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Effect of short time temperature alteration on two key reproduction hormone levels of Nile tilapia (*Oreochromis niloticus*)

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Master of Science

Effect of short time temperature alteration on two key reproduction hormone levels of Nile tilapia (*Oreochromis niloticus*)

Thesis submitted to the Norwegian University of Life Sciences (NMBU)for the degree of Master of Science

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Declaration

I hereby declare that this thesis has been composed entirely by myself and has not been previously submitted for any other degree or qualification.

The work of which it is a record has been performed by me, and all sources of information have been specifically acknowledged.

Name of the student

Nowshim Sultana

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Content

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Glossary of Terms

CO_2	carbon dioxide
cu	cubic
cm	centimetre
DO	dissolve Oxygen
E_2	17β-estradiol
FAO	Food and Agriculture Organization
FSH	Follicle stimulating hormone
g	grams
GnRH	gonadotropin releasing hormone
GtHs	gonadotropic hormones
hrs	hours
HPG	hypothalamic-pituitary-gonadal
IMM	inner mitochondrial membrane
kg	kilograms
mL	millilitre
mg	milligram
mm	millimetre
mg/l	milligram/litre
$NaH_2PO_4.H_2O$	Sodiumdihydrogenphosphate
L	litre
LH	luteinizing hormone
ppm	Parts per million
ppt	parts per thousand
PVC	polyvinylchloride
Rpm	Revolutions per minute
T	Testosterone
RIA	Radioimmunoassay

Abstract

This study investigates the effects of rapid and short temperature change on the levels of the hormones testosterone and estradiol-17β and the subsequent effect on egg maturation in Nile tilapia (*Oreochromis niloticus*). The purpose is better synchronisation of a group of Tilapia when applying stripping and controlled reproduction. Short-term temperature manipulation, drop from 28^0C to 22^0C for three hours, was applied to 24 female Nile tilapias in glass aquaria. The baseline water temperature was maintained at 28 ± 0.5^0C, and the temperature alteration was obtained by partly exchange of the aquaria water at day 8 of the experimental period, which lasted for 14 days. Eight control fish were kept at the same temperature (28^0C) throughout the experiment. Blood serum was collected for hormone analysis at three specific times during the total experimental period: 24 hours after spawning, after temperature alteration, and immediately before stripping. The fish were fed 1% of their body weight, 2 times a day, with a 12/12 hrs photoperiod. The groups of Tilapia that were tested were on average 800 g. Number of fertilised eggs were counted. Also, the length and width of sample eggs from each fish were measured, along with number of unfertilised/dead eggs. Finally, the numbers of hatched fry were counted manually and fry with deformities were recorded and sorted out. Changes of testosterone and estradiol-17β levels in blood serum were examined by immunoassays.

The results show that both the estradiol-17β level and the testosterone level were significantly higher ($P<0.01$) in treated fish than in the control group at the time of stripping. Also, most of the fecundity parameters measured did improve by temperature treatment, especially egg mass, which increased with almost 30%, and the reduction in deformities was ca 40%, both differences being significant ($P<0.05$). The results observed in the experiment show that the manipulation of an environmental factor (temperature) could be significant for greater control on fry production of Nile tilapia.

Introduction

By the year 2050 the human population will likely reach more than 9.3 billion, (Béné et al., 2015) and it will become a big challenge to meet the needs for animal protein to feed this many. Up to now, fish and livestock has been the main source of animal protein (Yue et al.,2016). The contribution from aquaculture and capture fisheries were 171 million tons in 2016, and the aquaculture part of this has increased constantly, reaching 47 percent in 2016 (FAO, 2018). Globally fish provides people with ca 17 percent of their intake of animal protein (Waite et al., 2014).

More than 420 types of fish species are cultured in the world now, among these, tilapias are by many considered to be one of the most important future aquaculture species (Webster & Lim, 2006) . Tilapias are mainly a freshwater fish, from the cichlid family (M. S. Azaza et al.,2008); they are commercially important and grown in almost 120 countries, China, Indonesia, Egypt, Philippines, Thailand, Honduras, Ecuador and Costa Rica being the main production countries (Fitzsimmons, 2016; Paessun & Allison, 1984; Pullin et al., 1982). Tilapia, also termed the aquatic chicken due to its mild taste and ability to feed on a pure vegetable diet (Yosef, 2009), has for a long time been economically profitable for the producer as well as attractive for a large market worldwide.

According to El-Sayed (2006), the characteristics that make tilapia an ideal candidate for aquaculture are: rapid growth, it is omnivorous and can feed on low-priced plant sources, and grows well in a wide range of environmental conditions (i.e. temperature, salinity, low dissolve oxygen, etc.). It also seems tolerant to stress and diseases, has a short generation interval and low supplementary feed requirement (El-Sayed, 2019).

A recent re-classification of tilapia taxonomy was reported by (Dunz, 2013) and is based upon differences in terms of feeding habits, bio-geographical distribution and principal mode of reproduction. The three generic groupings are: Oreochromis (arena-spawning maternal mouthbrooders), Sarotherodon (paternal or biparental mouthbrooders), and Tilapia (substrate spawners) (Le François et al.,2010).

Nile tilapia aquaculture

In freshwater, the culture of Nile tilapia dominates among the tilapia species. The Nile tilapia is strong and capable of surviving in difficult conditions. The natural distribution of Nile tilapias is quite wide; covering the Nile river basin, Eastern and Western rift valley lakes in East Africa, and Chad, Niger, Benue, Volta, Gambia and Senegal rivers in the west. (El-Sayed, 2006); (Lind et al., 2019).

