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# Histopathological observations of gonads and liver of African catfish *Clarias gariepinus* induced by a commercial insecticide Thalis 112 EC

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## Abstract

*Thalis 112 EC, a binary insecticide consisting of synthetic molecules Emamectin benzoate (48g/l) and Acetamiprid (64g/l) is used in cotton growing in Benin. In several ways, this insecticide ends up in waterways. However, the harmful effects of this biocide on the aquatic biotopes, ultimate receptacles of these pollutants and especially on the aquatic biocenosis are not well studied and reported. Our study aims to investigate the histopathological observations of the gonads and liver of a well-represented species in the ecosystems of Benin, the African catfish (Clarias gariepinus). Fish (42.3 ± 15.73g) were submitted to six sub-lethal concentrations (in triplicates) of Thalis (0.00, 0.03, 0.6, 0.12, 0.25 and 0.49ppm) for 28 days. Fish gonads and liver were sampled on days 28 of Thalis exposure. Observations of samples revealed a down regulated spermatogenesis while oogenesis was upregulated with atretic follicular oocytes up to 60% in females, indicating an estrogenic effects related to Thalis exposure. Degenerative alterations such as necrosis, vacuolation, fibrosis, lobes with immature cells within their lumen, lobular disorganization, have been observed in the testis, while necrosis, cytoplasmic vacuolation and cytoplasmic retraction have been identified in the ovaries. The histopathological abnormalities observed in liver were necrosis, vacuolation, melanomacrophagic centres, hydropic changes and nuclear hypertrophy. The present survey indicates that Thalis can affect the gonads and liver of African catfish. Therefore, this pesticide should be cautiously handled in agriculture away from any natural sources of water.*

**Keywords:** Thalis 112 EC, toxicity, gonads, livers, African catfish, histology.

## Introduction

In Benin, agricultural sector contributes to almost 32% to gross domestic product[1]. Among the many speculations produced, cotton has taken an important and essential place in the national economy. In fact, according to the latest publications of the General Directorate of Economic Affairs, cotton production experienced an increase of 32.4% in 2018 compared to 2016, which makes the country the first cotton producer in Africa[2]. The sector has long benefited from the support of the State, and served as a means of combating poverty and the promotion of development[3]. But that was not done without consequences especially on the environment because approximately 90% of all the pesticides imported in Benin are used in cotton production[4]. Up to 98% of active substances used in agriculture or public health are dispersed in the environment by air, infiltration, and runoff, reaching foods chain and threaten aquatic species[5]. Several studies have already shown that pesticides used in cotton fields contaminate aquatic ecosystems and have harmful effects on living fish in these biotopes in northern Benin[6-9]. The least low dose of these substances are likely to disturb the nervous system, the hepatic system, hormonal regulation, reproduction, embryonic development and fish growth[6-8]. Therefore, exposure to such pesticide is potentially toxic and alter the structural integrity of cells, tissues and organs of fish[10]. In recent years, several pesticide molecules have been introduced into the phytosanitary cotton plant program in Benin to strengthen production. Current practices consist to mix several chemical substances to have a more effective pesticide against resistant cotton pests[8]. Among these combinations, we have Thalis 112 EC which is an emulsifiable concentrate based on Emamectin benzoate and Acetamiprid at respective concentrations of 48g/l and 64g/l[8,11]. Thalis is an aphycidal insecticide used to kill biting-sucking insects and first-generation carpophagous butterflies such as *Helicoverpa armigera*[4].

Emamectin benzoate belongs to the Avermectin family[12]. Juvenile African catfish *Clarias gariepinus* are strongly sensitive to Avermectin, with 15µg/l as LC50 under conditions of non-renewal of the test solutions[13]. Emamectin benzoate is commonly used in aquaculture to treat fish against parasites but its effects on the integrity of cells, tissues or fish organs like gonads and the liver are not known.

Acetamiprid, a first generation molecule of Neonicotinoid family, has its LC50 estimated at 265.7ppm for juveniles African catfish[14]. Acetamiprid is found to be reprotoxic in male mice[15] and impairs endocrine functions with feminization and transgenerational effects in Zebrafish *Danio rerio*[16].

The isolated effects of molecules being largely different when combined, this survey aims to assess in controlled conditions, the harmful effects of Thalis on the reproductive and hepatic systems of African catfish, a extensively used species in the Benin's cotton basin. It is a histological analysis of samples collected from gonads an liver of African catfish reared in a Thalis contaminated environment.

