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REPRODUCTIVE ALTERATION AS INDICATOR FOR ENVIRONMENTAL HORMONAL DISRUPTION IN *OREOCHROMIS NILOTICUS* INHABITS LAKE MANZALA

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Abstract

The presence of testis-ova (TO) in gonochristic fish has been used as biomarker for detecting xenoestrogens exposure e.g. polyaromatic compounds (PAHs) and Polychlorinated biphenyl (PCBs). In late 2011, a pilot screening was performed to detect signs of estrogenic compounds on Nile tilapia *O. niloticus* inhabit Lake Manzala 31° 16′ 0″ N, 32° 12′ 0″ E; followed by other one in 2012.

Hepatosomatic index (HSI) and Gonadosomatic index (GSI) values variation were noted in both sexes and seasons in all subjected areas. The presence of oocytes within the testicular tissue of males were explicit. Hence, severity index ranking (0-4) was developed based on the number of oocytes per testicular tissue section. The number of histological section needed to clearly detect intersex in tilapia was statistically verified by using samples collected from 2011 to 2012.

Seasonal differences in severity and occurrence *O. niloticus* male were detected by calculating occurrence percentage in the two consecutive seasons. The TO percentages ranged from 30-60% in first season and 24-45% in second season in all sampling areas. The highest diffusion and severity were noted during postspawn – prespawn season, when compared with spawning season.

On the other hand, females' gonads showed seasonal differences. In the first season, spent stage (V) was the common stage with 100% of the collected samples. While, in the second season mature stage (III) and ripping oocyte stage (IV) where detect.

Keywords: Xenoestrogens, Lake Manzala, Testis-ova, Endocrine disrupting chemicals (EDC), *Oreochromis niloticus*.

INTRODUCTION

Environmental pollutants are widely spread all over the world because of the industrial evolution. This rapid evolution produced enormous amount of natural and synthetic substances that have the ability to disrupt the normal physiology and endocrinology of vertebrate and invertebrate classes (Panter *et al.*, 2006). Environmental pollutants are derived from pesticides, herbicides, fungicides; polyaromatic compounds (PAHs), Polychlorinated biphenyl (PCBs), organic oxygen compounds (phthalates, bisphenol A), surfactants, drugs, metals and phytoestrogens (Multigner *et al*., 2008). Khan (2013), PAHs could induce decrease in gametogenesis, decrease in gonad size, low egg production and low hatching success. Otherwise, PAHs disrupted endocrine function in teleosts by altering testicular steroid production by affecting lipid metabolism through reducing the amount of physiologically available energy for gonad maturation.

The testis-ova or as referred by other researchers ovo-testis or intersex which are described as the presence of female germ cells, or oocytes, within the male gonad (Blazer *et al*., 2007). The presence of TO is usually associated with other histological, hormonal and behavioral alterations such as; alterations in blood sex steroid hormone concentrations, sperm or oocytes quality, decreased fecundity rate, testicular and ovarian histological alterations and/or damage, vitellogenesis process impairment, gonads maturation delay (Banaee *et al.*, 2008 and Jobling *et al.*, 2002). Reproductive and parental behavior alteration and impairment in olfactory response and disorder in reproductive migrations has been also mentioned by Scholz *et al*. (2000) and disruption in coordinating courtship behavior of male and female fish and time of spawning (Jaensson *et al.*, 2007).

Such alterations have been noted in several fish populations and several species by Khan, 2013; Balch and Metcalfe, 2006**;** Japanese

medaka (*Oryzias latipes*), cunner (*Tautogolabrus adspersus*), winter flounder (*Pleuronectes Pseudopleuronectes* americanus), male roach (*Rutilus rutilis)*, salmon (*Salmos alar),* walking catfish (*Clarias batrachus*), freshwater eel (*Monopterus albus*). Numerous researchers have mentioned the same alterations in other geographical areas due to exposure to domestic effluents containing estrogenic compounded such as UK, USA rivers downstream (USA EPA, 2012 and Jobling *et al.,* 1998) and Danish streams (Danish EPA, 2012). Testis-ova have been induced experimentally by wide variety of chemicals that mimic estrogen effect such as, NPEs, bisphenol A and EDC e.g., DDT, endosulfan, methoxychlor, malathion, diazinon, fenitrothion (Mahdi Banaee, 2012). Odum *et al.* (2000) showed that, alkylphenols including NP and NPE family in laboratory was mimicking the effects of estrogen in vitro and in vivo studies.