In fresh water, the culture of Nile tilapia represents a major fish species for aquaculture worldwide, global production amounted 4.2 million tons in 2016 (Dietz & Liebert, 2018). The (Mutayoba et al.,1990) Nile tilapia has the world's fourth highest production among all aquaculture species and the production is steadily growing (Biswas et al.,2005).

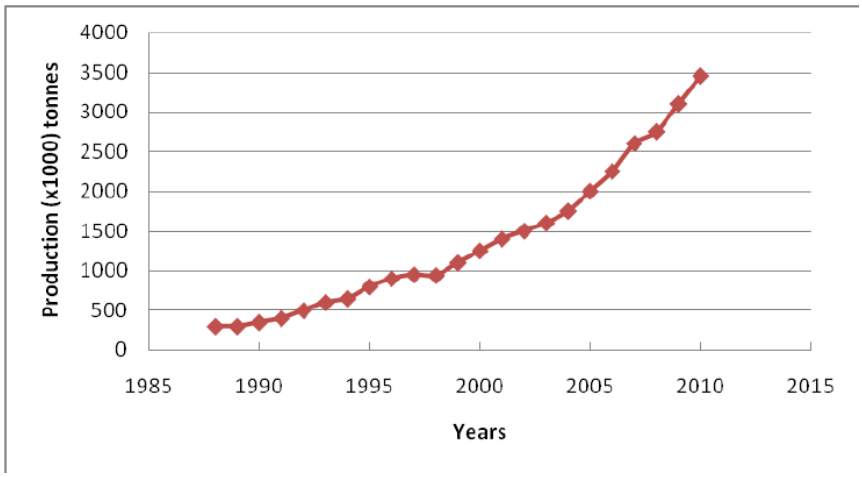

Figure 1 : Global aquaculture production of Oreochromis niloticus

(Adapted from FAO Statistics and Information Service of the Fisheries and Aquaculture Department. Top 10 species groups in global aquaculture 2017)

Breeding and reproductive biology of Nile tilapia

Breeding habit and natural spawning

Nile tilapia have deep and laterally compressed bodies with moderately large cycloid scales. (Nazir & Ijaz, 2017). When sexual mature and ready to spawn, both sexes show a red blush on the belly and lower flanks. The dorsal and caudal fin margins of the males become bright red. The males will normally be 20-30% heavier in weight in comparison to the females (Hussain, 2004). The genital papilla is also larger than that of females and has two openings, whereas the female has three openings. At the age of 3 months, with body weight of around 40-60g, the Nile

tilapia normally becomes sexually mature (Rajaee, 2011). During spawning, the female exhibits the movement of touching the nest. The ripe and fertilised eggs are pale yellow orange in colour, ovoid in shape, and their size ranges between 1 - 3 mm in diameter and 2 – 3 mm in length. Depending on the size and age of the female, fecundity fluctuates widely, from a few hundred to several thousand eggs (El-Sayed, 2019).

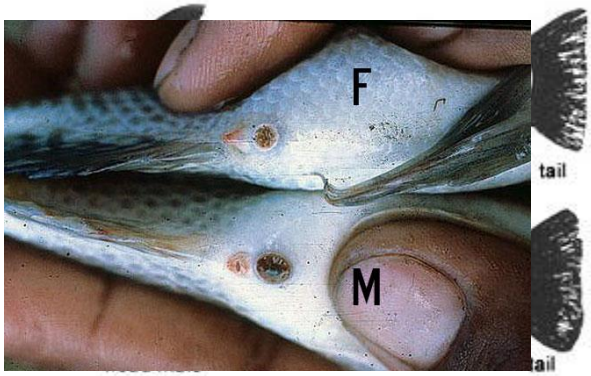

Figure 2:Some morphological characteristic of Nile tilapia Oreochromis niloticus

Adapted from https://ep.franphil.com/the-biology-of-tilapia/

Process of gonad development:

The development of gonadal products (egg and sperm) in Nile tilapia is a complex prosses. Early egg development starts with the oogonia in the female fish, and then primary oocytes give rise to secondary oocytes. A high-energy yolk (vitellogenin) deposition occurs in the growing oocytes in this pre-ovulatory period. After that, secondary oocytes complete such yolk deposition by the process of vitellogenesis and remain as mature oocytes in the ovary for a variable period of time until their final maturation or ovulation. The ovarian development phases are defined differently by various authors: (Wallace & Selman, 1981) states that in the teleost phase, four main stages of the oocitary growth are documented; stage A (immature), stage B (in maturation), stage C (mature), and stage D (empty). Six stages are also characterised in the ovarian development (Vazzoler, 1996) . Phase I – germinative young cells; Phase II – stocked oocytes; Phase III – oocytes with lipidic vitellogenesis; Phase IV – Oocytes with lipidic and proteic vitellogenesis; Phase V – oocytes with complete vitellogenesis; and Phase VI – oocytes in hyalinization. (see also Figure 3).