## Materials and methods

**Animal acquisition:** Three hundred African catfish with an average weight of 42.3±15.73g, were collected in a fish farming complex equipped with a closed circuit located in Parakou in Benin (50% females and 50% males). Fish were appropriately in aerated water, packed in polythene bags, and transported to the University of Parakou in Research Laboratory in Aquaculture and Aquatic Ecotoxicology (LaRAEAq).

**Acclimation:** The fish were acclimated under laboratory conditions for 21 days according to OECD 215 guidelines, before the start of the test and fed twice a day with commercial GOUESSANT dry food (Protein 46%; diameter 2mm). The glass tanks were connected to a constantly operating aeration system with a light-dark cycle of 12±1:12±1. The temperature was maintained between 26.2°C and 30°C and the pH between 7.0 and 7.8. During this acclimatization period, the water in the stock was renewed once every 48 h to avoid the deposit of waste such as decomposing food residues.

**Chemical:** Thalis 112 EC, an insecticide consisting of synthetic molecules Emamectin benzoate (48g/l) and Acetamiprid (64g/l) and used by cotton growers in northern Benin was buy at "*Société de Distribution des Intrants*" for the test. This pesticide is yellow in color and viscous liquid in nature. Test solutions were obtained by mixing the insecticide, which is an emulsifiable concentrate, directly with chlorine-free water. All of the test solutions were prepared immediately before testing.

**Experimental design:** The test was carried out in accordance to OECD directions 215 with some modifications. Based on the value of 96 h LC50 = 4.9 ppm[17], five sub-lethal concentrations (T1, T2, T3, T4 and T5) i.e. 0.03ppm (0.6% LC50), 0.06ppm (1.2% LC50), 0.12ppm (2.5% LC50), 0.25ppm (5% LC50) and 0.49ppm (10% LC50) respectively were chosen for the experiment. The T0 control solution is made from single dilution water. Eighteen glass aquariums (30 L capacity) were used for these six treatments in triplicate. Fourteen fish were randomly assigned to each glass aquarium containing 25 L of test solutions. The experiment was carried out for 4 weeks (28 days) with a renewal of half of the experimental solutions every two days. During the experiment, the fish were fed ad libitum morning, noon and evening each day with an artificial granulated food GOUESSANT (Proteins 46%; diameter 3mm). The glass tanks were connected to a constantly operating aeration system with a light-dark cycle of 12±1:12±1. Physicochemical data such as pH (6.87±0.05) and temperature (27.02±0.32°C) of water were recorded every 2 days before renewing the experimental solutions. The glass tanks were protected with small-mesh nets to prevent fish escaping. No mortality was noted in any treatment during this experiment.

**Organs sampling:** The samples were taken on the 28th day (D28) at the end of the exposure. Per treatment, 12 individuals (06 males and 06 females) were sampled. The fish were dissected after anesthesia with the MS 222. The left gonads and the liver were then removed and preserved in acetic formaldehyde 4% solutions for fixation and histopathological examinations.

**Histopathological analysis:** Specimens preserved in formaldehyde were sent to the Laboratory of Histology of the University Joseph Ki Zerbo (Burkina Faso) for histological treatments. In this laboratory, the tissues underwent conventional treatments (dehydration by solutions of increasing concentrations of methanol and toluene) then embedded in paraffin.

In male individuals, the testes were sectioned through a series of 5µm sections and one paraffin section was mounted every 50µm. For histological analyses, the sections were colored with the classic dye mixture made of Hematein and Eosin (HE) and examined using an Olympus brand Cx23 light microscope at a range of magnifications (10–40X). The stages of spermatogenesis such as spermatogonia A, spermatogonia B, spermatocytes I, spermatocytes II, spermatids and spermatozoa, were assessed by counting the cells in a bounded area using the microscope, which was randomly repeated three times in each section of testis[6,7]. These six steps of spermatogenesis have been identified depending on the structuring of the OECD[18].

In female individuals, the ovaries were cut in 5µm and mounted on glass slides. Some cross sections were colored with HE and examined under a light microscope at a magnification range (10–40X). Maturation stages of oocytes such as nucleolar chromatin oocytes, perinucleolar oocytes, cortical alveolar oocytes, early vitellogenic oocytes, late vitellogenic oocytes and spawning oocytes, were estimated according to OECD[18]. The percentage of each stage was determined on one hundred cells counted per ovary[6,7].

A sample (mid-section) of each liver fixed in acetic formaldehyde 4% solutions was treated as gonad tissues and carve at 6µm. Sections were placed on glass slides and colored with conventional HE stains.