Lake Manzala by far is the largest with an area of approximately 500 km^2 representing 35% of the total production of natural lakes in Egypt (GAFRD, 2013). However, Lake Manzala have been subjected to wide variety of environmental pollutants which caused decrease of species diversity; total catch and organ malformation Engineered (1997). Furthermore, Barakat *et al.* (2012 and 2002), indicated that PCBs are major contaminants in Lake Manzala especially in El-Gamil that contained relatively high concentrations of PCBs suggesting a possible localized pollutant discharge sources due to the use of PCBs in transformers, electrical equipment, and other industries. Moreover, Badawy and Wahaab (1997) demonstrated that fish samples collected from Lake Manzala are contaminated with low levels of PAHs.

The main objective of the percent study is detecting reproductive alteration caused by environmental pollutants for both male and female fish inhabits Lake Manzala.

METHODS

The flow of water into Manzala comes from several drains. The lake is exposed to high inputs of pollutants from Agriculture, industrial and domestic drains. Each area has its distinctive water traits due to the type of wastewater input; hence, the five sampling areas were chosen according to the type of waste drained in the area; the five areas are El-Bashteer, El-Gameel, El-Serw, El-Enania & El-Sayala and Temsah (Fig. 1).

Twenty five to thirty five wild-mixed age *O. niloticus* (average weight ranged from 50-118 g) were taken from the above-mentioned locations by a fishing boat on two different seasons December 2011 and August 2012. Fish samples were transferred into plastic ice container until submitted for manual sexing and sex ratio were calculated (Table 2); then fish total length and weight were measured. At least 10 male were dissected; gonads and liver were taken and GSI and HSI were calculated.

The fish gonads were removed, dissected into small pieces and fixed in Davidson's modified solution, then dehydrated through a series of ascending concentrations of ethanol, cleared with xylene solutions, embedded and blocked in paraffin wax according to (Fournie *et al.*, 2000). Fine transverse sections 5µ were cut, mounted and stained with hematoxylin and eosin (H&E) (Johnson *et al.*, 2009). From each fish (4-6) transverse sections and (3) microscopic fields were examined to determine the developmental stage and 100% of three section tissues were examined for tests-ova count and expressed as means ± S.E. The tissue slides were examined for abnormalities by light microscope and photographed by fluorescence microscope Leica DM2500 Germany. The examined sections were classified into several distinct spermatogenic stages according to the most developed germ cell types which were used as reproductive biomarker according to histological gonadal staging adopted by (Kosai *et al*., 2011) as follow: Stage I: Immature testes were

recognized by the absence of spermatogenic activity in the interstitial tissue and the presence of primarily spermatocytes. With no spermatozoa were present in the lobules. Stage II: Early spermatogenesis was characterized by mostly thin interstitial tissue and the presence of primarily immature cells (spermatocytes to spermatids); however, some spermatozoa were also present. Stage III: Midspermatogenesis, the interstitial tissue was moderately thick and some proliferation and maturation of the sperm could be observed; spermatocytes, spermatids and spermatozoa were present in roughly equal proportion. Stage IV: Late spermatogenesis, the interstitial tissue was thick. Although all cell types were represented, spermatozoa predominate in this stage.

On the other hand, Female gonads were classified of being in one of six maturation stages according to the classification scale proposed by Srijunngam and Wattanasirmkit (2001) and Kosai *et al*. (2011); I Chromatin nucleolar stage, II Perinucleolar stage, III Cortical alveoli formation stage, IV Vitellogenic (yolk) stage, V Ripe (mature) stage and VI Spent stage. The developmental stages of the ovarian structures were categories according to the following criteria. Stage I, Chromatin nucleolar stage; the oocyte was small spherical cell containing a central nucleus. The nucleus contained one to four nucleoli together with chromatin network. Cytoplasm was thin layer and strongly basophilic. Stage II, perinucleolar stage; the number of nucleoli increased and were arranged along the inner side of nuclear membrane. Nucleus was large and surrounded by increased mass of cytoplasm, which appeared less basophilic. Follicular cells were monolayer of simple squamous lining surrounded the oocyte. Stage III, cortical alveoli formation stage: this stage is characterized by the appearance of clear vesicles (cortical alveoli) in the cytoplasm. The vesicle was accumulated from the periphery of the oocyte. Although the nuclei were still perinucleolar. The nuclear membrane started to be convoluted. In this stage, a thin acidophilic zona radiata or a primary envelope became visible for the first time. Follicular

