KASDI MERBAH UNIVERSITY, OUARGLA

FACULTY OF NATURAL AND LIFE SCIENCES
DEPARTMENT OF BIOLOGICAL SCIENCES

Memorial end of studies

In order to obtain the diploma of

Professional MASTER

Domain: Natural and life sciences

Field: Marine and continental hydrobiology

Specialty: Aquaculture

Presented by: - **Miss Adjabi Saada**

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Theme

Effect of using Neem extract "Azadirachta indica" on the sexual differentiation of Tilapia "Oreochromis niloticus"

Publicly defended on: 2022 / 09 / 15

In front of the jury:

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College year: 2021/2022.

Acknowledgments

This work would not have been possible without the expert guidance of our esteemed supervisor **Ma'am HIDOUCI S. (MCB; Department of Biological Sciences –K.M.U.O)**, to her insightful comments and constructive criticisms at different stages of this research were thought-provoking and very helpful. We are deeply thankful to her.

Cannot be overlooked on our co-supervisor **Mr. HMIDAT M. (NCRDFA Director)**, and how he put in his expertise to bring this work to its best.

We also wish to acknowledge the jury members' efforts for accepting to read examine and enlighten this work with their valuable expertise: **Mr. CHOUANA T. (MCA; Department of Biological Sciences –K.M.U.O)** the president of the jury,

Mr. BENSALEM S (MCB; Department of Biological Sciences –K.M.U.O) the examiner.

Special thanks are destined to **Mr. BOUAL Z.** for under covering us on polyphenols extraction.

We are also truly grateful to **Dr. MESSAID F.** for helping us with the histological slides.

Without forgetting to mention, all the thanks to **Mr. KHABAB F. (technician superior in plant protection, El Oued)** who was kind enough to provide us with the material biological Neem leaves.

To all and everyone who in their way nurtured this work, there is no better way to reveal our respect and gratitude than seizing the chance you all contributed to and doing our best in the making of this work. Thank you.

ADJABI S

ADJINA K

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Introduction

Introduction

Fish and other aquatic animals are important sources of animal protein and other important nutrients in people's diets. Between 1961 and 2016, the average annual increase in global food fish consumption (3.2%) outpaced population growth (1.6%) (FAO., 2018). As a result, food fish consumption increased to 20.5 kg capita-1in 2017 from 9 kg capita-1in 1961. This increase in per capita fish consumption has been largely attributed to the rapid development of global aquaculture since the 1980s. Aquaculture currently supplies 52% of food fish for direct human consumption globally (FAO., 2020).

Tilapia surpassed salmonids to become the second most important farmed finfish group by quantity in 2005 when its farmed production first reached 2 million tons. By 2018, the world production of farmed tilapia reached 6.03 million tons, which further consolidated the position of tilapia as the second-most important farmed finfish species group (Miao and Wang, 2020).

Because of their large size, rapid growth and palatability, a number of Tilapiine cichlids are at the focus of major aquaculture efforts. The main species cultured in ponds, cages and pens is the Nile tilapia (*Oreochromis niloticus*). The problem with this fish is their early maturation (5-6 months) and ability to breed every month. These characteristics result in the overpopulation of stocked tilapia ponds and the stunting of growth because of the crowding of the fish (Fashina-Bombata and Megbowon, 2012). Another problem associated with a mixed sex of tilapia is the sizes of the fish at harvest, varying from small to large due to the faster growth of males, they generally reach a weight of 1/2 pound (200 + grams) in 3 to 4 months. This makes it more difficult to establish uniformity of product. For producers wanting high yields of large-sized fish in 6 months, all male fry are preferred (Popma and Masser, 1999; Megbowon and Mojekwu, 2013; Jensi *et al.*, 2016).

All male culture of tilapia is preferred because of their faster growth than females (Megbowon and Fashina-Bombatta, 2010). Production of all male tilapia can be accomplished by such techniques as separating the males and females manually, hybridization, chromosomal manipulation and hormonal sex reversal. The most efficient and least expensive method is sex reversal with the use of 17α-methyl testosterone. However, the use of steroids in aquaculture is undesirable and is avoided in many countries, on account of its effects negative environmental factors (Baroiller *et al.*,

2009). In addition, 17-α methyltestosterone is quite expensive and not readily available in many developing countries.

The use of YY males (Abucay and Mair, 2004; Alcántar-Vázquez *et al.*, 2014) is much less widespread, according to a recent survey (Baroiller and D'Cotta, 2018a), only 5% of producers use YY males (high cost, complexity of managing this brood stock not available for many breeding strains), while 95% of them favor hormonal inversion.

The development of non-hormonal approaches for sexual control respecting the consumer and the environment first requires a better knowledge of the determinism and sex differentiation in fish and in particular in tilapia.

Many plants have been used to produce predominantly male tilapia in aquaculture (Gabriel *et al.*, 2017; Gabriel, 2019; Ghosal and Chakraborty, 2020; Ghosal *et al.*, 2021).

The main objective of this work is to study the effect of polyphenols extracted from Neem leaves "*Azadirachta indica*" used in food on the sexual differentiation of Nile tilapia "*Oreochromis niloticus*" in order to obtain a mono-sex male population.

The experiment was conducted at the university level, Kasdi Merbah University, Ouargla, faculty of natural sciences and life.

Four main axes constitute the skeleton of our work:

Axe 1: General information on the biological model chosen, sexual differentiation, and inversion sexual.

Axe 2: Concerns the experimental part and explains the management of breeding, and the treatments carried out on the different amounts of fish.

Axe 3: Includes the results obtained.

Axe 4: Discussion and conclusion.

Literature review

I. Literature review

1.1. Status of Fish Production in Africa and Algeria:

Several authors have emphasized the great importance of fish in global food and nutrition security, claiming that it provides around 20% animal protein, fatty acids, and micronutrients to over 3.1 billion people worldwide (Quaas *et al.*, 2016). Fish proves to be a good source of high-quality protein and essential fatty acids leading to a surge in demand for high-quality fish (Bank, 2013).

African governments under the tutelage of the African Union, identified the great potential of fish farming and are determined to encourage private sector investment, stating that potential exists for fish farming to make a difference. He further reiterated that, the fish sector provides income for over 10 million people engaged in fish production, processing and trade in Africa (Busari, 2018).

Aquaculture development in Algeria as well as Africa has been reported to be insignificant compared to Asia and Europe (Sogbesan and Ekundayo, 2014).

Aquaculture development in most African countries is primarily focused on socioeconomic objectives such as nutrition improvement in rural areas, income generation, diversification of farm activities (integrated farming) and creation of employment especially in rural communities where opportunities for aquaculture in northern part of Africa especially Egypt is economic activities are limited. This approach over the years has resulted in sustained aquaculture growth in some African countries such as Ivory Coast, Egypt, Ghana, Malawi, Nigeria and Zambia (Ayoola, 2010).The growth expansion and production of aquaculture in northern part of Africa especially Egypt is more advanced in techniques and technicalities in comparison to Algeria.

Annual aquaculture production in Algeria is estimated at 5100.12 tons according to FAO statistics for 2018 (FAO., 2018). This production remains low, despite efforts to promote this sector; it is still at the primary stage, while the country has significant water resources constituting significant potential for the development of aquaculture. Several constraints hinder the development of fish farming in Algeria. These include the stocking density, survival rate and growth rate of the fish, which in turn depend on the management techniques of the fish farms, the feeding of the fish, the systems practiced, type of culture, species choice and environmental factors. The low availability of food at lower cost remains one of major constraints of the weak development of fish farming.

1.2. Tilapia as aquaculture candidate for improved fish production:

Oreochromis niloticus is an African cichlid endemic to Burkina Faso, Cameroon, Chad, Ivory Coast, Egypt, Gambia, Ghana, Guinea, Liberia, Mali, Niger, Nigeria, Senegal, Sierra Leone, Sudan, Togo and Uganda and have been introduced to many other countries (Froese and Pauly, 2004, 2012). Nile tilapia has proven to be an important food fish that has been introduced to many different parts of the world by man with wide spread occurrence on all continents except Antarctica (Senanan and Bart, 2009).

They are omnivorous by nature, but feed on a wide range of natural food organisms, such as plankton, planktonic and benthic aquatic invertebrates, larval fish, bacterial film and detritus (FAO., 2011). Tilapia is a tolerant species, tolerant to high salinity, high water temperature, low dissolved oxygen, and high ammonia concentrations, compared to most of fresh water species. It is reported that this species can tolerate oxygen levels lower than 0.3 mg/l and grow well in temperatures between 25 °C and 35 °C with an optimum temperature range of 25 °C to 27 °C (Makori *et al.*, 2017).

Oreochromis niloticus is a cichlid species which sexual maturity depends on age, size and environmental conditions and generally have early sexual maturity before reach market size (FAO., 2016). Tilapia is susceptible to change in temperature as evidenced by their increased ratio of male to females as temperature rises with sex separation caused by 1ow temperatures (Fuentes-Silva *et al.*, 2013). It is evidenced by researches that at thecritica1 stage of sex differentiation, temperature is very important especially in very young fish ((Baroiller *et al.*, 2009). At very high temperatures during sex differentiation, obstruction of aromatase that enhance the conversion of androgens to estrogens happens which leads to a population having more males than females(Baroiller and D'Cotta, 2018a). This is evidenced in masculinizing tilapia at 32 0C. Growth of fish significantly increases with increasing dietary protein (cp) up to 46% where an optimum growth and feed utilization of *Oreochromis niloticus* is obtained (Ahmad and Abdel-Tawwab, 2011). The digestible protein requirement for Nile tilapia in the sexual reversion phase is 38.6 % where the growth was maximum and the survival decreased linearly as digestible protein levels increased (Ahmad and Abdel-Tawwab, 2011).

1.3. Sex determination in fish

Controlling the sex ratio is crucial in aquaculture as a balanced sex ratio is a good management tool for broodstock management and fish seed production. However, the production of mono sex populations is highly desirable in some species due to the existence of sexual dimorphism in this species, primarily in growth or sexual maturation.

Gonadal development are all developmental processes directed at transforming an undifferentiated primordial gonadal cell into a mature ovary or testis (Martínez *et al.*, 2014).

According to (Hayes, 1998), sex determinism is the set of genetic and/or environmental mechanisms that govern the process of sexual differentiation. Sexual differentiation, on the other hand, refers to the genetic and physiological processes of the development of gonads not yet differentiated into ovaries (female gonads) or testes (male gonads) (Fig.1) (Hake and O'Connor, 2008; Piferrer *et al.*, 2012).