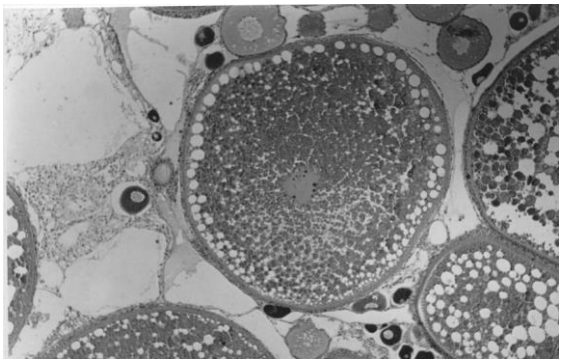

Figure 3: Histological section of an ovary shows different stages of development at peak maturation in *O. niloticus*. (Source: Adapted from https://www.researchgate.net/figure/Histological-section-of-an-ovary-shows-various-stages-of-development-at-peak-maturation_fig5_283348879)

Fish reproductive endocrinology:

In fish, reproduction is controlled by the hypothalamic-pituitary-gonadal (HPG) axis. A stimulus of the hypothalamus produces and secretes the gonadotropin releasing hormone (GnRH). The pituitary gland is responsible for the production and secretion of the gonadotropic hormones (GtHs), and the gonads are responsible for the production and secretion of the sex steroid hormones (Chehade, 2012). GnRH travels through direct innervations between the pituitary and hypothalamic neurons, stimulating the gonadotrophs to release GtHs (Carr & Norris, 2005). These feedback mechanisms are regulated by primary stimuli and environmental cues, such as photoperiod and temperature. These cues stimulate various sensory receptors, including those in the pineal gland, and are integrated within the brain to initiate and regulate reproductive endocrine signalling (Ankley et al., 2009).

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are the two different forms of the GtHs. Follicle-stimulating hormone (FSH) is responsible for gonadal growth and gametogenesis, and luteinizing hormone (LH) is responsible for gonadal maturation and spawning. LH acts to stimulate the production of the sex steroids: estrogene, androgens and progesterone. 17β-estradiol is the main estrogene in females (MacLatchy et al.,1997).

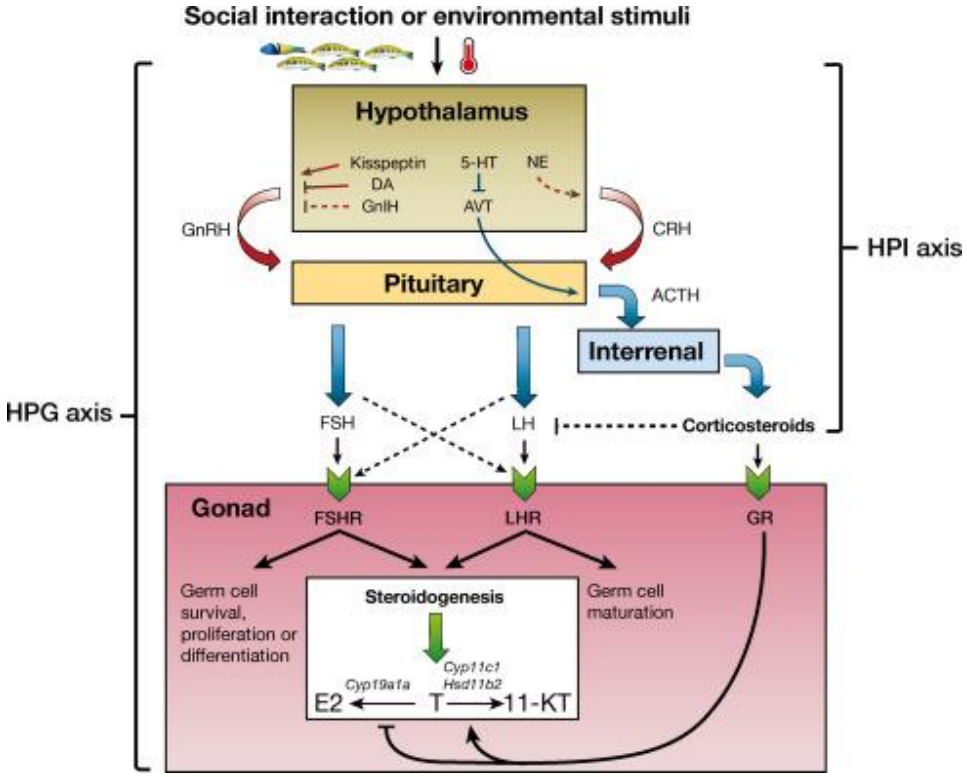

Figure 4: Histological section of an ovary shows different stages of development at peak maturation in *O. niloticus.*

(Source: Adapted from https://www.sciencedirect.com/science/journal/00702153)

The sex steroid hormones are produced after completing a complex biosynthetic pathway relating a number of intermediates and enzymatic conversions. The pathway is reliant on the delivery of the substrate, cholesterol, from the cytoplasm, across the mitochondrial membrane, to the inner mitochondrial membrane (IMM). Once in the IMM, the cytochrome P450 side-chain cleavage enzyme (P450scc) hydroxylates carbons 20 and 22 and removes a six-carbon residue side chain (C22-C27), giving rise to pregnenolone. Pregnenolone is then the originator for the synthesis of the steroid hormones T, E_2 and 11-KT (Biswas et al., 2005). The following authors describe the macroscopic analysis of ovarian development in details: (Latif & Saady, 1973) and (Srisakultiew, 1993).