For these three organs analyzed (testis, ovaries and liver), a qualitative histopathological evaluation was carried out to

identify the lesions using a multifocal Olympus in order to make the results more objective. Results were also assessed semi-quantitatively using a modified scoring protocol from Bernet et al.[19]. The value of the score and the importance factor of each lesion were multiplied and these results for all the lesions found were then added together to engender a testicular indice (It), an ovarian indice (Io) and a liver indice(IL) respectively depending on the organ for each fish. The following formula from Bernet et al.[19] enables these indices to be determined:

$$Iorg = \sum_{rp}.\sum_{alt}(\alpha\ org\ rp\ alt\ \mathrm{X}\ \omega\ org\ rp\ alt$$

Where: org = organ (constant), rp = reaction pattern, alt = alteration, α = score value and ω = importance factor.

The index thus calculated were classified to assess the intensity of the lesions of each organ depending on a grading system of Van Dyk et al.[20,21] based on the scoring scheme proposed by Zimmerli et al.[22].

Class 1 (index <10): Normal organ structure with slight histological alterations.
Class 2 (index 10–25): Normal organ structures with moderate histological alterations.
Class 3 (index 26–35): Pronounced alterations of organ tissue.
Class 4 (index >35): Severe alterations of organ tissue.

**Data analysis:** The data was entered into the Excel 2013 spreadsheet and processed using the Minitab 18 statistical software. The fish is the experimental unit. In order to make weak the effect of gender, the data were processed by sex. The differences between the means were evaluated with an ANOVA 1 using the concentration of Thalis as single factor for each gonadal stage. The data plotted are means ± SD normalized by Log(x+1). P-value≤0.05 was considered statistically significant. The histological analyzes of the tissues being qualitative, the methods of Bernet et al.[19], Zimmerli et al.[22] and Van Dyk et al. [20,21] were used for the comparisons.

## Results and discussion

**Testis histology: Maturation stages and alterations:** The test is removed after 28 days of exposure show the first three stages (Spermatogonia A, Spermatogonia B and Spermatocyte I) in various proportions according to the treatments (Figure-1). Spermatogonia A are more represented in all individuals, including controls, but in higher proportions in individuals exposed to Thalis compared to controls. The percentage of Spermatocytes I is significantly lower in all exposed individuals compared to unexposed individuals (P<0.05).

The alterations identified in individuals exposed to Thalis are: infiltration of adipocytes, fibrosis, necrosis, lobes with immature cells within their lumen, lobular disorganization. In unexposed individuals, there were only rare necrotic cells. Some lesions observed in contaminated individuals are shown in Figure-2.

**Testis index:** The histopathological indices of the test is calculated from the alterations identified and their occurrences are summarized in Figure-3. The indices of the testis of Thalis exposed fish vary between classes 3 and 4, while that of the control is class 1.