layers were also seen at the first time consisting of a simple cuboidal or a columnar layer surrounded with stratified squamous thecal layer. According to Kosai *et al*. (2011); from Stage III, onward a number of ova undergo a process of resorption. At this stage, the whole follicle loosed its shape and was described as an atretic follicle. Stage IV, vitellogenic (yolk) stage: the oocyte size increased. Small yolk granules were visible as a ring of deep eosinophilic in the cytoplasm and later incorporated the whole cytoplasmic area. The nucleus was still convoluted. The zona radiata was clearly visible as a noncellular deep eosinophilic band. Follicular layers were well developed simple cuboidal or columnar layer surrounded by stratified squamous thecal layer. Stage V, Ripe (mature) stage: the stage was characterized by the enlargement of both cortical alveoli and yolk granules. The oocyte size markedly increased. The peripheral migration of the nucleus was observed. The zona radiate was clearly visible. Follicular cells were cuboidal or low cuboidal surrounded by a thin thecal layer. Stage VI spent, this stage was typical of females after spawning. Ovaries were large and containing few large oocytes that probably belonged to the same batch of ova that had just been spawned. They were likely to be rapidly eliminated. Large atretic follicles were observed in the spent ovary; according to Shalloof and Salama (2008); atretic state characterized by liquefied cytoplasm, disintegrated membrane, fibrosis, vacuoles and granulose cells attached to yolk matter become degenerated gradually until the oocyte disappear. Also hypertrophied cells surround oocyte vacuole and erosion of oocyte membrane which gave abnormal shape of oocyte noted. All experimental samples were subjected to two-way analysis of variance test to investigate their significance differences ($p < 0.05$).

Water parameters were adapted from (EEAA, 2011 and 2012) annual report for northern lakes environmental monitoring program (Table 1).

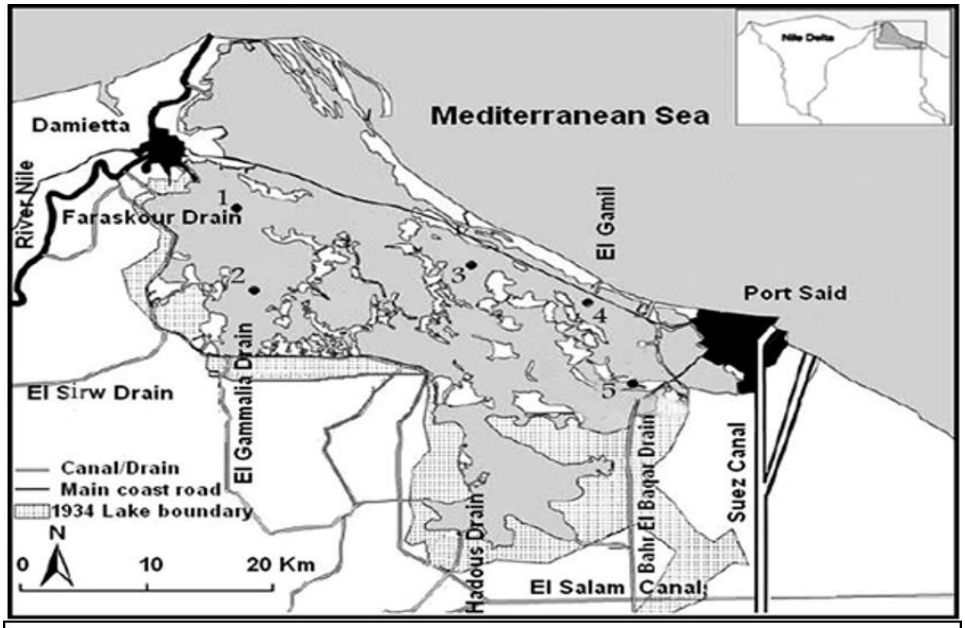


Figure 1. Locations of sampling sites at Lake Manzala. 1: El-Inaniya, 2: El-Sirw, 3: El-Temsah, 4: El-Gamil, 5: El-Bashtir

RESULTS

The present results of Sex ratio was not in the same proportion for both sexes; male and female occurrence in the first season were roughly close; while, in the second season male occurrence was dominant (table **2**). The total lengths in both seasons were slightly changed for both male and females, ranged 15 -17 cm in male and 14 -17 cm in female in the first season and 14-17cm and 14-18cm for male and females respectively in the second season (Table 3 and 4).

Variations in HSI values were noted in all subjected areas in both seasons. While male and female highest recorded values were in El-serw area were 3.08 and 3.6 respectively; first season had the highest HSI in all sampling areas compared to second season.