The interaction of these two mechanisms is essential for the development of a phenotypic sex (male and/or female) corresponding to the genotype (Piferrer, 2001). In gonochoric teleosts, physiological and environmental factors can influence sexual differentiation and consequently lead to phenotypic sexes different from genotypic sex. Because of the diversity of their mode of reproduction and the system of sex determination which can be genetic or environmental or mixed (Devlin and Nagahama, 2002), gonochoric teleost fishes are of great interest in terms of the study of determinism and differentiation of sex among vertebrates (Baroiller and d'Cotta, 2001).

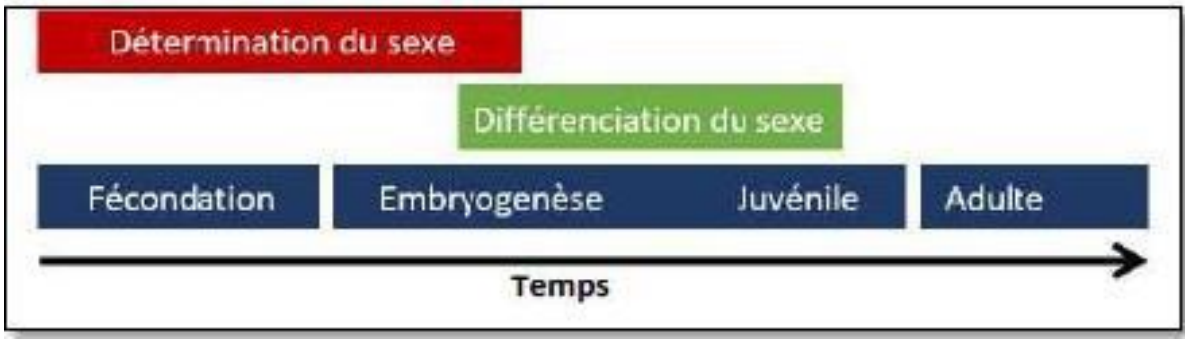


Figure 1: Time-dependent process of sex determination and differentiation in fish (modified from Degrange, 2019).

1.4. Induction of sex inversion

1.4.1. By hormones

The use of hormones to alter the sex ratios offish was first demonstrated in species other than tilapia. Sex hormones, in addition to modification of secondary sex characteristics, also affect the gonads (Yamamoto, 1969). Androgen induced masculinization and estrogen resulted in feminization (Phelps and Popma, 2000).

Hormone treatment does not alter the genotype of fish but directs the expression of the phenotype. As a result of hormone treatment, it is possible to have phenotypical male fish which are genetically female or phenotypically female fish that are genetically male (Sreenivasa and Prabhadevi, 2018). Production of all male population through administration of androgen (17 methyltestosterone) is considered to be the most effective and economically feasible method for obtaining all male tilapia populations (Fig.2) (Guerrero and Guerrero, 1988). There are three methods for steroid administration: Injection (either intramuscular or intraperitoneal), immersion in a static bath, and hormone supplementation in the diet (Judycka *et al.*, 2021).

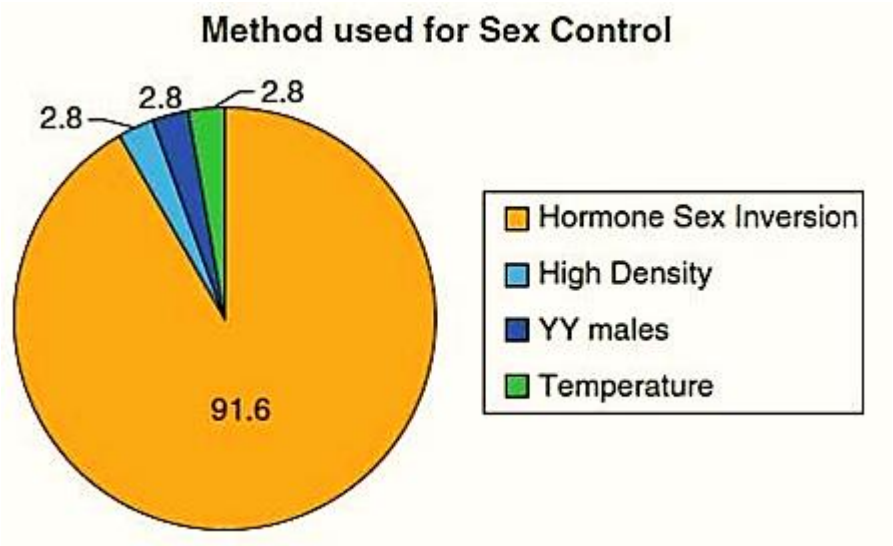


Figure 2: Methods used by 83% of producers who control the sex of tilapia, showing that 91.6% use sex hormone reversal (Baroiller and D'Cotta, 2019).

1.4.2. By using temperature

Sexual determination (SD) is a process by which a vertebrate decides which direction to take when it initiates its sexual differentiation as male or female (Capel, 2017). SD allows for a sex ratio that is adapted to each species and crucial for population viability (Ospina-Alvarez and Piferrer, 2008). The main environment sex determination (ESD) factor reported in fish is temperature (TSD) Since the first evidence of TSD in fish in Atlantic silverside, Menidiamenidia, in 1981 (Conover and Kynard, 1981), almost 60 species displaying TSD or genetic sex determination with a temperature effect (GSD+TE) have been described (Ospina-Alvarez and Piferrer, 2008).

Nile tilapia (*Oreochromis niloticus*) has a masculinizing effect of high temperature (above 32°C) that can override the GSD (Baroiller *et al.*, 1995), Thermal treatments with temperatures above 32°C to 36.5°C for at least 10 days during the gonadal differentiation period (from 10 to 30 days post fertilization (DPF for Nile tilapia) induce masculinization (Baroiller and D'Cotta, 2018b). The magnitude of masculinization strongly depends on parental effects, from no effect to almost 100% males (Mair *et al.*, 1991; Bezault *et al.*, 2007).

1.4.3. Hybridization

Hybridization has been reported to produce offspring that perform better than both parental species (Mbiru *et al.*, 2016) by combining valuable traits from two species into a single strain. Several tilapia species have been used to produce hybridizations (Hickling, 1960; Pruginin, 1968).

Wami tilapia (*Oreochromis urolepishornorum*) male x Nile tilapia (*O. niloticus*) female were the crosses used by (Pruginin, 1968; Chakraborty *et al.*, 2014; Mbiru *et al.*, 2016) while *O. urolepishornorum* male x Mozambique tilapia (*O. mossambicus*) female were used by (Hickling, 1960) and crosses between *O. niloticus* female x *urolepisurolepis* male were done by (Mapenzi and Mmochi, 2016). These crosses produced 100% male hybrids (Mtaki *et al.*, 2022).

With this technique, it is difficult to achieve 100% sterility of a population because of difficulty in maintaining pure parental stocks that consistently produce 100% sterile offspring, and difficulty in producing adequate number of fry due to spawning in compatibility between parent species (Pompa and Masser, 1999).

1.4.4. Manual sexing

Based on the anatomy of the genitals, it is possible to sort tilapia into males and females when they have reached 50-80 g (Mair *et al.*, 1991). The females and males can easily be sorted through the anal papilla by visual inspection (Omasaki *et al.*, 2016) or with the aid of dye (Fortes, 2005; Fuentes-Silva *et al.*, 2013).tow orifices are present in the female but one in the male (Fig.3).

Although manual sex sorting is feasible, it is tedious and can stress the fish through too much handling (Popma and Lovshin, 1996; Beardmore *et al.*, 2001), and it is difficult even for skilled workers to achieve 90% of success in sex sorting, as a result, reproduction is rarely controlled. This method may have negative impacts on economic returns because you need to employ skilled workers or train them at a cost, and female fish are all discarded after sorting stage (Popma and Masser, 1999).

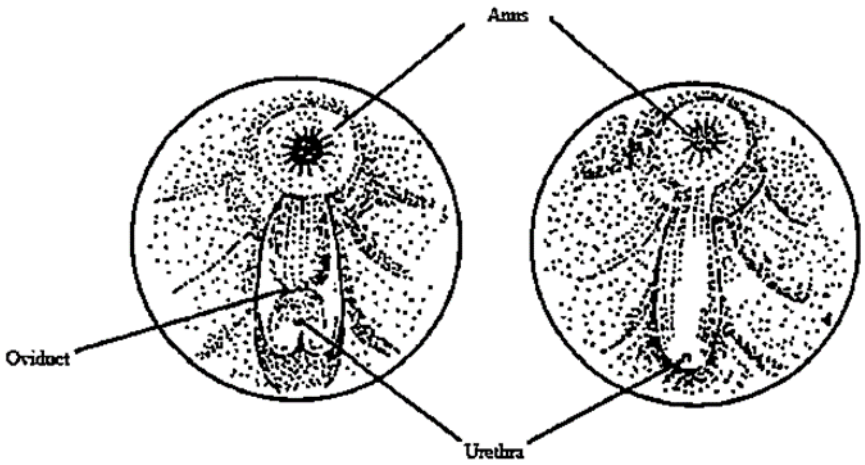


Figure 3: Male and female genital organ of tilapia (FAO, 1976).

1.4.5. Sterilization methods

1.4.5.1. Genetic manipulation: triploidization

In this method, the main aim is to produce sterile fish by using normal spermatozoa. Triploidization technique that sterilization can be achieved by administration of an

environment shock shortly after post fertilization. Therefore, degradations due to sexual maturation are overcome by triploidy technique (Fig.4) (Piferrer *et al.*, 2009).

Many workers successfully used both temperature (cold and heat) and pressure stocks as effective agents in inducing triploidy in several fishes. In a thorough investigation in Nile tilapia, *Oreochromis niloticus*, (Hussain, 1998).

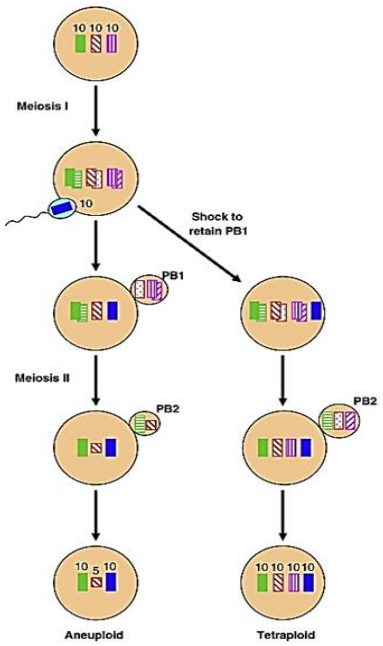


Figure 4: Production of 3n triploid individuals (Piferrer et al., 2012).