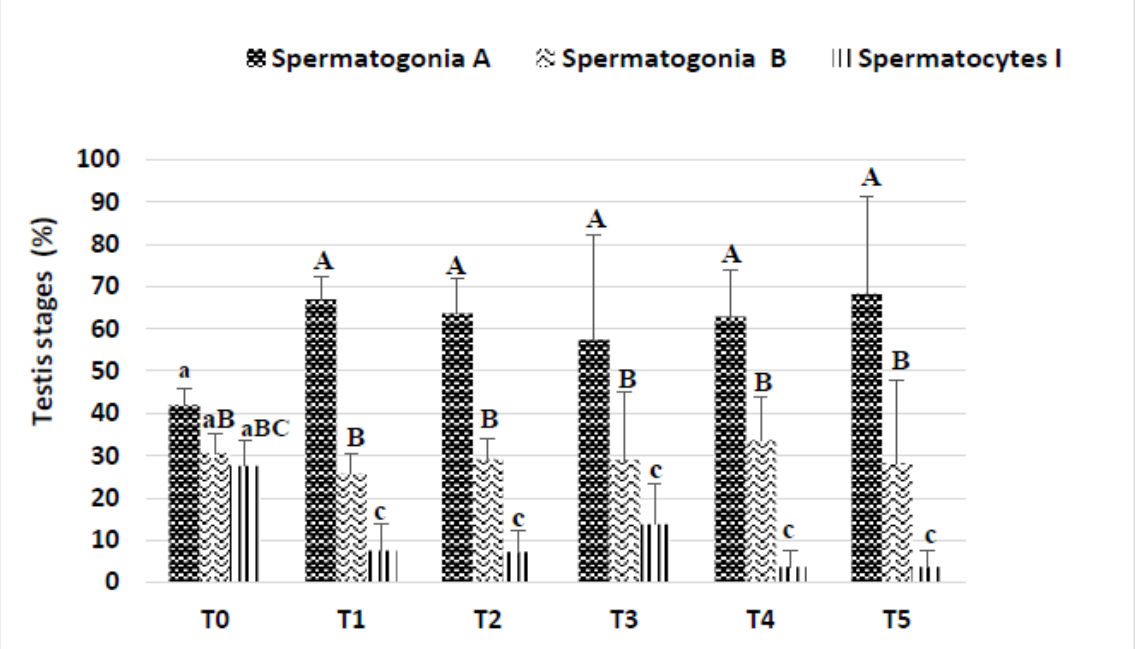


**Figure-1:** Percentages of different testis stages in *Clarias gariepinus* submitted to sub-lethal concentrations of Thalis.

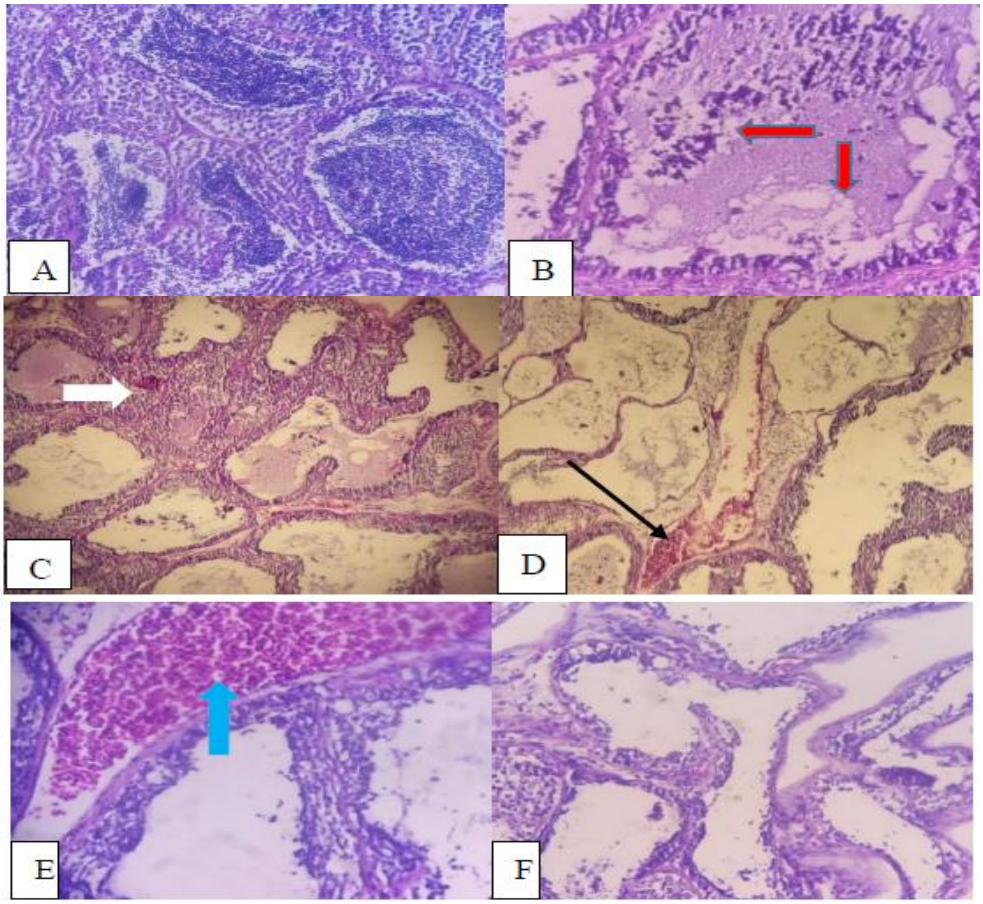


**Figure-2**: Histological micrographs of testis section of *Clarias gariepinus* colored with HE indicating (A) Normal testis; (B) Adipocyte infiltration (arrows); (C): Fibrosis (arrow); (D): Focus of necrotic cells (arrow); (E) lobes with immature cells within their lumen (arrow); (F): lobular disorganization, Magnification: 20X.