Furthermore, GSI showed variation in all sampling areas in the first season males and female showed slight differences in GSI value with the highest value in El-Gameel area 1.26 and 1.96 for male and female receptively. While the second season had the highest value, 1.8 for males in El-Serw and 2.7 for females in Bashteer (Table 3 and 4).

Gonadal staging (figures A-F) showed seasonal differences in the first season were the spent stage (V) was the common stage with 100% of the collected samples also some ripe oocytes were found with upnormal membrane shape and vacuolated yolk, which indicate oocyte atresia. While, in the second season mature stage (III) and ripping oocyte stage (IV) where detected in almost all case. Atretic oocyte were observed probably due to the multi-cyclic reproduction of tilapia (multi-group synchronous). Males showed stages II, III and IV were characteristic of sexually mature fish. With the least activity occurring in offseason (stage II) and the most activity taking place immediately prior to and during the spawning season (stage IV). Moreover, testis-ova (To) was found in samples collected in both seasons and all collection sites with different occurrence percentage and severity ranged from 30-60% in first season and 24-45% in second season in all sampling areas.

Discussion

The variation in sex ratio could be due to male mating and food search behaviors characteristics. Offem *et al.* (2007) and Peña-Mendoza *et al*. (2005) mentioned that, it is common that male cichlid populations migrate from spawning areas to feeding grounds in shallow parts of the lakes. On the contrary females, go to ward submerged vegetation and rocky areas to avoid predation and/or capture by fishermen to incubate offspring.

The changes of HSI index values from first season, which has the greatest values compared to that of second season; it could be due to the continuous changes in the hepatic cells glycogen storage and/or lipids.

Which accumulated during resting period and mobilize to the oocyte with the start of yolk deposition into oocytes at the onset of spawning seasons. This assumption was promoted by; the notes of Saeed (2013); Sudarshan and Kulkarni (2013); who mentioned that; fish hepatic tissue store large amount of glycogen during preparatory phase as HSI increases. The decrease in HSI during prespawning phase indicates the storage of hepatic contents to be readily available for gonadal development. Moreover, the common morphological response of fish liver to stresses (spawning and reproduction) may enhance utilization of glycogen as an immediate source meeting the energy demand during spawning seasons. The GSI increased slightly from preparatory to pre-spawning phase, while maximum decrease occurred during spawning and post spawning phases.

The presence of atretic oocytes in the spawning season could be due to certain pattern of gametes development and their association with both internal and external causes, that create unfavorable conditions for reproduction e.g., LH surge failure, stress, low feeding intake, water quality, heavy metals and endocrine disrupting chemicals (PAHs, PCBs bisphenol A).

As shown in Table (1) PAHs, PCBs were detected in Lake Manzala with annual average values 0.74µg/l and 2.96 ng/l respectively. Barakat *et al.* (2012) mentioned that PCBs are major contaminants in Lake Manzala especially in El-Gamil that contained relatively high concentrations of PCBs. Other authors such, Hose *et al.* (1989); Thomas (1989) and Banaee *et al.*, 2008; indicated that, heavy metals, PAHs and PCBs have been related to wide variety of physiological disorders in fish e.g., alterations in blood sex steroid hormone concentrations, oocytes quality, vitellogenesis process impairment, gonads maturation delay which eventually lead to oocyte resorption or atretic oocyte. It could be interpreted from that, the alterations in blood sex steroid hormone concentrations caused by such pollutants. Affect the hormonal path way

illustrated by Amer *et al.* (2001) and Nagahama (1997) which showed that the ovulatory surge of LH (GtH-II) results in a shift in steroidogenesis, which increases the production of the maturation-inducing hormone 17 alpha,20 beta-dihydroxy-4-pregnen-3-one (17,20-P); action of GnRH is effectively blocked by LH, which is produced in increasing amounts during late vitellogenesis. Hence, GnRH induction of apoptosis in the fish ovary of fish will only be possible if the ovulatory surge of LH fails to happen, due to either of inappropriate environmental cues or the presence of endocrine disrupting chemicals in the environment.