Endocrine hormone profiles

The most essential hormones in the process of sexual maturation and gonad development are vitellogenin, 17β-estradiol, testosterone and 11-ketotestosterone. Sex steroid hormones control the development of secondary sexual characteristics such as bright and shiny body colour, blush fins with reddish margins, prominent urogenital papilla, etc. in fish. In female fish, egg yolk generally results from a precursor of lipophosphoprotein-calcium complex called vitellogenin,

synthesised by the liver, after that is released into the blood and finally sequestered by the oocyte (Tyler et al.,1987). The yolk precursor is synthesised during the vitellogenesis time of ovarian growth under the influence of oestrogenic control (stimulated by 17β- estradiol hormone). Finally, vitellogenin and all other sex steroid hormones have interlinked functions to control oocyte maturation. Serum calcium concentration (index of vitellogenin) and sex steroid hormone levels in sexually matured female and male *O.niloticus* have been determined respectively by using the atomic absorption spectrophotometry and radioimmunoassay techniques (Coward, 1997).

Reproductive hormones

Over the past few years, study on the molecular characteristics of fish reproduction has progressed quickly. Through the accessibility of new wide-ranging molecular tools, fish researchers can operate in the brain, pituitary, and gonad. This investigation has exposed new variations of reproductive hormones and their receptors, which lights on the mechanisms of many different systems. What has been revealed, is that the three sex steroid hormones, 17β-estradiol , 11-ketotestosterone (11-KT) and 17α20 β- dihydroxy-4-pregnen-3-one (DHP), are abundantly produced in gonadal tissues under the control of pituitary gonadotropins (GTH) (Taghizadeh et al., 2013).

In the present study, two sex hormones, testosterone and estradiol-17β excretion levels are measured.

Estradiol

Estradiol is abbreviated E2 as it has two hydroxyl groups in its molecular structure. Estradiol is secreted from both the female gonads and inter-renal tissues. Estradiol is responsible for stimulating vitello genesis and development of the secondary sexual characteristics, also secreted by female gonads during the pre-spawning period (Pankhurst, 2008) .

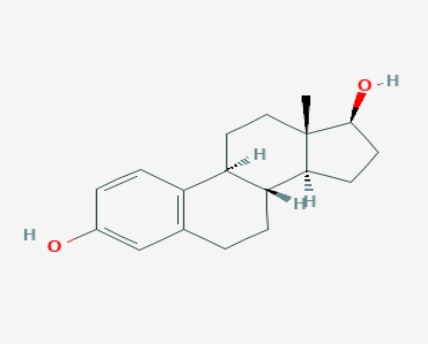

Figure 5: Structure of estradiol

(Source: Adapted fromhttps://pubchem.ncbi.nlm.nih.gov/image/imgsrv.fcgi?cid=5757&t=l)

Testosterone

Testosterone is a steroid hormone belonging to the androgen group of hormones. It is found in both males and females and in mammals. Testosterone is primarily secreted from the testes of males and from the ovaries of females. Small amounts are also secreted by the adrenal glands.

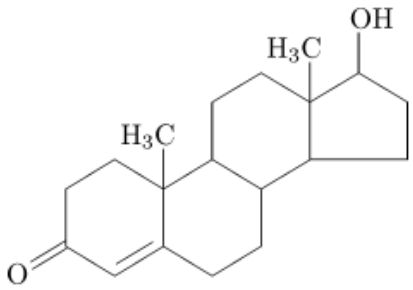

Figure 6: Structure of testosterone

(Source: Adapted fromhttps://dailymed.nlm.nih.gov/dailymed/image.cfm)

The environmental effects on reproduction

Several environmental and physiological factors are actively involved in fish gonadal maturation and reproductive behaviour. The external factors, such as temperature, photoperiod, salinity, pH, DO and lunar cycle, are held to have an influence on seed production in fish (Watanabe, 2000). These exogenous factors and a mixture of social factors are perceived by the brain and translate into neural impulses serving to excite the endocrine pathways of the HPG to act in a suitable fashion, and in this way, reproduction only occur during those times of the year when the most favourable survival of fry can be expected. The frequency of tilapia spawning varies considerably with environmental factors (Baggerman, 1980; Bromage et al., 1992; Hokanson, et al.,1998).

Fish is an ectotherm animal and highly dependent on temperature. In teleost fish, temperature, seasonal rainfall and other factors are known to regulate reproductive cycles. These factors are significant and precisely timed annual fluctuations in temperate regions. Climate warming thus will influence the relative phenology of annual migrations and spawning (Simpson, Blanchard, & Genner, 2013). Temperature is a crucial physical regulator and an influencing factor that effects and is expressed mainly in the control of all reproductive processes (Pankhurst & Munday, 2011), (Remen et al., 2015). For instance, changing the temperature in *D. albisella* reduced the inter-spawning interval (Hilder & Pankhurst, 2003). Reproductive endocrine homeostasis in fish is thus responsive to changes in temperature (Pankhurst, 1997). Alternations

in the secretion and action of hormones are associated with all the mechanisms of the HPG-axis, controlling the reproductive processes. Temperature affects hormone synthesis, secretion and metabolism (Pankhurst, 1997). Temperature also has a remarkable effect on the speed of spermatogenesis (Alvariño, 2000). Under aquaculture conditions, the timing of gonadal maturation and spawning frequency can be adjusted by modifying the water temperature (El-Sayed & Kawanna, 2007), and the main environmental factor that leads to the development of fish eggs is held to be temperature (Pankhurst, 1997). Also hatching is influenced by temperature in *Oreochromis niloticus (Bhujel, 2000)*.