1.5. Use of plant extracts to control tilapia reproduction

Various interventions have demonstrated the possibility of utilizing plant extracts as possible options to synthetic steroids in aquaculture in response to the increasing consumer demand for organically produced agricultural products, including fish (Leet *et al.*, 2011).

The acceptance of plants for use in aquaculture is linked to ease of access and being relatively safer for the environment and humans, than synthetic hormones (Logambal *et al.*, 2000; Chakraborty *et al.*, 2012; Olusola *et al.*, 2013; Chakraborty *et al.*, 2014; Reverter *et al.*, 2014). To date, several plant extracts are predominantly used to improve fish growth, enhance innate immune responses, and control disease in aquaculture, as compared to reproduction control (Baluran *et al.*, 2018).

Nevertheless, the androgenic compounds present in some plant extracts could be used to control unwanted reproduction in tilapia production systems (Chakraborty *et al.*, 2014; Gabriel *et al.*, 2017; Gabriel, 2019; Ghosal *et al.*, 2021).

The Phyto androgens, for example, testosterone, androstenedione, and dehydroepiandrosterone, have been implicated in sex reversal of fish (Godwin *et al.*, 2003).

Extracts from 20 plant species, belonging to 17 families are reported to control reproduction in tilapia culture systems. The geographical distribution, usable parts phytochemical composition, androgenic and fertility impairment, one of this plant is Neem tree, *Azadirachta indica* (Abaho *et al.*, 2022).

1.6. Neem tree, Azadirachta indica

A. indica is commonly known as “Neem tree” belongs to order Rutales, and is amember of mahogany family known as Meliaceae (Hashmat *et al.*, 2012). It is a broad-leaved plant that grows up to 30 m tall and with a girth of 2.5 m (Fig.5) (Ndodo *et al.*, 2013). The tree is native to the tropical and semi-tropical regions (Dos Santos and Kiwango, 2010).

The extracts of various parts of neem tree have antimicrobial, anti-inflammatory, and spermicidal effects, as well as antifertility activity and abortifacient properties (Biswas *et al.*, 2002; Jegede and Fagbenro, 2007; Priya *et al.*, 2012).

This is attributed to the high concentration of fertility regulating-saponins and tannins in neem extracts (Biu *et al.*, 2009; Kapinga *et al.*, 2018), in addition to sodium nimbinate, “a spermicide” (NRC, 2002). Consequently, the neem tree's antifertility activity has been harnessed to control prolific breeding in tilapia in culture systems. The inclusion of 2.0 g kg^{-1} of A. indica leaf powder rendered the testes and ovaries of Tilapia zilli devoid of spermatids and oocytes after 60 days of treatment (Jegede and Fagbenro, 2008). Likewise, Nile tilapia, fed on diet containing 1.0–8 g kg^{-1} of crude *A. indica* ethanol-based leaf extracts reduced the number of hatchings, with no spawning by the fifth week (Obaroh and Achionye-Nzeh, 2011; Obaroh *et al.*, 2012; Obaroh and Nzeh, 2013). However, *A. indica* only lowered the spawning of Nile tilapia by 76% at the highest inclusion of 8.0 g of the leaf powder kg^{-1} diet for 90 days (Kapinga *et al.*, 2018). This inconsistency in performance of *A. indica* extracts could be attributed to differences in extraction media and seasons (Isah, 2019).

These discrepancies hamper the wide-scale utilization of neem extracts to control tilapia reproduction.

Figure 5: Neem tree and Neem leaves (Suraphol, 2018).

1.7. Chemical Constituents of medicinal plants

Phytochemicals are non-nutritive components that have protective or disease preventive properties found in plant-based diets (Arendt and Zannini, 2013). They are non-essential nutrients; they are not required by the human body for sustaining life. It is well-known that plant produce these chemicals to protect themselves but recent research demonstrate that they can also protect humans against diseases. Phytochemicals are naturally occurring in all parts of medicinal plants that have defense mechanism to protect humans and animals from various ailments and these phytochemicals are primary and secondary compounds in plants (Ahmad *et al.*, 2016). Phytochemicals have been broadly classified into six major categories based on their chemical structures and characteristics; alkaloids and other nitrogen containing compounds, carbohydrate, lipids, phenolics, terpenoids (Huang *et al.*, 2016).

Primary compounds of plants are chlorophyll, proteins and common sugars while secondary compounds are phenolic compounds such as flavonoids, tannins, and lignins, terpenoid, and alkaloids (Wadood *et al.*, 2013) many of which act as antioxidants.

Flavonoids make up the largest class of phytochemicals of these phenolic compounds (Du *et al.*, 2016). Flavonoids are molecules with a low molecular weight. Flavonoids include flavones, isoflavones, flavonoids, flavonols, flavanones, anthocyanins, and proanthocyanidins, according to the flavonoid categorization system (Altemimi *et al.*, 2017). Terpenoids exhibit various important pharmacological properties while

Alkaloids are used as anesthetic agents and are found in medicinal plants (Wadood *et al.*, 2013).

Tannins are natural products found in many plant families and have large amounts of phenolic rings in the structure (Altemimi *et al.*, 2017). Saponins are a diverse group of compounds that is diverse in the plant kingdom having varying structures, physiological and biological effects (Addisu and Assefa, 2016). Saponins in herbs have been found to be very low levels to have any harmful effects when consumed (Francis *et al.*, 2002).

Saponins act as both anti-nutrient and antioxidant in humans stating that saponin in cultivated plants is terpenoid saponins while those in plants not cultivated (herbal) are steroidal saponins (Adeniji, 2013).

Twenty percent of known plants have been used in parts or wholly in pharmaceutical studies, leading to positive impacts on the healthcare system such as treating tumors and other harmful diseases and may supplement the needs of the human body by acting as natural antioxidants to protect cells against oxidative damages (Altemimi *et al.*, 2017). Others have hormonal actions and stimulate enzymes while others have antibacterial effects (Lillehoj *et al.*, 2018).

Materials and Methods

II. Materials and Methods

2.1. Experimental site

The experimental part was conducted between 2022-03-15 and 2022-06-28 in the aquaculture-breeding laboratory in the Department of Biological Sciences, Faculty of Nature and Life Sciences, Kasdi Merbah University, Ouargla.

The extraction of polyphenols from neem leaves was carried out at the Physico-chemical analysis research center, Ouargla annex.

The histologic slides, implicated in the Laboratory of anatomic-pathology of Dr. MESSAID F. -Ouargla.

2.2. Acquisition of Fish:

Oreochromis niloticus fry were obtained from the Hatchery Farm of National Center for Research and Development of Fishing and Aquaculture, Ouargla (NCRDFA).

Newly hatched *Oreochromis niloticus* fry have been collected (Fig.6) and transferred to the aquaculture-breeding laboratoryat the university. Afterward, we placed them in one aquarium with the intention of adaptation before distributing them to the rest of the aquariums in order to start the experiment.

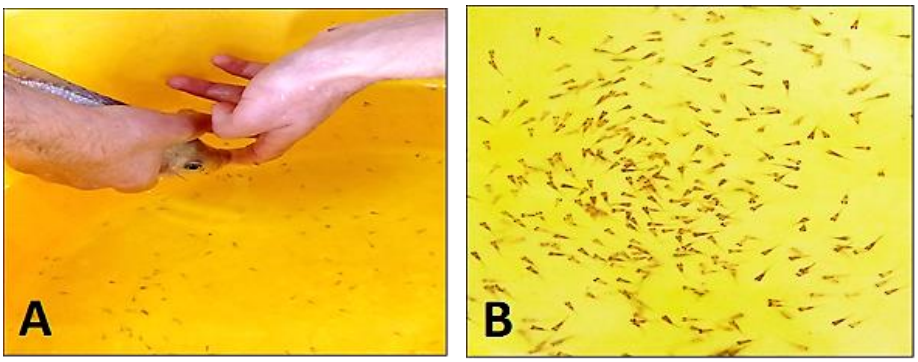


Figure 6: Collecting fry, A: taking fry out from the mother mouth, B: Fry.

2.3. Masculinization treatment:

Two masculinization treatments were exercised on the fish larvae for 30 days, the first treatment, we incorporated into the diet of the *Oreochromis niloticus* larvae a TN concentration of Neem leaves powder "*Azardirachta indica*" (Fig.7).

For the second we incorporated a TPoly concentration of polyphenol extract from Neem leaves (Fig.8), an active ingredient suspected of sexual inversion in *Oreochromis niloticus*.

Masculinization treatments were prepared with imported commercial food presented in 20 kg bags, in the form of powder with 45% crude protein. The larvae of the two treatments are fed 6 times a day for 30 days.

Neem leaves "*Azadirachta indica*" were provided by the Aquacade farm, Mr. Khabeb Allal, Oued Souf, where it was cultivated and adapted. After treatment period, fingerlings were maintained for a period of 2 months, after which the identification of the sex and the evaluation of the effectiveness of the treatment will be carried out by calculating the male and female proportions.

Figure 7*:* Preparation of masculinization food with Neem powder.

Figure 8: Preparation of masculinization food with Neem extract (Polyphenol).

2.4. Experimental Design

A total is 240 *Oreochromis niloticus* larvae were divided into 2 groups representing the treatments (TN and TPoly, with TN representing Neem powder treatment, TPoly representing polyphenol extract treatment). Each group was further divided into 3 replicates consisting 40 fishes per replicate. Each group of 40 fishes were stocked in aquarium (71×45×51cm) supplied with 140 liters of water (Fig.9). The fish were fed 11% of their body weight, seven times a day, at the beginning of the experiment, and 6.5% at the end, five times a day. Diet ratio were adjusted based on the weight obtained bi-weekly. Water in each tank were replaced daily.

The study lasted for 90 days with 30 days treatment and 60 days for fish growth.

