highly significant decreased absolute fecundity (Sperm density and live %) in both groups. where group.2 B was somewhat better than 2A. The authors approved these results by the vacuolar degeneration of spermatocytes together with thickening of seminferous tubules lumen containing very few sperms according to [15] who permitted that *Fusarium* and its toxins decreased the pituitary response to gonadotropin – releasing hormone.

Treatments trials were held out using two common medicinal plants: garlic and black seeds owing to their immunestimulants prosperity and antifungal activities, [16]. Garlic was defined by[17] as the promising treatment of fungal – associated diseases and proved its' ability to inhibit growth of fungi.

Among both sexes, garlic and black seeds promoted growth thus B.L., B.W., $W_{G,}$ F.B.W., F.B.L., F.O.W. markedly increased as compared to infected groups which accommodated with [18] on *Oreochromis niloticus*.

Concerning absolute fecundity, (the T. Egg No. and sperm density) although was obviously raised with both trials, still remained far beyond the control. Moreover, histopathological examinations showed that females' ovaries of those treated with *Nigella sativa* contained a large number of big sized empty ova and males' testes contained several testicular necrotic areas with majority immature or dead sperm cells. [32] proved that *Fusarium* and its toxins reduced ovarian development and sperm number due to its estrogen like activity which the authors found a logic elucidation. In addition, the authors believe that the severe degenerative testicular changes presented by [31] might not be completely reversible.

Liver histopathologically changes were not completely treated by *Nigella sativa* which was a natural proof for the fungal reisolation from livers of some females. Consequently, their serum T.P. level was nearly unaffected. Furthermore, T. Egg No. remained far away from control since their majority was empty big – sized ova (free from vitellogenin which is formed in the liver). The resulted highly significantly increase in serum T.P. among both sexes treated with garlic might be associated with the stronger innate immune response [36]. Garlic had reduced liver enzymes through enhancing activity of non specific defense mechanism, [18] which elucidated the highly significant decrease of liver enzymes in this research.

It could be concluded that *Fusarium moniliform* has a drastic effect on fish fecundity and it affect fish food contamination. Immune stimulants such as garlic and black seeds failed to result in complete remedy in both sex. It is recommend to use garlic addition to fish diets for prophylaxis not for treatment.

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