Tilapia are thermophile fish and can tolerate a wide range of temperatures from 8 to > 40^0C (Nivelle et al., 2019), but Tilapias become inactive when temperature is below 16^0C (Chervinski, 2008). According to Hatikakoty & Biswas (2004) reproductive performance becomes very poor at temperatures higher than 35^0C.

In tilapia, the effect of light has also long been investigated. Photoperiod has a great influence in the development of biological rhythms in fish, both in their reproduction and metabolism are very variable and can change over a great range, often very rapidly (Okorie, 1973; Boeuf & Le Bail, 1999). Sexual maturation is thus strongly reliant on photoperiod and the dependent androgen secretion has an effect even on somatic growth (Le Gac et al., 1993). Campose et al. (2004) showed that photoperiod manipulation also influences egg size. In regulating the daily endogenous rhythms in fish, photoperiod acts as an artificial synchroniser (Bromage et al., 1993), and photoperiod manipulation is thus a functional tool for controlling the reproduction of tilapia culture. The early development of tilapia eggs is strongly influenced by the immediate difference in photoperiod and light quantity (Blanco-Vives et al., 2011).

Photoperiod manipulation is generally accepted as the most important factor synchronising sexual maturation and reproduction in fish (Bromage et al.,2001). A 12L: 12D cycle has been suggested for the most favourable reproduction performance in Nile tilapia, and this photoperiod produced the highest spawning performance in a study by (El-Sayed & Kawanna, 2007).

Tilapia can tolerate poor water quality and can even survive in a low content of oxygen dissolved in water; they can tolerate a DO level as low as 1 ppm (Pullin & Lowe-McConnell,

1982). But the growth rate of tilapia of course diminishes in low DO levels (Buentello et al.,2000). Tilapia can tolerate oxygen super saturation up to 400% (Morgan, et al., 1997)

pH is one more environmental factor that influences the growth performance of tilapia, and the tolerable range for tilapia is 6.5 to 8.0 (Hussain, 2004).

Salinity is also an important environmental factor for reproduction in fish and many Tilapias can tolerate a high salinity level as well as freshwater (Stickney, 1986). The optimal range for growth, however, is 0–20 ppt (Suresh & Lin, 1992), whereas the range of salinity tolerance is 0-25 ppt for Nile tilapia (Hussain, 2004). At 14 ppt Nile tilapia spawned but their spawning stopped at 28 ppt in study by (Fineman-Kalio, 1988).

The existence of 'natural toxicants', such as ammonia, are yet another factor known to play a key role on the physiological functions (Boeuf & Le Bail, 1999). Ammonia has been described a significant factor for growth and survival. By breaking down of organic matter, ammonia is produced. It is also a by-product of protein metabolism in fish. Fish excrete nitrogenous wastes via the gills in the form of ammonia. For toxic levels of ammonia, different physiological changes occurs (Ahmed & Rab, 1995). Ammonia can exists in two forms, ionized NH_4^+ and unionized NH_3 (Francis-Floyd et al., 2009), and the toxicity of ammonia depends on DO, CO2 and pH. An increase of ammonia levels in water affects fish growth, gives symptoms for caused by various fish disease and can cause severe mortality (Bhakta, 2006) (Foss et al., 2009). Consequently, high ammonia levels will also be negative for fecundity.

Depending on environmental conditions, female tilapia spawn asynchronously every three to four weeks (Campos-Mendoza et al., 2004).

Temperature modulate all physiological processes and endocrine regulations in teleost fish. It can arrest or delay some gametogenesis stages, particularly the most advanced ones including maturation and ovulation (Billard, 1986). Temperature control is a natural method not requiring the purchase of veterinary medicines and is in addition very effective for certain species. It therefore has economic advantages for the fish farmer, as well as for the image of aquaculture in the eyes of the consumer.

The effect of temperature alteration on spawning behaviour and hormone changes during the ovulation period is still not too well documented, the aim of the present study is thus to assess the effect of short time temperature alteration on the hormone level of Nile tilapia and to formulate some practical guidelines for obtaining synchronous spawning in a group of fish. The present study thus was designed to investigate the effect of short time temperature shock treatments on synchronisation of the artificial spawning of Nile tilapia and observe the rate of hormone changes during the period of ovulation.

Materials and methods

Fish:

Healthy and sexual mature females of Nile tilapia (n=32) were collected (originating from the 16th generation of the GenoMar Supreme Tilapia strain (GST®), the Philippines) for this experiment from the fish lab at the Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences.

Experimental fish treatment protocols

The experiments were carried out in 8 glass aquaria containing water with approximately the same physicochemical characteristics and with continuous aeration. Due to limited number of aquaria available, the experiment was divided in two time periods, to increase the total number of observations and thus the statistical power. Each round included 16 fish, i.e. 2 in each aquarium, separated with a transparent divider (Figure 8). Fish were randomly assigned to one of two groups: Treated (n =12) or Control (n=4) in each round, i.e. n=32 (24 treated and 8 control) in total.

Spawning activities were initially observed for 7 days, and fish that had spawned were moved to their experimental aquarium within 24 hours after this event.

Blood samples were taken three times during the experimental period:

1. On day 3, after being moved to the aquaria, at12:00
2. On day11 at12:00
3. On day 14 at 12:00, i.e. 2 days before stripping.