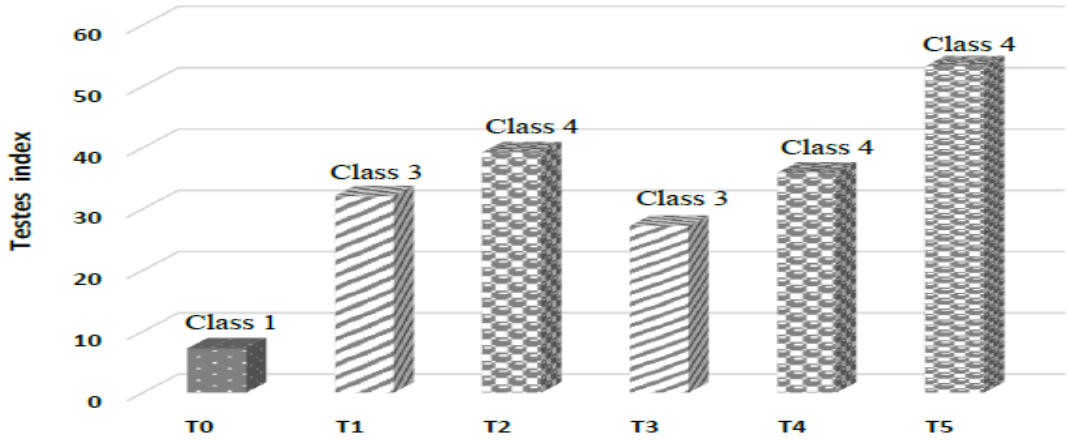


**Figure-3:** Testes index in *Clarias gariepinus* submitted to Thalis.

**Ovary histology: Maturation stages and alterations:** The four first oocyte stages (perinucleolar oocytes, cortical alveolar oocytes, early vitellogenic oocytes and late vitellogenic oocytes) were identified after 28 days of exposure in all female fish including controls but in different proportions (Figure-4). The ovaries of female fish from T2 to T5 show a significantly very low percentage of perinucleolar oocytes, cortical alveolar oocytes and early vitellogenic oocytes compared to controls, with late vitellogenic oocytes almost very abundant in these exposed fish ($p<0.05$).

The most important lesions identified in the ovaries of Thalis contamined female fish are: necrosis, vacuolization, decrease in the volume of the cytoplasm with peeling off the cytoplasmic membrane of the perinucleolar oocytes and pre-ovulatory atretic oocytes up to 60% in T5 (Figure-5). In uncontaminated female fish no lesions were found.

**Ovary index:** The histopathological indices of the ovaries calculated from the alterations identified and their occurrences gave class 2 values for T2, T3 and T4 females and class 3 for T5 females but class 1 values for the non-contaminated females (Figure-6).

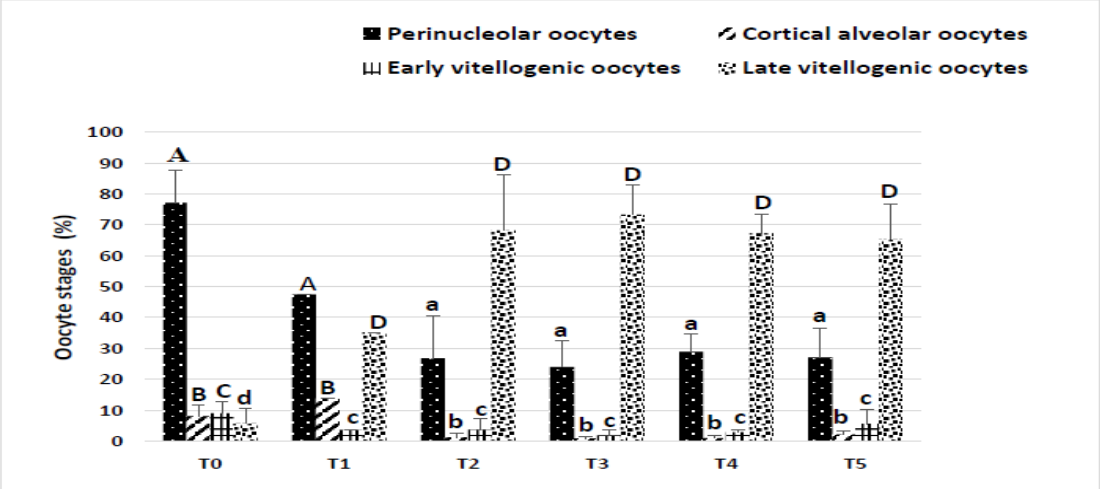


**Figure-4:** Percentages of different oocyte stages in *Clarias gariepinus* submitted to increasing concentrations of Thalis.