The presence of testis-ova displayed the influence of environmental pollutants and /or heavy metal on male testis; which differed in severity from null to sever according to sampling area and the type of effluents agricultural; swage, agricultural and industrial effluents drainage. These could lead to a complex interaction among those chemicals overtime and the accumulation additive effect on the reproductive process of the entire fish population inhabit Lake Manzala in general. So, the findings of this study is in agreements with the findings of Sadekarpawar and Parikh (2013); Testis of fish represents the most dynamic organ having a high cell turnover during the reproductive period which makes it vulnerable to a wide variety of chemical toxicants. Moreover, Kosai *et al*. (2011) pointed that, exposure to estrogenic chemicals or xenoestrogens during the critical periods of sex differentiation has induced testis-ova in several fish species. Johnson *et al*. (2009); stated that, elevated occurrence of intersex in wild fishes populations has been associated with exposure to human wastewater effluent. Blazer *et al*. (2007) noted that estrogens 17b-estradiol, and the synthetic estrogen 17a-ethinylestradiol used in birth control and hormonal medication were found in these effluents. Furthermore, Bakke (2003) showed that agricultural runoff has also been associated with endocrine disruption or reproductive abnormalities due to the presence of natural and synthetic hormones, pesticides, and

herbicides. Bin-Dohaish (2012); mentioned that, synthetic products such as biphenol A, polychlorinated biphenol, dioxins, phtalates, pesticides, heavy metals, alkyphenols, polycyclic aromatic hydrocarbons, ethinylestradiol and estradiol. Such compounds, among others, that seeping from sewage water to aquatic environments are associated with observation of testis-ova in males and changes in secondary sex characteristics of male and female.

CONCLUSION

The present data highlighted the reproductive alteration caused by environmental pollutants for both male and female fish. This may influence the number of oocytes released in the up-coming spawning seasons; the developmental rate of next oocyte generations; the rate of sex cycles or reproductive failure on the long run. Such changes can inflict a significant damage on the economic revenue of Lake Manzala. Thus, it is recommended to increase water circulation through inlet purgation to mitigate pollutants and restocking the lake until it regains its natural balance.

Table 1. Water Parameters.

Parameter	Reading*
pH	8.2
Oxygen (mg / L)	8.5
Temperature range (C°)	22.9
Salinity ‰	4.6
Ammonia (mg / L)	0.96
Nitrite (mg / L)	0.005
TOTAL Nitrogen TN (mg / L)	4.79
PCBs (ng)	2.96
PAHs (Mcg)	0.74

Adopted from EEAA Lake Manzala annual report 2011-2012;* All readings are expressed as annual average

Table 2. Sex ratio in different investigated areas For Season1 and 2 Samples**.**

Area	♂ Sex ratio S1	♂ Sex ratio S2	♀ Sex ratio S1	♀Sex ratio S2
Bashteer	80.00	85.29	20.00	14.71
El-Enania	46.67	64.29	53.33	35.71
El-Gameel	66.67	68.42	33.33	31.58
El-Serw	46.67	73.53	53.33	26.47
El-Temsah	51.61	91.30	48.39	8.70

Table 3. First and Second Sample males anatomical Data expressed as averages ± SE.

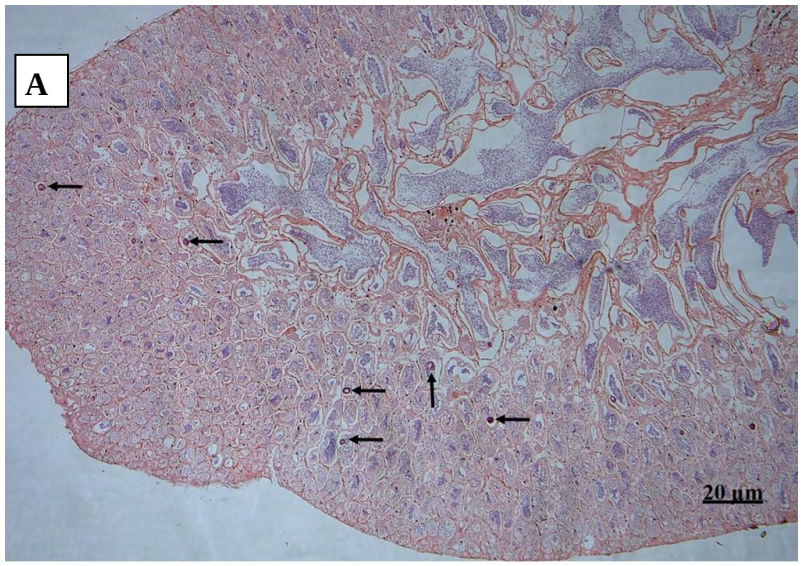
Area	TW	TL	GW	LW	G.S.I	H.S.I
Bashteer S_1	87.21 ±6.9	17.25 ±0.42	1.05 ±.020	2.60 ±0.35	1.18 ±0.21	2.95 ±0.2
Bashteer S_2	50.83 ±1.71	14.02 ±0.16	0.53 ±0.1	1.01 ±0.19	0.98 ±0.18	2.00 ±0.4
El- Enania S_1	87.56 ±11.47	17.61 ±0.64	0.78 ±0.18	1.77 ±0.36	0.92 ±0.23	1.88 ±0.19
El- Enania S_2	118.63 ±14.49	17.81 ±0.67	0.29 ±0.09	2.06 ±0.32	0.29 ±0.11	1.73 ±0.19
El- Gameel S_1	76.46 ±12.65	16.17 ±0.89	0.81 ±0.15	2.17 ±0.38	1.26 ±0.29	2.83 ±0.30
El- Gameel S_2	36.89 ±3.02	12.39 ±0.30	0.20 ±0.07	0.58 ±0.05	0.67 ±0.29	1.62 ±0.18
El- Serw S_1	96.92 ±3.28	17.07 ±0.30	0.62 ±0.11	3.00 ±0.30	0.66 ±0.13	3.08 ±0.29
El- Serw S_2	57.54 ±4.46	14.88 ±0.26	0.92 ±0.10	0.84 ±0.10	1.83 ±0.22	1.53 ±0.17
El- Temsah S_1	66.09 ±7.43	15.57 ±0.63	0.57 ±0.23	1.81 ±0.37	0.93 ±0.49	2.63 ±0.35
El- Temsah S_2	31.20 ±2.33	11.32 ±0.22	0.05 ±0.01	0.35 ±0.03	0.17 ±0.04	1.23 ±0.11