Aquaria treatment

Eight glass aquaria (100 x 50 x 50 cm; 200 L capacity) were used for the experiment. A filter pump (620 l/h) was used for treatment and circulation of water. The aquarium had PVC covers to maintain the water temperature and prevent fish from jumping out. An electric water heater (300W thermostatically controlled) was applied to maintain the water temperature at 28±0.5°C in each aquarium. Each aquarium was aerated with a low-pressure blower (Airtech AT-40, 0.13kgf/cm^2).

Figure 7: Aquaria system used throughout this experiment; each aquarium containing 2 fish.

Water quality parameters:

Water quality was monitored two times a day : Water temperature pH and DO,) were monitored by the instrument WTW pH/Oxi 340i. Nitrate, ammonia and CO_2 level were checked by water quality kits (la Motte, model PCO-DR). The water temperature was maintained at 28±0.5°C.

Photoperiod:

A digital timer kept a day light length (12 D: 12 L).

Feed:

Fish were fed twice a day (at 08:00 and 18:00) providing at total of (1% body weight per day. The feed given was a standard commercial pelleted feed (Chemical composition is given in Appendix, Table 5).

Measurements of fish

Before starting the experiment, the fish were anesthetised with MS 222 (0, 5g/L), and length were measured by using a metric ruler (cm) and weighted using weighting balances (g). For complete recovering, fishes were allowed to swim in clean water before releasing them back to the aquarium. Fish were weighed again at the end of the experiment.

Body pigmentation

Fish were checked twice a day during the initial experimental period to observe the behaviour of spawning and the changes of genital papilla, redness of the tail and body. The main sign of imminent spawning is changing of the body colour to red (Figure 9 and Table 1).

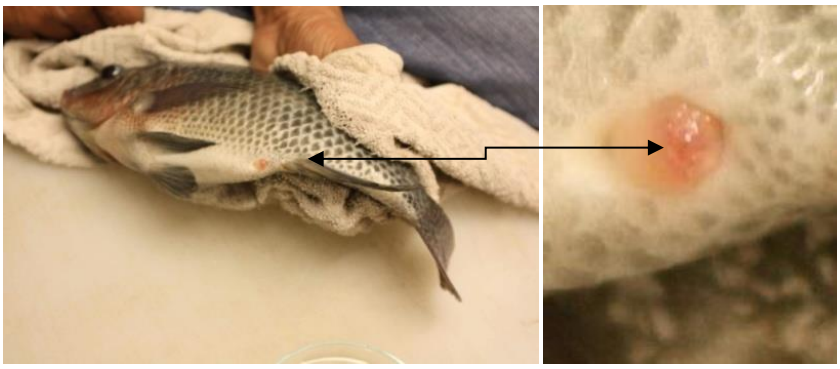

Figure 8: Before stripping papilla shows redness in colour and enlarged size

Table 1: Sexual changes of pigmentation in *O. niloticus*

Area of pigmentation	Pigmentation
Whole body	light orange to purple
Edges of the dorsal fin	Black
Head	red-purple
Belly	Red
Caudal fin	black strips

Temperature treatment

In the Treated group, an environmental stimulus was achieved at day 10 by a sudden drop in temperature, from 28^0C to 22^0C, by adding colder water. The reduced temperature was kept at 22^0C for 3 hours, from12:00 to 15:00, before the temperature was elevated back to 28^0C by introducing warmer water again.

Control fish were kept at the same temperature (28^0C±0.5°C) throughout the experiment. Eggs from all fish were stripped and fertilize at day16.

Stripping and artificial fertilisation

The fish was wrapped in a cotton cloth to avoid scale damage, after being anesthetised with MS-222 as described above. The eggs were stripped into a 300-mL petri dish by applying gentle squeezing the pectoral region between the thumb and forefinger and stroking in a head-to-tail direction (Figure 10). Milt was collected from the male fish in the same way. The milt and eggs were stirred for 1-2 minutes with a feather, and few drops of water were added to start activation. The mixture was left for a minute to complete the fertilisation, and the fertilised eggs were then placed in a controlled temperature at 28^0 C ±1^0 C in the hatchery (Figure 11).

Figure 9 : Artificial spawning of the brood fish by gentle squeezing of the abdomen between the thumb and forefinger.

Artificial Incubation and Nursing

Egg incubation was facilitated by using a basin with 8 individual conic plastic jars, each having an individual tap, set to a constant water flow rate of 0.17 L per min, causing a suitable water movement in the bottom of the jar, similar to the natural movement of eggs in the female's mouth (Figure 11). After hatching (Figure 12), the fry is led by the water flow into a nursing basin (Figure 13). Sufficient oxygen supply and optimal temperature ($28^{O}C$) was maintained in the hatchery.