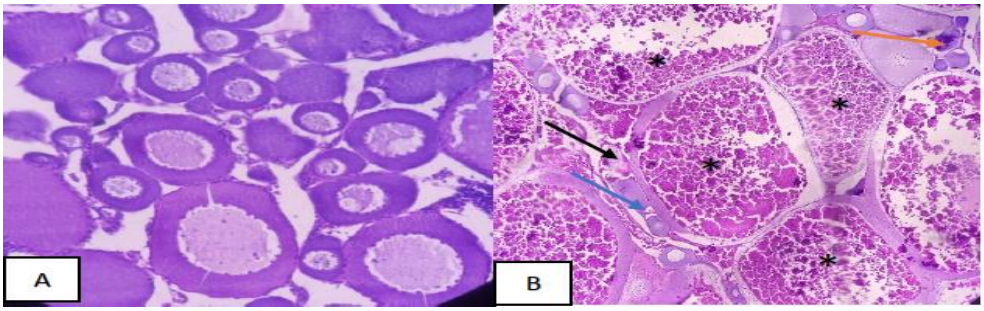


**Figure-5:** Histological micrographs of ovary section of *Clarias gariepinus* colored with HE indicating (A) Normal ovarian tissue; (B): Necrosis (yellow arrow), Vacuolization (black arrow), Peeling off the cytoplasmic membrane of the perinucleolar oocytes (blue arrow) and pre-ovulatory atretic follicles (black asterix); Magnifications: 20X.

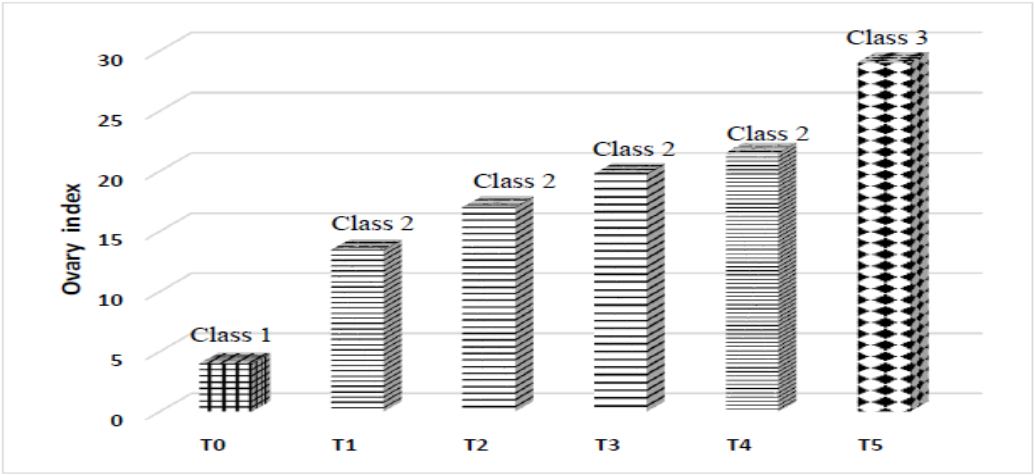


**Figure-6:** Ovary index in *Clarias gariepinus* submitted to Thalis.

**Liver histology: Alterations identified:** The most frequent alterations identified in the liver of fish exposed to Thalis for 28 days regardless of sex are progressive (Focal zone of hepatocyte hypertrophy (dropsic change), nuclear hypertrophy) and regressive (vacuolation of the cytoplasm of hepatocytes, melanomacrophagic centre (MMC), focus of necrotic cells). Only a few rare vacuoles and MMCs were observed in control individuals regardless of sex (Figure-7).

**Liver index:** The indices were calculated according to the type of alteration and the severity of the alterations. The indices calculated for the contaminated fish, whatever the sex, varied between class 3 and 4, while those of the controls remained in class 1 (Table-1).

**Discussion:** The variation in sperm and ovarian stages levels in exposed fish indicates that Thalis insecticide had an inhibitory effect on spermatogenesis and stimulatory effect on oogenesis. Indeed, Agbohessi et al.[7] had also demonstrated that Endosulfan had a delaying effect on the spermatogenesis and oogenesis-promoting effect of this same species African catfish. Agbohessi et al.[6] had also revealed in the cotton basin in northern Benin the inhibitory effect of the agricultural runoff on spermatogenesis and the stimulatory effect of oogenesis in *Coptodon guineensis* and in African catfish. These authors have shown that the pollutants which have the capacity to decelerate the process of spermatogenesis and accelerate oogenesis are xenoestrogens. This effect of Thalis revealed in the present study is surely due to its Acetamiprid component which has already proven its feminizing effect in *D. rerio*[16] and in male rats[23]. The estrogenic effect of Thalis revealed in the present study materialized in females by the high rate of pre-ovulatory atretic oocytes. But we did not have, conversely in the males, testis-ova. The absence of testis-ova is surely linked to the low concentrations of the insecticide tested and especially to the very short duration of the experiment.