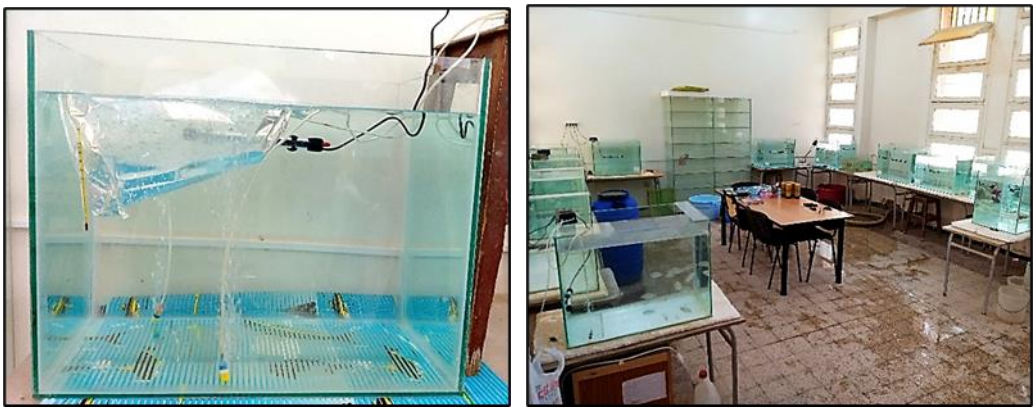


Figure 9: Experimental breeding device.

2.5. Monitoring of water quality:

Water temperature, pH and salinity were measured daily. According to the electrometric method (Rodier *et al.*, 2016), using a multi-parameter "water quality meter 8603". Measurements were taken after calibration according to the instruction manual provided by the manufacturer, by immersing the probe in water for approximately 1-2 minutes, and then the readings were recorded, in degrees Celsius for the temperature and g/L for salinity.

2.6. Growth performance assessment:

In order to assess the growth performance of the fishes, lengths andweights were taken in each aquarium. An electronic balance was used to measure the weights. Sampling was done after every 14 days.

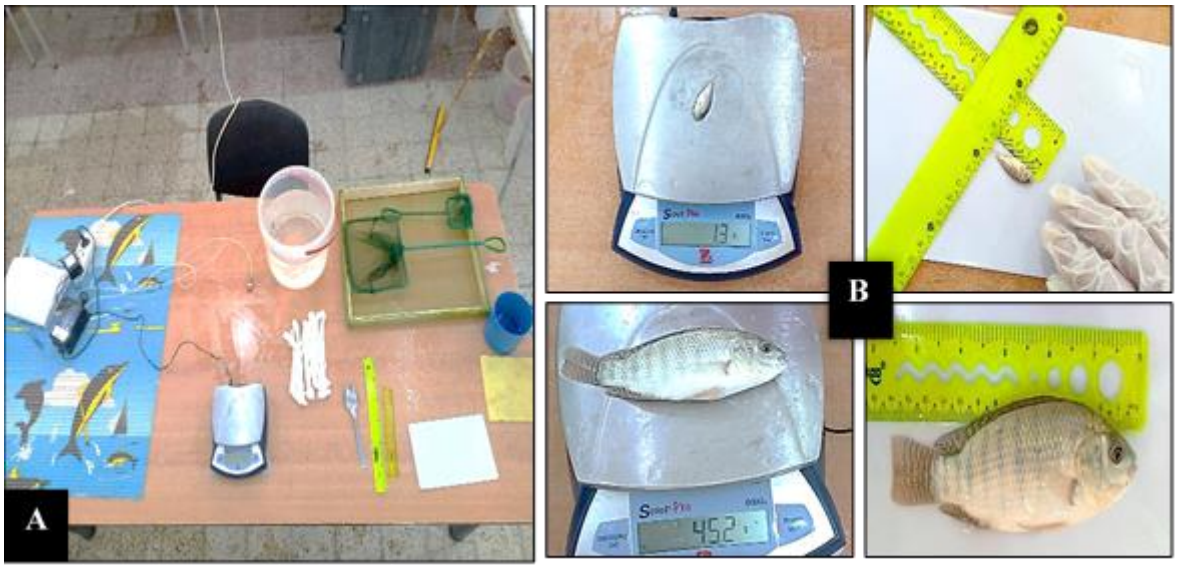


Figure 10: weight and height measurement, A; work table, B; measurement.

Initial average weight (IAW):

IAW (g) = Initial biomass (g) / Initial number of fish.

Final average weight (FAW)

FAW (g) = Final biomass (g) / Final number of fish.

Survival rate (SR). This rate made it possible to know the effect of the substitution on the Follow-up fish.

SR in % = (Number of individuals at the end of the experiment / Initial number of individuals) x 100.

Specific growth rate (SGR). The SGR gives the instantaneous speed of fish growth. It is expressed by the following formula:

SGR in % / d = [Ln (FAW (g)) - Ln (IAW (g)) x 100 / Duration of experiment]

2.7. Fish sex determination:

Sex determination is applied manually by condescending to the external sex organs of fish (Fig.11 A and B), followed by confirmation with the so-called gonadal squash technique of (Guerrero and Shelton, 1974). We dissected 111 fish, 52 for the polyphenol treatment (TPoly) and 59 for the Neem powder treatment (TN) in order to determine their sex by their gonads (Fig.12) as well we took their height and weight without viscera and gonads weight.

The ovaries appear stockier while the testicles are filiform and occupy the entire length of the cavity (Fig.12). The gonads were saved on formalin solution (with 10% concentration) and were used to make histologic slides.

Figure 11: Male and female fish (phenotypic), A; Male, B; Female.

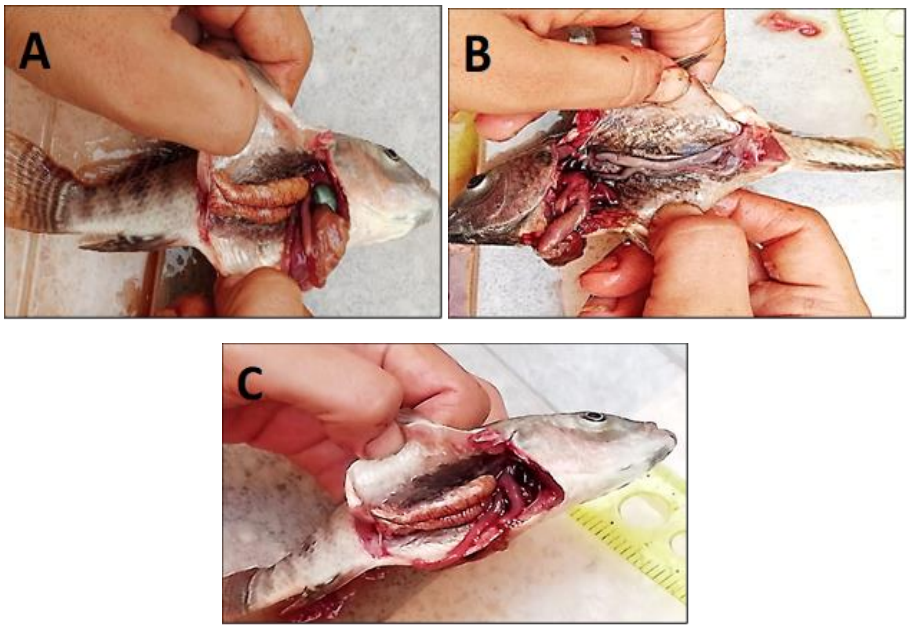


Figure 12: Nil tilapia fish gonads, A; ovary, B; testicles, C; hermaphrodite fish.

2.8. Statistical Analysis:

In all cases, descriptive statistics (mean ± standard deviation) are used to describe all of the results. Before any statistical analysis, we checked the homogeneity of the variances. Tests were carried out to compare the means. The significance level was determined at 0.05. All statistical tests were performed using XLSTAT statistical software version 2014.5.03.

RESULTS

III. Results:

3.1. Water Quality Parameters of Fish Culture Water:

3.1.1. Temperature:

Temperature was not significantly varied (p = 0.05) across all treatments, moreover it ranged between 26.81±1.06C and 28.29±1.18 °C (Fig.13).

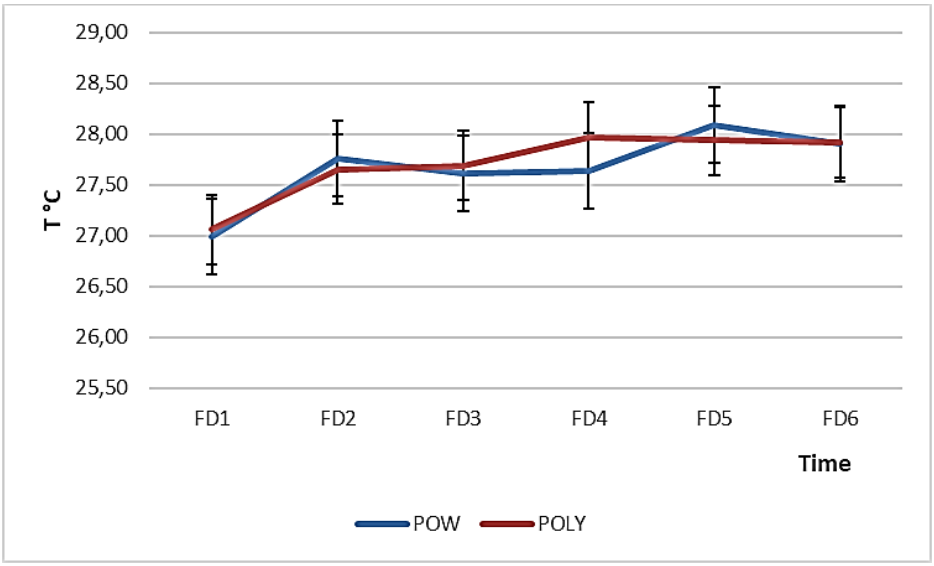


Figure 13: Temperature variations of water breeding

3.1.2. PH:

PH ranged from 7.32±0.10 to 7.89±0.07. However there are no significate varied across all treatment aquarium (p = 0.32) (Fig.14).

Figure 14: pH variations of water breeding (FD: forty days)

3.1.3. Salinity :

Salinity of water is relatively similar for both treatments throughout the experiment (p = 0.238), it varies between 3.2 and 3.9 ‰ with an average of 3.47± 0.08 ‰ for the TN and for TPoly varies between 3.2 and 3.7 ‰ with an average of 3.48 ± 0.086 ‰ (Fig.15).