Figure 10 : The Hatchery system in the experiment room

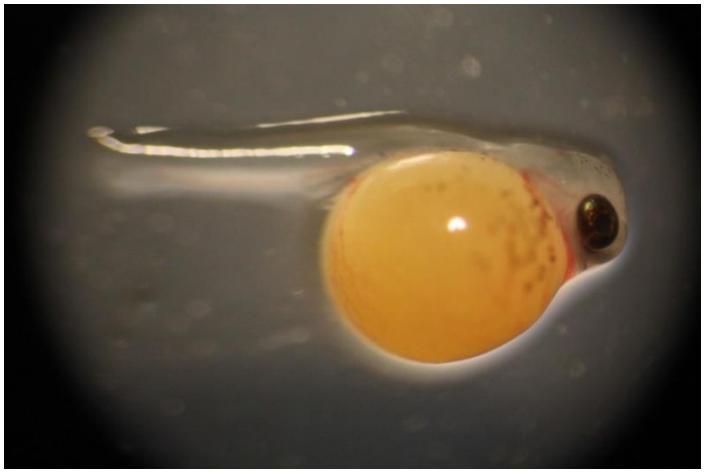

Figure 11: One day old fry with yolk sack

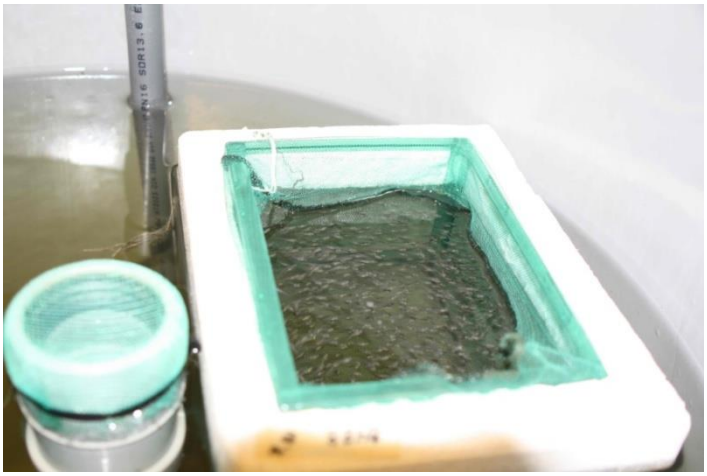

Figure 12: Nursing of tilapia fry in a net box

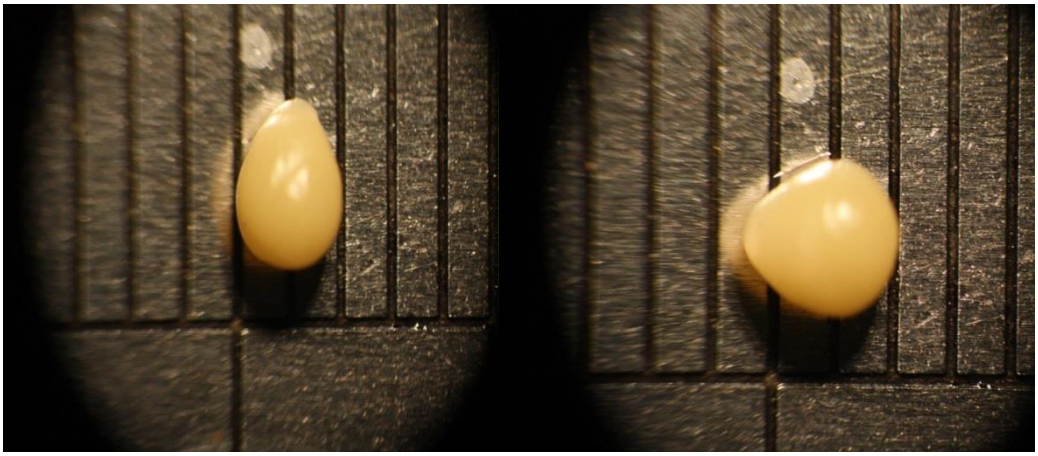

Figure 14: Measurements of the fish were taken both horizontally and vertically

Figure 15: Example of unfertilised eggs

Fecundity records

Eggs from the fish were taken out by artificial stripping into a petri dish and the total weight of the eggs were determined and recorded. Fifty eggs were counted and taken out from the petri dish and re-weighted. By multiplying the egg per gram in this sample with measured grams of the whole stripped material, an estimate of the total number of eggs was obtained.

Both the length and width (Azaza et al ,. 2008) of some sample egg from each fish were measured (Figure 14), along with number of unfertilised/dead eggs (Figure 16):

Finally, the numbers of hatched fry were counted manually and fry with deformities (Figure 16) were recorded and sorted out.

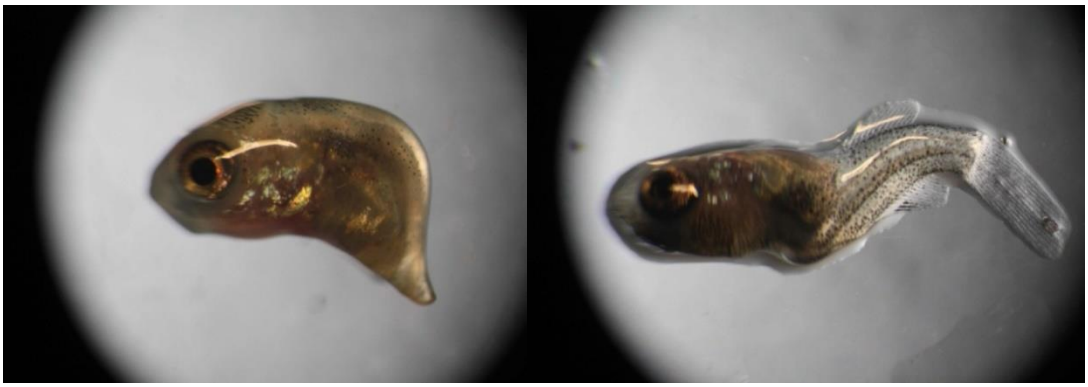

Figure 13: Deformed tilapia fry

Blood sampling and analysis

Before blood sampling, the fish were anaesthetised by keeping them in MS222 solution for 3-4 minutes. Blood was collected from the caudal vain with heparinised syringe 22G needles within 10-20 minute after capture. Eppendorf tubes were used to prevent proteolysis. Plasma was obtained by centrifuge at 5000-6000 rpm for 15 minutes. The serum was stored at -80^{0}C and transported to the laboratory in a box with dry ice until time of doing radioimmunoassay.