S1= first season, S2= second season, Tw = total body weight (g), TL = total length (cm), Gw = gonad weight (g), LW = liver weight (g), GSI = Gonadosomatic index (%), HSI = Hepatosomatic index (%).

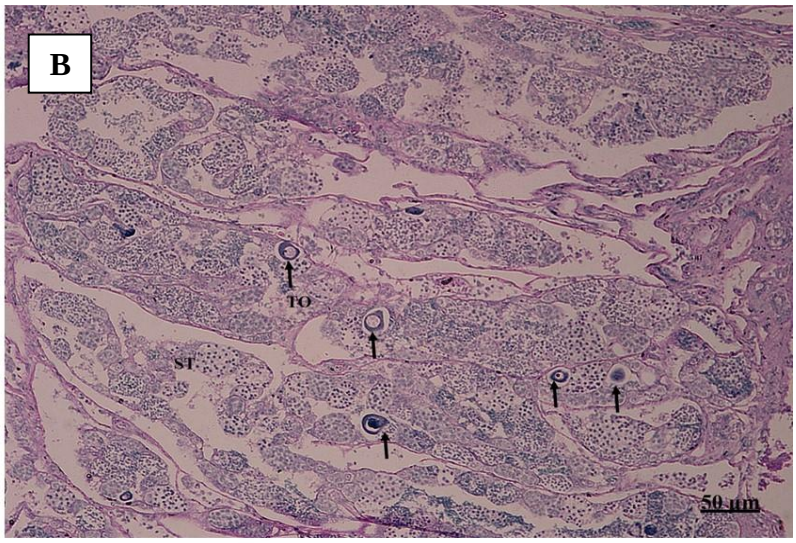
Table 4. First and Second Sample females anatomical Data expressed as averages ± SE.

Area	TW	TL	GW	LW	GSI	H.S.I
Bashteer S_1	50.92 ±4.42	14.52 ±0.37	0.61 ±0.21	1.44 ±0.43	1.35 ±0.49	2.73 ±0.57
Bashteer S_2	53.38 ±2.31	14.94 ±0.42	1.36 ±0.42	0.72 ±0.15	2.69 ±0.97	1.40 ±0.35
El- Enania S_1	69.12 ±13.7	16.34 ±2.78	0.40 ±0.06	1.62 ±0.28	0.60 ±0.09	2.41 ±0.40
El- Enania S_2	115.17 ±14.27	18.28 ±0.68	2.66 ±0.46	1.59 ±0.25	2.60 ±0.51	1.44 ±0.23
El- Gameel S_1	51.77 ±6.53	14.33 ±0.61	0.82 ±0.30	1.40 ±0.23	1.96 ±0.88	2.72 ±0.23
El- Gameel S_2	31.07 ±2.48	11.85 ±0.32	0.45 ±0.13	0.57 ±0.09	1.55 ±0.52	1.84 ±0.33
El- Serw S_1	108.50 ±16.32	17.38 ±0.8	1.12± 0.34	3.82 ±0.56	1.01 ±0.31	3.60 ±0.32
El- Serw S_2	57.77 ±6.42	14.81 ±0.42	0.77 ±0.19	0.58 ±0.09	1.47 ±0.41	0.98 ±0.11
El- Temsah S_1	52.84 ±1.66	14.72 ±0.15	0.58 ±0.17	1.78 ±0.29	1.08 ±0.31	3.31 ±0.48
El- Temsah S_2	31.90 ±7.52	11.50 ±0.50	0.50 ±0.00	0.45 ±0.25	1.66 ±0.39	1.30 ±0.48