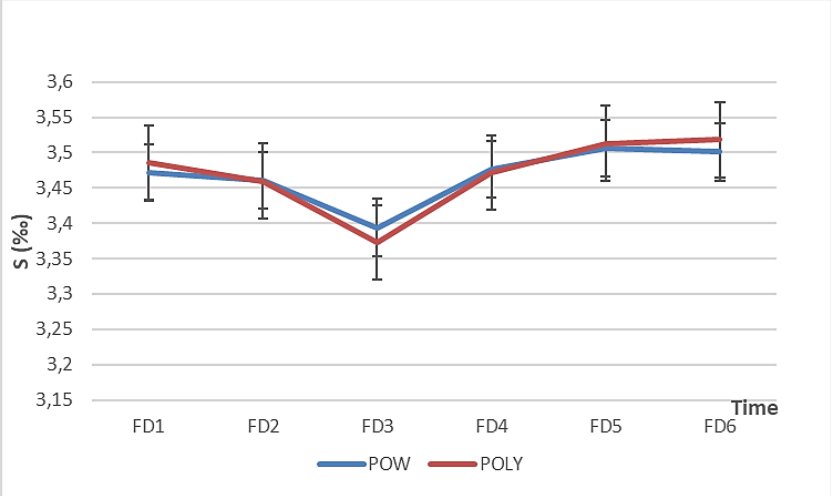


Figure 15: salinity variations of water breeding

3.2. Growth performance indices and survival:

Results for growth performance of *Oreochromis niloticus* fed diets TN and TPoly presented in Tables 1.

Table 1: Growth Response of *O. niloticus* Fed with Neem powder treatment and polyphenol extract treatment (TN: Neem powder treatment, TPoly: polyphenol treatment).

Parameters	TPoly	TN
Survival rate (%)	37.50 ± 22.22	45.83 ± 10.10
Initial weight (g)	0.035	0.035
Final weight (g)	25.19 ± 10.23	28.23 ± 6,29
Initial biomass (g)	1.4	1.4
Final biomass (g)	962.01	1517.30
Mean weight gain (g)	25.15 ± 10.23	28.20 ± 5.13
Specific growth rate (S.G.R) (%/j)	7.31 ± 0.34	7.43 ± 0.19

3.2.1. Survival rate:

The survival rate for both treatments was calculated at the end of the experiment.

In addition, it obtained at the end of the treatment is appreciable; by reaching 70.83±18.76% for TN and 56.66 ±32.62% for the TPoly. At the end of the experimentation, we obtain 45.83±10.10% for TN and 37.50±22.22% for TPoly treatment.

Survival rate shows no significant difference between treatments (p= 0.58), but is highly significant between aquaria (p = 0.001).

3.2.2. Growth performance indices:

3.2.2.1. Average weight :

Oreochromis niloticus fry shows a continuous evolution of weight according to age (fig. 16). Final mean weight of fish fed diet containing Neem powder (TN) shows no significant difference from that of the Neem extract treatment (Tpoly) fish, (p =0.05), where we record respectively 28.23 ± 6,29g and 25.19 ± 10.23 g (Tab. 1).

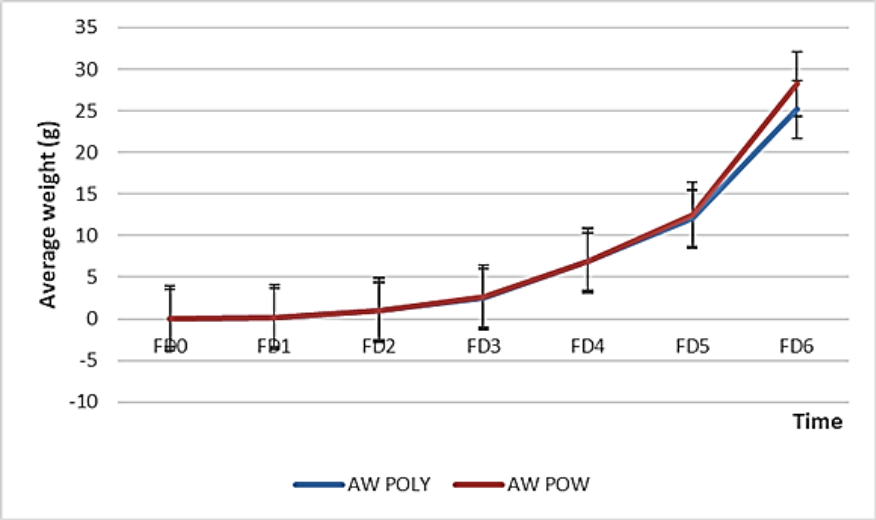


Figure 16: Evolution of the average weight of Nil Tilapia

3.2.2.2. Specific growth rate (SGR):

The evolution of specific growth rate shows no significant difference between the two treatments (p= 0.91). We notice an SGR with an average of 7.31±0.34%/d for the TPoly and an average of 7.43 ± 0.19 %/d for TN (Tab. 1). Furthermore, there is a significant difference very high in temporal variations (p=0.0001).

We note a specific growth rate at the beginning of the experiment significantly higher than at the end (p = 0.04), where it went from 15.18%/d to 3.10%/d for TPoly and from 14.73 to 3.97%/d fot TN (Fig. 17)

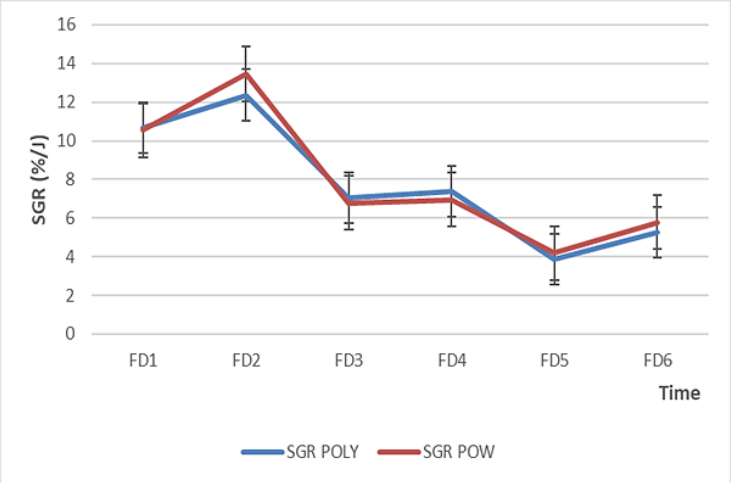


Figure 17: Fluctuations of specific growth rate during study period

3.3. Sex reversal changes in O. niloticus larvae:

Results of manual sexing and gonadal squash performed on *Oreochromis niloticus* and confirmed by microscopic observation (Fig. 20, 21; A and B), are presented in Table 2.

Figure shows the results of different treatments obtained. The rate of males in the fry treated with Neem powder is 67.8% that of polyphenol treatment is 77 % (Fig. 18).

Moreover, we discovered an undefined sex fish with the percentage of 5.75% on TPoly and 8.47% on TN, as well as hermaphrodites with rates of 1.99% and 8.47% (Fig. 19). Further there is no significant difference noted between the two experiments (p=0.42).

Table 2: Reversal rate proportions in treated *Oreochromis niloticus.*

Treatments	Initial headcount	Population dissected	Gonadal squash							
			Nbr M	Nbr F	Nbr UND	Nbr HER	M (%)	F (%)	UND (%)	HER (%)
TPoly	120	52	40	8	3	1	77	15	5,75	1,92
TN	120	59	40	9	5	5	67.8	15.25	8.47	8.47

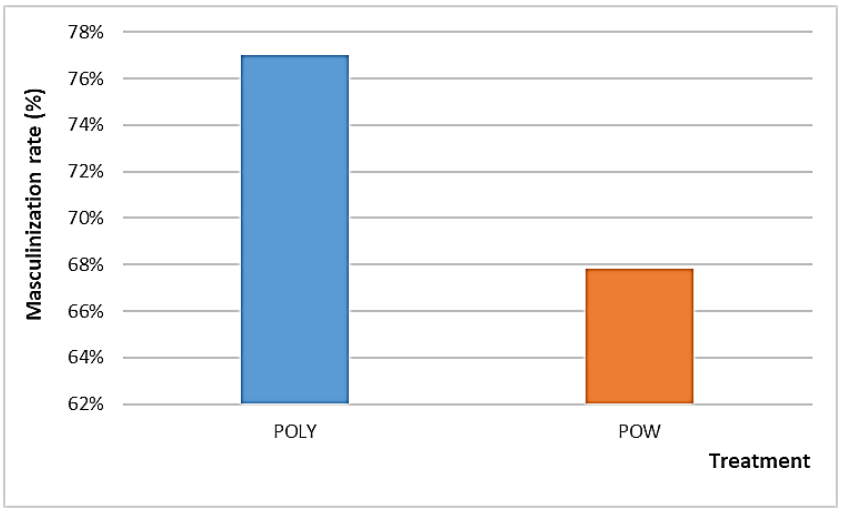


Figure 18: Proportion of males in treated *Oreochromis niloticus* (POLY: polyphenol treatment; POW: Neem powder treatment).

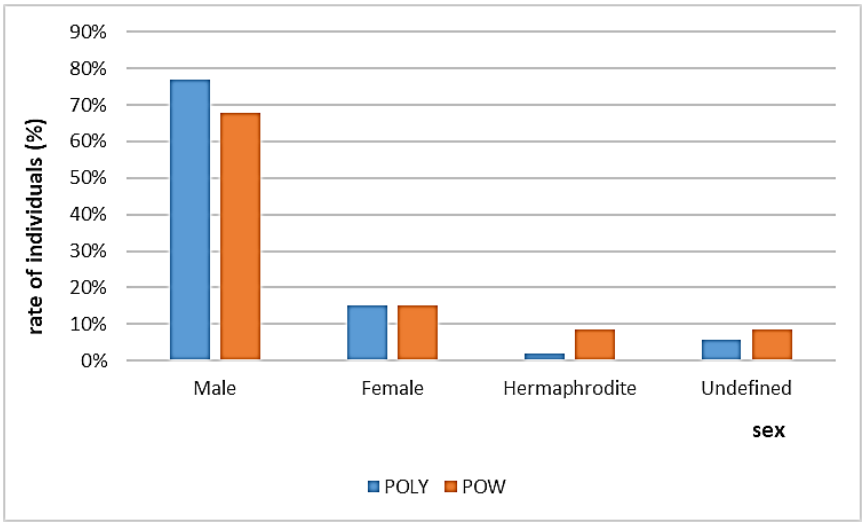


Figure 19: Reversal rate proportions in treated *Oreochromis niloticus* (POLY: polyphenol treatment; POW: Neem powder treatment).