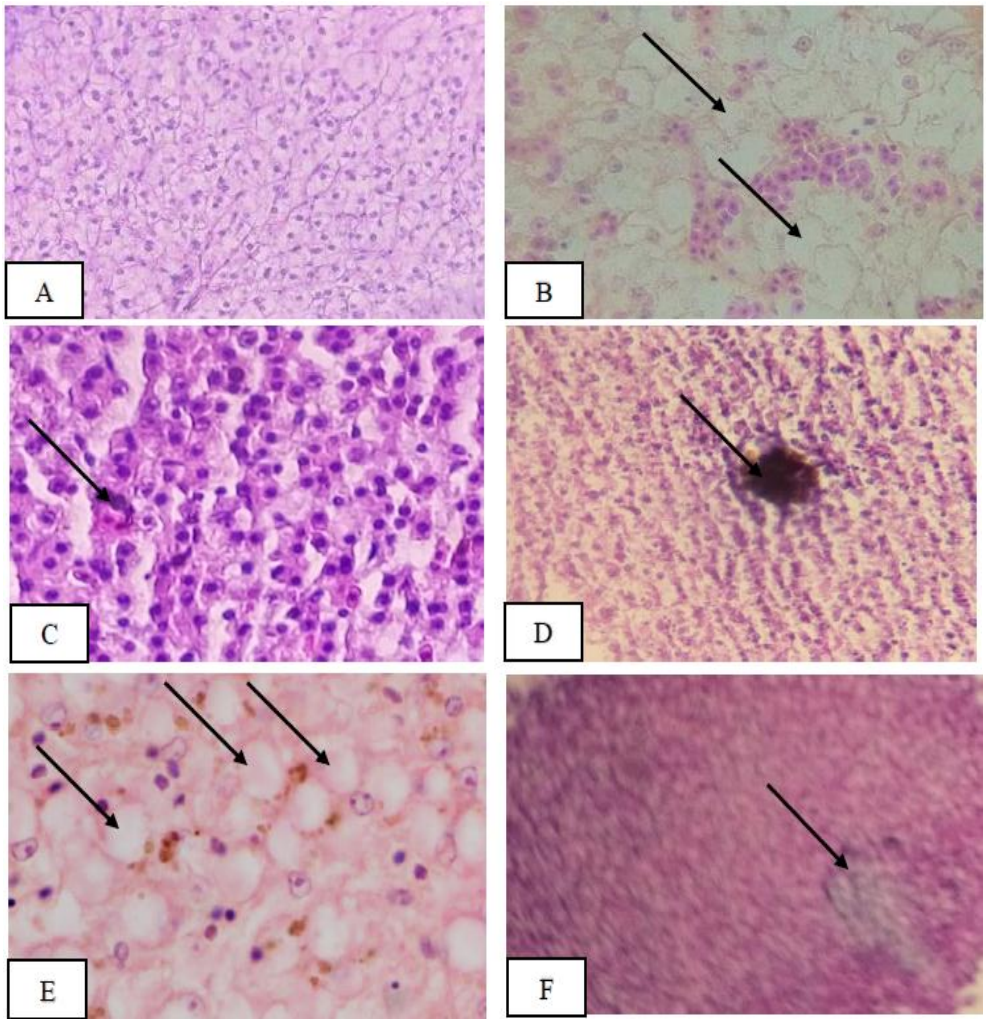


**Figure-7**: Histological micrographs of representative liver sections stained with HE indicating (A) Normal tissue; (B): Focal zone of hepatocyte hypertrophy, dropsic change (arrow); (C): Nuclear hypertrophy (arrow); (D): Melanomacrophagic centre (arrow); (E): Vacuolization (arrows); (F): Focus of necrotic cells (arrow). Magnification: 20X.

**Table-1***:* Liver index of *Clarias* gariepinus submitted to Thalis.

| Treatments | Liver index males | Liver index females |
|---|---|---|
| T0 | 5.11 ± 3.72 (1) | 8.78 ± 6.82 (1) |
| T1 | 39.33 ± 6.75 (4) | 40.89 ± 9.49 (4) |
| T2 | 37.75 ± 7.74 (4) | 42.56 ± 6.73 (4) |
| T3 | 30.5 ± 13.98 (3) | 31.11 ± 6.43 (3) |
| T4 | 30.5 ± 13.98 (3) | 41.11 ± 11.58 (4) |
| T5 | 28.55 ± 5.65 (3) | 34.63 ± 20.30 (3) |