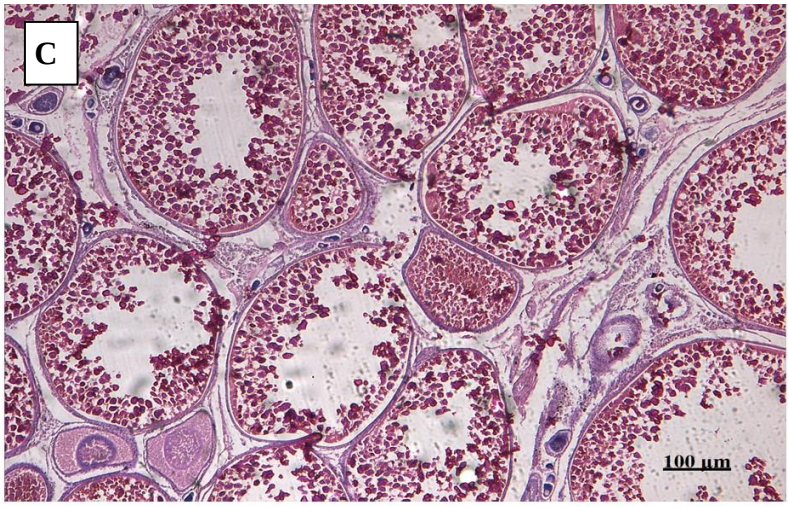
S1= first season, S2= second season, Tw = total body weight (g), TL = total length (cm), Gw = gonad weight (g), LW = liver weight (g), GSI = Gonadosomatic index (%), HSI = Hepatosomatic index (%).



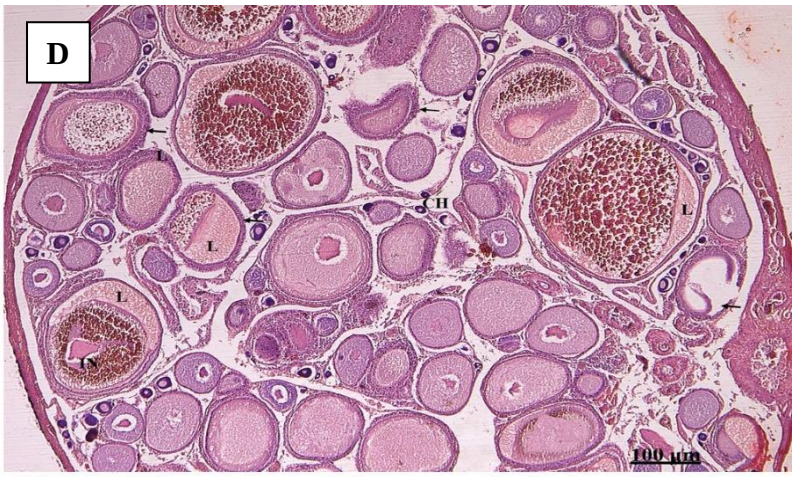
T.S of *O. niloticus* testis general view arrows referring to testis-ova.



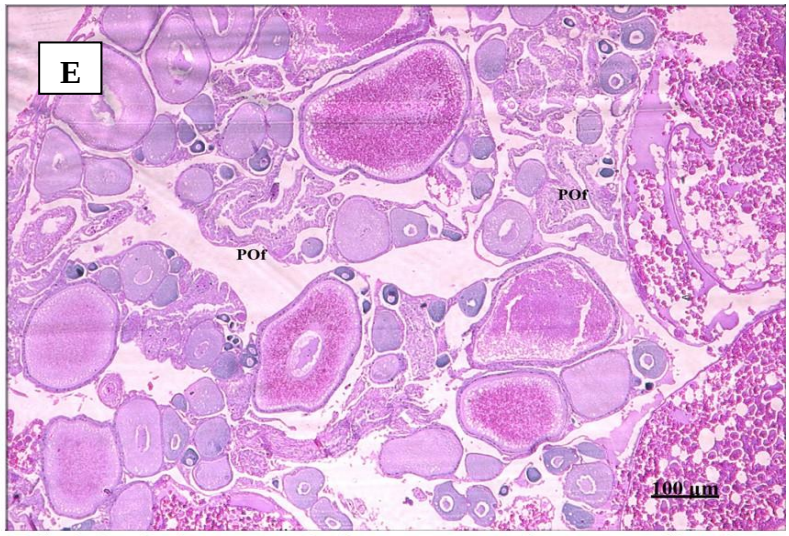
T.S of *O. niloticus* testis arrows referring to testis-ova (TO).