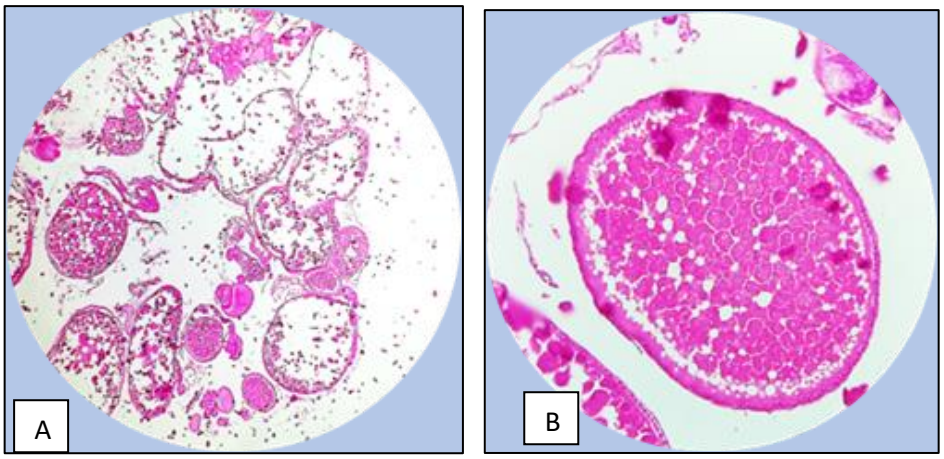


Figure 20: Microphotographs of histological sections of tilapia ovary (×10 and ×40).

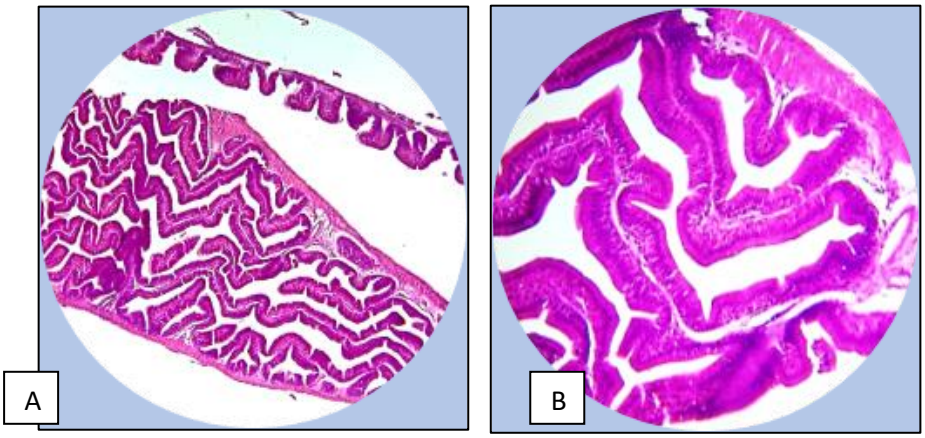


Figure 21: Microphotographs of histological sections of tilapia testes (×10 and ×40)

Discussion

IV. Discussion:

Concerning the quality of breeding waters, generally, the water's physical and chemical parameters are within the ranges of optimal values recommended.

Temperature values (27.8 ± 0.92°C and 27.76 ± 1 °C) recorded during this experience comply with European regulations for fish farming waters (2006/44/EEC and 2006/113/EC) which is 8 to 30°C. (Melard, 1999). However, the best growth performance for *Oreochromis niloticus* was observed between 24 and 28°C (Lacroix, 2004). The breeding optimum is between 28 and 32°C (Lazard, 2009).

PH variations (7.58 ±0.29 and 7.58±0.39) are well within the optimal limits for the growth of tilapia *Oreochromis niloticus* according to El-Sherif and El-Feky (2009), the optimal water pH for the culture of Nile tilapia, *Oreochromis niloticus* L., is 7 – 8. In general, the suitable range of water pH for aquaculture is 6.5 - 9.0 (Boyd, Tucker, & Somridhivej, 2016).

The salinity rate recorded value of 3.4 g/L, the water used is nature brackish, the value is following the recommendations of (Kirk, 1972; Kestemontet al., 1989), the salinity of Nile tilapia for the growth performance are between 0–8 g/L, respectively (Alvarenga et al.,2018; El-Sayed, 2006; Likongwe et al., 1996). Nevertheless, it has been suggested that Nile tilapia is also suitable for brackish water aquaculture with a salinity level up to 15 g/L (Popma & Masser, 1999Rairat et al., 2022).

The survival rate. Initially the experience concluded fish fry which is very sensitive and weak in particular females, especially during the last phase of this stage (Cushing et al., 1996), although, it doesn't explain the law survival rate, 45% for TN (Neem leaves powder treatment) and a 37% for TPoly (polyphenol expect treatment). An unfortunate accident that occurs at the level of the aquarium of adaptation at the beginning of the experiment, which the level of tempter dropped from 28C° to 23C °, cold shock. Caused by an electrical problem.

The effect of cold shock exposure on fish is stronger as the rate and magnitude of temperature change are increased and as temperatures approach the limits of species-specific or ontogenetic thermal tolerance ranges (Donaldson *et al.*, 2008).

Rearing tilapia fry in recirculating systems must be practiced at the optimum water temperature, which is about 28C°, in order to achieve the best growth, feed conversion, and survival, and in turn, the highest portability (El-Sayed & Kawanna, 2008). Therefore, it explains the height mortality rates at the beginning of the experience, and it is widely acknowledged that cold shock causes stress in fish, that studies assess how this physiological stress is manifested through behavioural consequences that may ultimately affect survival and fitness (Donaldson *et al.*, 2008, Szekeres *et al.*, 2016).

Kapinga (2018) experience about studying *Aspiliamossambicensis* and *Azadirachta indica* medicinal leaf powders modulate physiological parameters of Nile tilapia (*Oreochromis niloticus*), they recorded an 85% of survival rate on *A. indica* treatment. And the leaves of Neem (*Azadirachta indica*) have been used in treating microbial and parasitic infections in livestock (Tibebu and Haile, &Kebede, 2017), as (Abarike, and Dandi & Ampofo-Yeboah, 2022) used Neem leaves with two other plants (Bitter leaves and Guava leaves) to improve haematology and resistance of Nil Tilapia. For the last addition (Putri*et al.*, 2018) reported that Neem leaves have compounds that are potential as immunostimulating ingredients for tilapia.

With previous research mentioned previously, the neem leaves cannot be considered a possibility of a decrease in survival rate. Moreover, it maybe explains survival rate is slightly higher with the treatment of Neem powder than polyphenol treatment since it has all compounds in neem leaves helped on keeping the fish health.

The growth results obtained for the two treatments are identical, with a similar result for both TN and TPoly. Compared to BERHANU (2020), our results are superior; the work was about three hormonal treatments and one control on the sexual inversion of Nil Tilapia (*Oreochromis niloticus)* with a final weight of 18.34g, 24.11g, 19g, and14.27for the same breeding period. And for another comparison (GhemamAli, 2020) recorded a medium weight to three Neem tree treatments and one control with 22.56 g, 13.40, 10.24 g, and 9.93g. However, our result is also considered excellent compared to (Khemis*et al.*, 2019) who recorded a maximum weight of 13.52g with hormonal treatment. Moreover, another one (Jegede and Fagbenro, 2008).

The obtained growth rate results for both treatments (TN and TPoly) were delighted and satisfactory. Moreover, they are no significant difference is noticed between TN and TPoly fish because the individuals were under the same conditions.

On the FD5, we separate the fish for both treatments for accelerating fish growth. The density affects the growth and maturation of fish regardless of the rearing system used, and it also provides the fish with more space that has allowed more freedom of movement and improves fish behaviour by reducing stress and competition (Rahman *et al.*, 2008; Narejo*et al.*, 2010; Daudpota*et al.*, 2014;Gangbazo*et al.*, 2018; Zaki et al.,2020). Lowering the fish density in every single aquarium affects fish growth positively, Moreover, we noted an augmentation in fish growth on the last FD.

We recorded a highly interesting result for the proportion of males in both experiments, with a result of 77% males for polyphenol treatment (TPoly), and 67.8% males for Neem powder treatment (TN), and they are no significant difference is noted between both the experiments.

For treatment Neem leaves powder by compared to a previous search by (Amira and Ammari, 2021), which proved that Neem powder incorporated into the larval diet promotes an increased masculinization rate, our result confirmed the same conclusion as theirs. Additionally, the result that polyphenol treatment gives shows that adding polyphenol to the larval diet has an affection on the masculinization rate by increasing it, which means it's probably the compound that is responsible for the increasing masculinization rate on Neem leaves.

The masculinization rate results are comparable to (Sani et al., 2019) who recorded 71.67% of males for treatment of *Nigella sativa*seeds. In addition, compared to (Sadek and Nady and Abou Zied, 2022) two treatments by hormones were 95.97%, 96.47%, and two *T. terrestris*treatments were 71.53%, 83.50%. Their hormone treatment is clearly higher than our result, but in *T. terrestris*treatment, the first result is comparable and the second is slightly higher than ours. Moreover, for (Khemis*et al.*, 2019) our result is comparable to their hormone treatment recorded by 65.3 % and higher than their thermal shock treatment recorded by 58.16%.

In addition, compared to (Amira and Ammari, 2021) recorded a result of an 80.18% on masculinization rate by Neem treatment on laver diet our results are less. Tilapia is a thermo-sensitive species, its male-to-female ratio increases with temperature and/or ovarian differentiation induced by low temperatures (Fuentes–Silva et al., 2013). Studies have indicated that temperature is influential at a critical stage of sex differentiation in larval fish relatively similar to the hormone-sensitive period (Brodie et

al., 1999; Baroiller and D'Cotta, 2001). Relying on previous references can be justified that our result is less high than the result of (Amira and Ammari, 2021) even though we have the same treatment, by saying maybe or probably the reason for that is the cold thermals shock that happened at the beginning of the experiment caused an ovarian differentiation or affected the fish hormones. Which contributed to a decrease in the effectiveness of neem leaves.

Conclusion

Conclusion

The main objective of this study was to investigate the effect of Neem leaves powder and polyphenols extracted from Neem leaves on the sexual differentiation of Nile tilapia « *Oreochromis niloticus*». To produce a mono-sex male population, our work recorded highly interesting results.

The physical and chemical parameters that were recorded in all the experiment periods for the stage larval intel they passed seemed to fit perfectly to *Oreochromis niloticus* since all the noted values are in the interval optimum, with the exception of the cold shock that existed before beginning the experience.