In this evaluation, we showed that Thalis induced variable level of gonadal alterations in both female and male fish. Necrosis (death of sperm cells, etc.), fibrosis (transformation of part of the testis into connective tissue), infiltration of adipocytes or lipid vacuolation, lobular disorganization and the lobes with immature cells within their lumen observed in exposed male fish, are inflammatory reactions associated with regressive changes[19]. These alterations were observed in the same specie contaminated with Endosulfan[24]. These different lesions in exposed male individuals were also observed in *Coptodon zillii*, *Neochanna diversus* and African catfish captured in a pond exposed to agricultural effluents[25], in African catfish subjected to Benzyl Butyl Phthalate[26] and in *D. rerio* contaminated with Mancozeb[27]. In female fish subjected to Thalis, alterations such as necrosis, vacuolation of cytoplasm and retraction of cytoplasm were identified in this survey. Similar disturbances have been identified by Bashir et al.[28] in *Catla catla* exposed to harmful materials e.g. heavy metals, pesticides, dyes and hydrocarbons, by Deka and Mahantan[29] in *Tilapia niloticus* contaminated with Malathion, and by Ramachandra[30] in *Lepomis macrochirus* subjected to Diazinon. The latter specifies that these findings and especially the pre-ovulatory atretic oocytes suggest that the histopathological changes in the ovary might be a reflection of the disturbance in the endocrine/hormonal imbalance.

The gonad indices (testis index and ovary index) indicate that the testis of the males most exposed to Thalis (T2 to T5) have reached class 4 (severe alterations) while the ovaries of T5 females are of class 3 (pronounced alterations). This observation confirms the feminizing effect of Thalis revealed in the present study since several studies have proven that when there is an estrogenic effect, the male gonads are the most affected[6,7].

The main detoxifying organ of xenobiotics is the liver[8,17,31] and in pesticides contained fish, it undergoes significant structural changes[32]. Due to its massive blood supply and involvement in metabolism, the liver is particularly vulnerable to these dangerous substances[33]. The present study found that after Thalis exposure, the liver of African catfish exhibits regressive changes (necrosis, vacuolation and MMC) and progressive changes (hydropic changes and nuclear hypertrophy), which are similar to those shown in other studies of fish exposed to different pollutants. When subjected to Diazinon, Banik et al.[34] observed comparable abnormalities (necrosis, vacuolation) in the liver of *Glossoglobius giuris*. In *Trichogaster fasciata* exposed to Thiamethoxam, Hasan et al.[35] noticed vacuolation and necrosis. In liver of African catfish exposed to Atrazine, Agbohessi et al.[8] observed degenerative alterations such as necrosis, hepatocyte vacuolation, nuclear degeneration, hepatocytes degeneration, sinusoids dilatation, etc. Abnormalies such as congestion, leukocytes infiltration, necrosis, hepatocyte degeneration, vacuolation, bleeding, MMC, sinusoids dilatation and glycogenic depletion were seen in the liver of African catfish after acute exposure to Thalis[17]. When exposed to Endosulfan, Agbohessi et al.[7] observed progressive abnormalities (hypertrophy cells, foci of hypertrophy cells) in African catfish. The cellular and nuclear hypertrophy of hepatocytes occurred as a consequence of high detoxification activity when fish were submitted to pollutants[36]. MMCs in reasonable proportions in tissues appear normal, but an increase in their extent and frequency becomes pathological[6]. Cell necrosis reveals irreversible morphological modifications coinciding with cell death. These changes affect both the nucleus and the cytoplasm. They are observable when the dead cell remains in a living environment and should be distinguished from autolysis[37]. Necrosis in the liver tissue were probably resulted from the excessive work required by the fish to get rid of the toxicant from its body during the process of detoxification by the liver[7]. The accumulation of lipids in liver cells forming lipid vacuoles is a vacuolation[37]. The vacuolation of hepatocytes could also indicate a loss of balance between the rate of synthesis of substances and the rate of their release into the circulation[38].

Liver indices indicate regardless of the sex of the fish that the liver of fish exposed to Thalis are between pronounced and severe alterations. This is due to the alterations identified. Such observations had also been made by Agbohessi et al.[7] in African catfish subjected to sub-lethal concentrations of Tihan 175 O-TEQ.

## Conclusion

These results clearly indicate that the insecticide Thalis disrupts the reproductive system of African catfish and induces damage to its liver. However, steroid profile (Testosterone, 11-Ketotestosterone, 17-estradiol) and aromatase activity were not analyzed in this study. These assays would allow us to confirm the feminizing effect of this binary pesticide on this fish. Eating fish caught from rivers receiving pesticide effluent seriously exposes humans due to the toxic effects linked to the bioaccumulation of pesticides in the body.

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