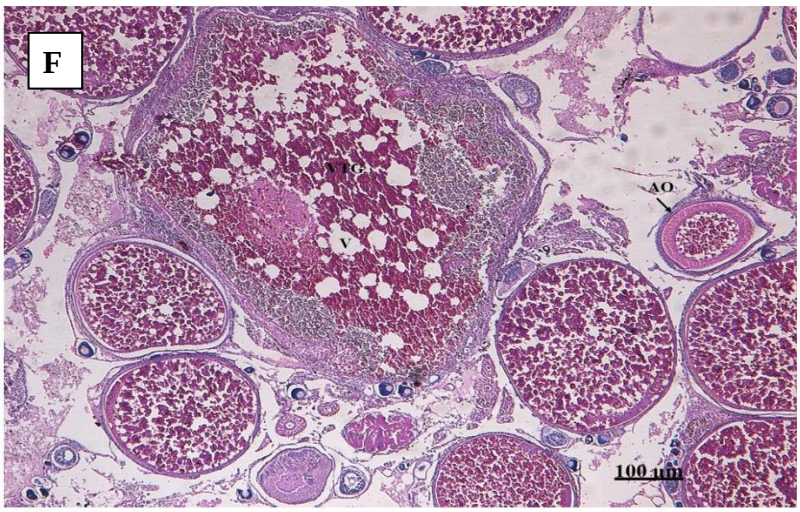
T.S of *O. niloticus* ovary during spawning season showing maturation phase



T.S of *O. niloticus* ovary general view arrows referring to atretic oocyte, chromatin stage oocte (CH), irregular nucleus (IN), cytoplasm liquefied in atretic oocyte (L).



T.S of *O. niloticus* ovary during post- spawning season. Showing different patterns of atresia of post ovulatory follicles (POF).



T.S of *O. niloticus* ovary during post- spawning season. atretic oocyte (AO), vacuole (V), vitellogenin (VTG).

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اضطرابات الغدد التناسلية كمؤثر ناتج عن الاستروجينات البيئية فى أسماك البلطي ببحيرة المنزلة

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الملخص العربى

يعد تواجد Tesits-ova في الأسماك منفصلة الجنس إحدى العلامات المرجعية لتعرض الأسماك لتلوث بالاستروجينات البيئية مثل المركبات العطرية المتعددة وثنائي الفينيل متعدد الكلور. فى اوخر عام ٢٠١١ تم عمل مسح استكشافي لعلامات التلوث بالاستروجينات البيئية في بحيرة المنزلة 31° 16′ 0″ N, 32° 12′ 0″ E ثم اتبع بأخر في ٢٠١٢.

وقد أوضحت النتائج المتحصل عليها وجود بويضات Tesits-ova بداخل النسيج الخصوي للذكر وكذلك وجود اختلافات في كل من دليل المناسل (GSI) والدليل الكبدي (HIS) في الجنسين وفي كلا الموسمين. لذلك تم عمل دليل إصابه (٠–٤) للذكور يعتمد على عدد البويضات داخل القطاع، كما تم التحقق من مدي صلاحية البيانات إحصائيا لكلي الموسمين.

كما تم أيضا حساب نسبة الإصابة في كلى الموسمين وتراوحت النسبة في الموسم الأول ٣٠–٦٠% بينما كانت في الموسم الثاني ٢٤–٤٥% في كل مواقع أخذ العينات. واتضح أن أعلي نسبة إصابة حدثت في موسم مابعد التفريخ وما قبل التفريخ مباشرة بينما أقل نسبة حدثت في أثناء موسم التفريخ.

على الصعيد الأخر أظهرت مناسل الإناث اختلافات موسمية ففي الموسم الأول كانت المرحلة السائدة من المبيض فى جميع العينات هي spent stage (V)، بينما في الموسم الثاني كانت المرحلة المبيضة السائدة هي mature stage (III) and ripping oocyte stage (IV).