Both sex ratios by the two treatments of polyphenols extracted and powder of Neem leaves, were determined by gonadal squash of fish obtained and then confirmed by histological slides. For both results of satisfaction with a masculinization rate of 77% for TPoly and 67.8% for TN. However, the survival rate for both treatments wasn't that satisfactory at 45% for Neem leaves and 37% for polyphenols, furthermore it was justified by an unfortunate accident caused by a cold shock.

This expert present that *A. indica* is important to the larval diet of *O. niloticus* leads to significant male sex inversion, moreover has a positive effect on fish growth and health. Furthermore, it also presents that polyphenol have an effect on masculinization as well and we suspect that polyphenol might be the compounds that are responsible for the male sex inversion of *O. niloticus.*

Utilization of this plant or its extract for masculinization will allow us to overcome many disadvantages in the other methods as masculinization by hormone, needs a high level of control and have a negative effect on the environment, and so by using tempter its effect on survival rate by decreasing it and it considerate harmful on fish growth. On the other hand, the treatments of Neem or its extract concentrate a good solution cause its natural and beneficial plant source that does not affect the environment or the health of fish and consumers.

Ultimately, this study confirmed that Neem leaves have an effect on the masculinisation of Nil Tilapia and that polyphenols could be the compounds responsible for it. Left us with the conclude of that it needed more effort to study the effects of Neem on sex-determining mechanisms, and specifies Neem extracts as well as environments and ecology. For a last word, it needed to minimize the utilization of chemical products in

aquaculture and replace it with biological choices for the sake of preserving the environment.

Annex

Annex 01

Polyphenol extraction from the Neem tree (*Azadirachta indica*):

The work was at CRPC, under the supervision of Dr. BOUAL Zakaria.

CRPC stands for SCIENTIFIC AND TECHNICAL RESEARCH CENTER IN PHYSICO-CHEMICAL ANALYSIS.

It is a laboratory for analyzing chime_physic, located at the third university pole

I.1. Material:

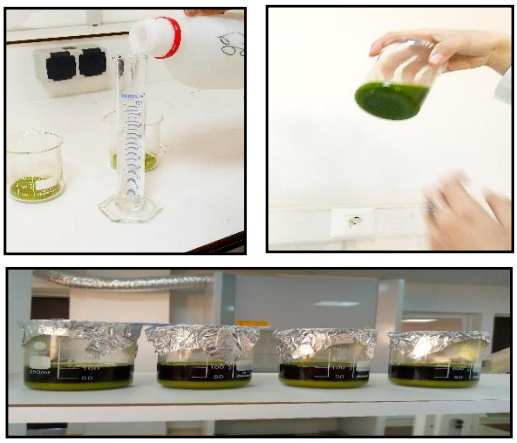
Four beakers 200 ml, Rotavapor., Graduated cylinder of 100ml/25ml, ethanol, tree Neem leaves powder, Eight glass bottles of 100 ml volume, Balance, 40 g of Neem leaves powder.

I.2. Method:

I.2.1. On the first day 06/03/2022:

- Pot Neem leaves powder + an amount of ethanol in one beaker.
- Mix it will.
- Repeated for the rest of three beakers.
- Close the beaker with silicon paper.
- Let it rest for 24 to 48 hours.

A. Balancing the neem powder.



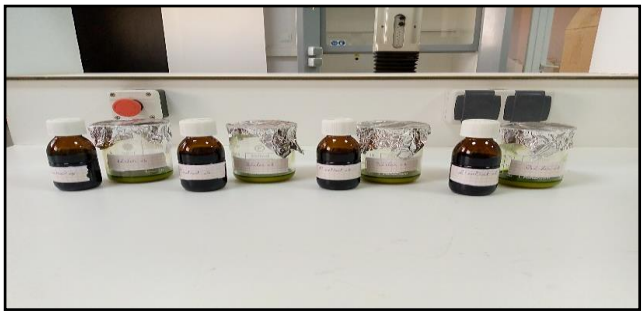
B. Preparing the sample.

Figure 01: explication of the method or working.

I.2.2. On the second day 08/03/2022:

- The solution rests for 44 hours.
- Pour the solution (just the ethanol liquid) into a bottle of a 100 ml volume, and pot it in the fridge until the next step (to separate the bottles used numbers 1, 2, 3, and 4).
- Fill up the beakers again with a graduated cylinder of ethanol.
- Mix it goodly.
- Let them set down for 24 to 48h.

A. Filtering ethanol.



B. Bottles and beaker after filtering.

Figure 02: explication of method on the second day of work.

I.2.3. On the third day 09/03/2022:

- The solution rests for 24 hours.
- Filtering ethanol from leaves, by pouring ethanol into the glass bottles (four bottles with tickets 1b.2b.3b.4b to separate from each other and lest bottles).
- The final step is by vaporing ethanol for that the left with polyphenol, for that we used Rotavapor (Fig.3).

- **Rotavapor:**

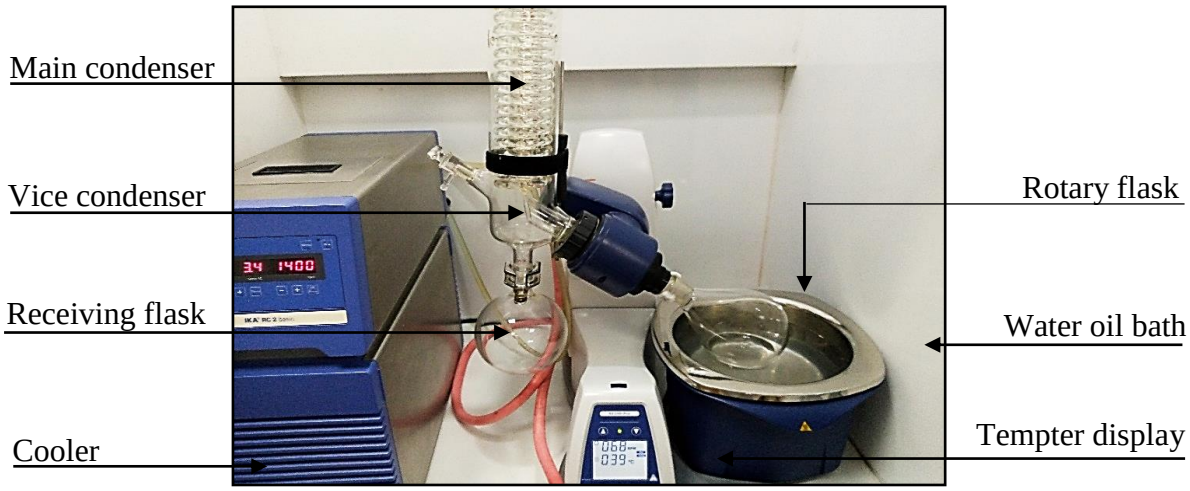


Figure 03: Rotavapor.

- **The steps of using Rotavapor:**

How it works: pot the sample in a rotary flask, sit the tempter afterward click ON the heat will vapor the solution (Ex: ethanol, methanol), the steam goes to the main condenser following to that the steam gets cooler and as result for that it goes back to a liquid again and goes to receiving flask.

For an amount of time, we going to be left just with required (depending on the solution, the experience, and the quantity of the sample) (Fig.4).

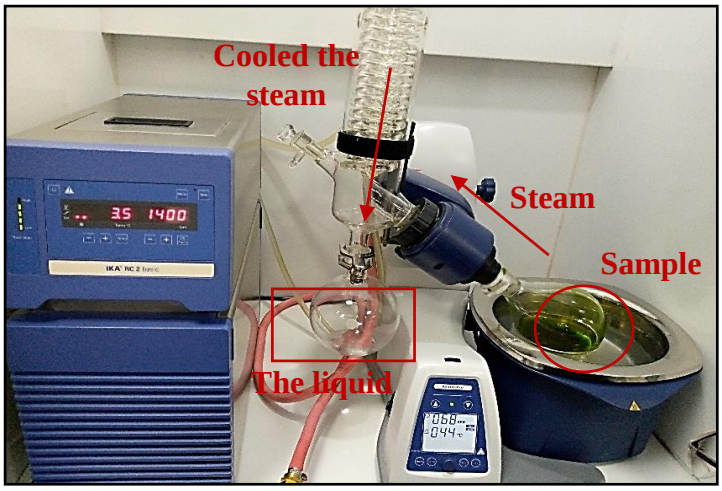


Figure 04: the work of Rotavor.

How we worked:

- Clean and sanitize the Rotaevor by that we mean the main condenser, vice condenser, rotary flask, and the receiving flask for that we vapor crud ethanol in Rotavoror.
- Pot our sample to its place on Rotavopr, pot the three bottles of the sample together (Ex: the bottles 1A, 2A, and 3A all together).
- The time and tempter setting of every sample (Table1).

Table 01: The time and tempter setting of every each sample.

Sample	Tempter °C	Time (min)
N1	51	11:10
N2	51	11:12
N3	52	08:54
N4	52	11:45

- We didn't evaporate all the ethanol we left a concentrated polyphenol.

I.2.4. Result:

We left with four filled glass bottles of concentrated polyphenol; there are two methods to use them:

II. Vaporing all the ethanol and leaving with a powder, the method for that:

- Pot the sample in a petri dish and expose it to a high tempter (50 -60°C) by using an oven.

III. Using the same at it is, means mix it with the food directly, and let it for a good time to vapors by itself. We used the second method.

Annexe 02

Histologic slides:

The histologic slides were implicated in the Laboratory of anatomic-pathology Dr.MESSAID F. Ouargla.

Method:

Gonads were transferred to the cassettes (Fig.5). The specimen should not be so thick to fill the cassette (it should be less than 4mm).

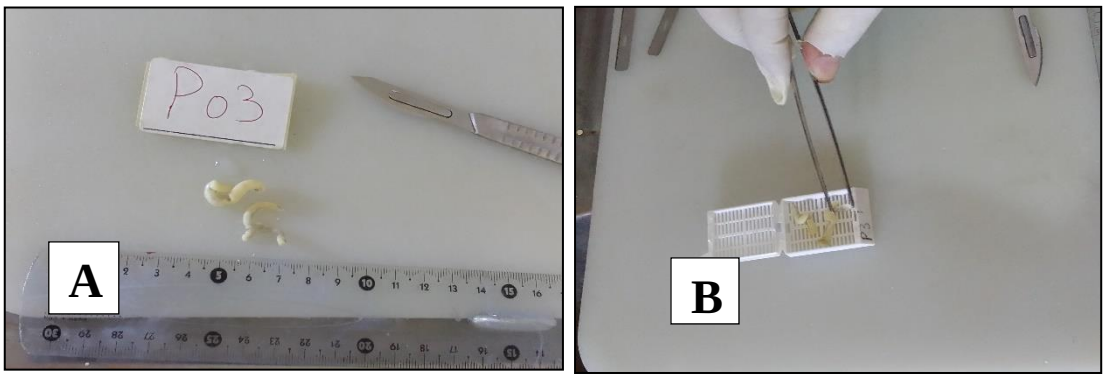


Figure 05: Placing the gonad in the cassette (**A:** Mesure of gonads, **B:** Placing the gonads).

The filled gonads cassettes are stored in formalin then they are placed on the automate to start the processing step as follows:

1. Dehydration, where it involves immersing the gonads in increasing concentrations of alcohol to remove the water and formalin.

2. Clearing, in which an organic solvent such as xylene is used to remove the alcohol and allow infiltration with paraffin.

3. Embedding, where specimens are infiltrated with the paraffin. The tissue becomes surrounded by a large block of molten paraffin. Once the block solidifies, it provides a support matrix that allows very thin sectioning (Fig.6).

Figure 6: Preparing blocks of paraffin.

4. Sectioning, with a microtome (Fig.7) The paraffin is removed from the surface of the block to expose the tissue. It's used to slice extremely thin tissue sections off the block in the form of a ribbon (mostly 5 µm).

Figure 7: Pictures of microtome.

Once cut, the tissue ribbons are carefully transferred to a warm water bath (Fig.8). Here they are allowed to float on the surface, and can then be scooped up onto a slide placed under the water level. Charged slides work best for this process, they improve tissue adhesion to the glass, and help to reduce the chance of sections washing off the slide during staining.

Figure 08: Picture of the water bath.

Slides should be clearly labeled, and then allowed to dry upright at 37oC for a few hours to gently melt the excess paraffin wax, leaving the tissue section intact.

5. Staining, histochemical stains are therefore used to provide contrast to tissue sections making tissue structures more visible (Fig.9).

This process is carried out by going through several stages (Fig.10) where the slides pass through several color reagents and fixaters for a specified period of time as follows :

Xylen 10min → Xylen 10min → Alcool 100% 5s → Alcool 100 % 5s → washing → Hematescylin 30s → washing → Lithium Carbonate 1s → Washing → Eosine 30s → Alcool 100% 5s → Alcool 100% 5s → Alcool 100% 1s → Xylen 5s → Xylen 5s → Xylen 5s.

Figure 09: Pictures of staining boxes.

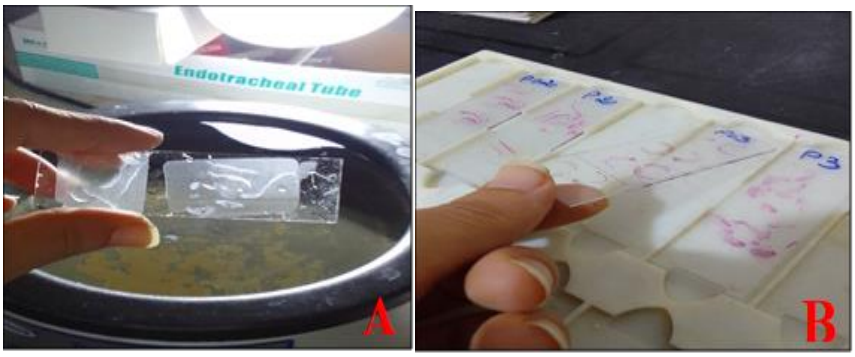


Figure 10: A; slide before staining. B; slide after staining.

A coverslip is mounted over the tissue specimen on the slide using optical grade glue to help protect the specimen. Then we put it under the microscope for observation (Fig.11).

Figure 11: Picture of the slide under the microscope optic.

Annex 03

Fry transport:

Newly hatched Nil Tilapia fry's collection and sterilization (Fig12).

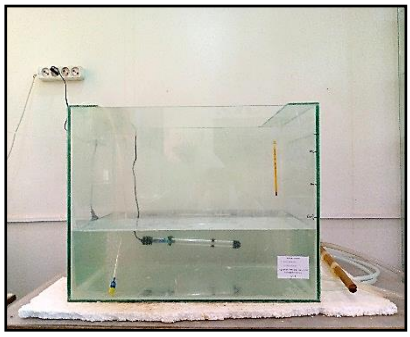
The fry transport was by transparent bag, compressed oxygen tablet (30mg/10L).



Annex 04

Fish measurement

The aquarium before fishing for measurement

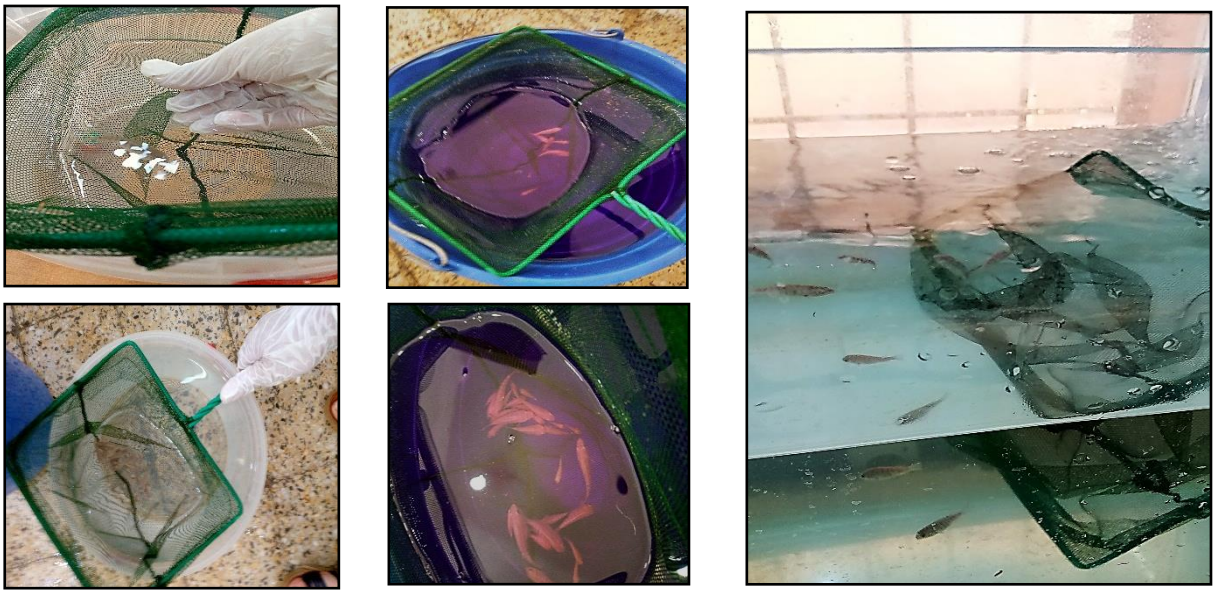



Fishing for measuring the height and weight



Caching the fish and measure them

Sanitizing the fish in permanent potassium before putting them back in their cleaned aquarium



Annexe 05

Making the barriers of aquariums

References

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Abstract

The object of this study is to investigate the effect of Neem (*Azadirachta indica*) leave on the sexual differentiation of Nile tilapia « *Oreochromis niloticus*», which consists in obtaining a population of phenotypically male individuals. This experiment was based on two treatments. Initially, by adding the quantity of Neem leaves powder (TN) to the fish food, and the second treatment by adding polyphenols extracted from Neem leaves (TPoly). Both treatments were performed on a larval diet. The results confirmed that the first treatment male population is 77%, on the other hand. The second treatment balanced at 67.8%. Statistical analysis shows no significant difference between them. The results recorded a survival rate in the first treatment which was equal to 45.83% with an average final weight of 28 .2g.While in the second treatment equal to 37.50% with an average final weight of 25.15g,

Keywords: Nil tilapia, Neem tree, Polyphenol, sexual inversion.

Effet de l'utilisation du Neem « *Azadirachta indica* » sur la différenciation sexuelle du Tilapia « *Oreochromis niloticus* »

Résumé

L'étude d'inversion sexuelle du tilapia du Nil (*Oreochromis niloticus*) qui consiste à obtenir une population d'individus phénotypiquement mâle, elle est basée sur un traitement de masculinisation avec une concentration dede poudre de feuilles de Neem *A. indica* (TI) mélangé avec l'aliment des larves comparé à une autre population des larves où une concentration polyphénols extrait de feuilles de Neem (TII) incorporer dans l'alimentation. Les résultats montrent que le 1ertraitement est confirmé par une population mâle de 67,8%, par contre le2ème traitement équilibré à 77%. L'analyse statistique montre aucune différence significative entre eux.Les résultats ont enregistré un taux de survie dans le premier traitement égal à 45,83 % avec un poids final moyen de 28,2g, tandis que dans le second traitement, il était égal à 37,50 % avec un poids final moyen de 25,15g.

Mots clés : Tilapia de Nile, Neem, Polyphenol, inversion sexuelle.

تأثير استخدام النيم على التمايز الجنسي لسمك البلطي

ملخص

الهدف الرئيسي من هذه الدراسة هو معرفة تأثير مسحوق أوراق النيم "*Azadirachta indica*" على التمايز الجنسي للبلطي النيلي "*Oreochromis niloticus*". تعتمد هذه الدراسة على إضافة نسبة من مسحوق أوراق النيم وادخالها في نضام الغذائي الخاص بيرقات البلطي النيلي من أجل الحصول على نسبة اعلى من الذكور ذات النمط الظاهري ومقارنتها بمجموعة أخرى من اليرقات تم معالجة علفها عن طريق إضافة نسبة من البوليفينول المستخلص من أوراق النيم بعلف الأسماك. أكدت النتائج فعالية مسحوق أوراق نيم بلغت67,8%، وفي المقابل نسبة المجموعة الثانية مستخلص البوليفينول بنسبة 77%, غير أن التحليل الاحصائي أظهر عدم وجود فرق معنوي بين النسب. سجلت النتائج معدل البقاء على الحياة في المجموعة الأولى يساوي 45.83% مع متوسط وزن نهائي 28,2غ. في حين أنه في المجموعة الثانية يساوي 37,50% مع متوسط وزن نهائي قدره 25,15غ.

الكلمات المفتاحية: البلطي النيلي، بوليفينول، التمايز الجنسي، النيم.