Radioimmunoassay

Plasma levels of testosterone (T) and 17β-estradiol (E_2) were measured by specific radioimmunoassay (RIA) (Mutayoba et al., 1990) . RIA is an immunological assay to analyse any antigen in the sample with high sensitivity. Prior to RIA, individual plasma samples (100-300µl) were mixed with RIA buffer (containing 0.05% NaN_3) in a ratio1:2 and heat-treated for 1h at 80^{O}C. After centrifugation, the supernatant was extracted and stored at 4^{O}C for hormone assay. Plasma samples were measured in duplicate. All tubes contained 50 µl of label (^{3}H-T; Perkin Elmer NET 370, 70 Ci/mmol, or ^{3}H- E_2; Perkin Elmer NET 317, 70 Ci/mmol), and 200 µl of steroid antibody (T and E_2 antisera were provided by Dr. Helge Tveiten, Nofima, Tromsø). Serum hormone values are expressed as µg/ml (Budzinski, Devier, Labadie, & Togola, 2006).

Further details of the hormone analysis:

RIA Buffer

$NaH_2PO_4.H_2O$	3.87gm
$Na_2HPO_42H_2O$	10.67gm
NaCl	9.00gm
Gelatine	1.00 gm
Na. acid	0.05 gm

Gelatine was dissolved in 200 ml hot distilled water. The other substances were dissolved in 700 ml of water. Ingredients were then combined, the pH adjusted to 0.7.

Dextrane-coated charcoal solution (DCC)

NoritA	1.0 gm
dextranT-70	0.1 gm

The dry powder was added to 100ml of RIA buffer and stirred for 1 hour at 4^{0}C, using a magnetic stirrer.

[^{3}H]-steroids

A stock solution was made up with ethanol (stored at 4^0C). For RIA, the stock solution was diluted with RIA buffer to obtain an activity of 30-35,000 dpm/50µl.

Antisera

For testosterone, antiserum was diluted to a final working solution of 1:2500

Standard curve

The standard curve is obtained from a series of steroid solutions in RIA buffer containing different concentrations of the respective non-radioactive steroid (cold). The steroid is first dissolved in ethanol to produce a stock solution of 100mg/50 µl. This stock solution is further diluted with RIA buffer stepwise to produce the following 9 dilutions (for the standard curve): 2000,1000,500,250,125,62.5,31.25,15.63,7.8pg/50 µl.

Hormone analysis procedure

For all steroid assays, duplicate 10x75 mm borosilicate tubes contained either 50 µl of standard or 100 µl of sample. Each vial received 50 µl[^{3}H]-steroids, followed by 200 µl antibody, total volume of all vials being 350 µl. The vials were then vortexed and incubated overnight at 4^0C. The following day, vials received 300 µl cold DCC, incubated for 5 min, then centrifuged for 5 min at 400 rpm at 4^0C. The supernatant was decanted into scintillation vials containing 4 ml of scintillation fluid. After vortexing, the vials were counted for 5 min in a liquid scintillation counter.

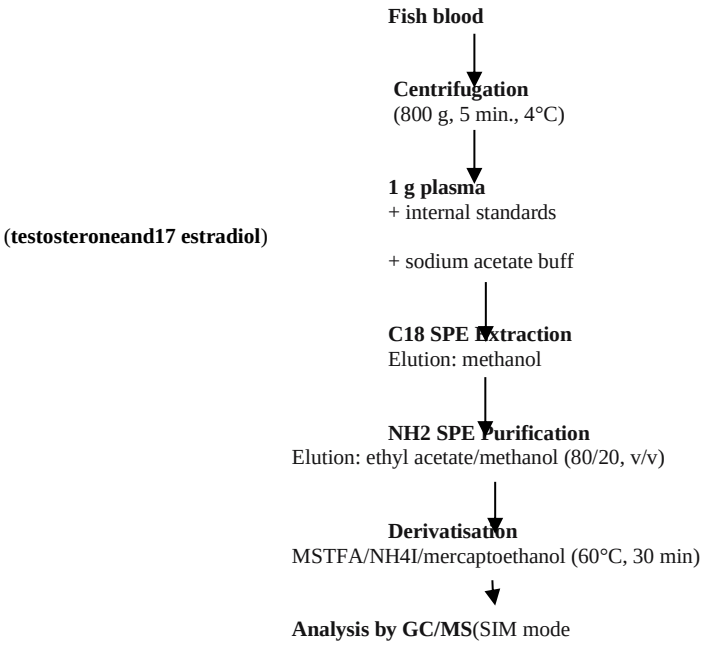

Figure 17: Analytical procedure developed for the determination of steroids in plasma sample

Results

Hormone levels

The Hormone levels in different time periods are shown in Figure 17 and 18, and in Table 2. A two-sided Student's t-test was used to see if the groups, Treated and Control, were significant different from each other at each time or period. Only in the last period, 24 hours before spawning, did the two groups significantly differ ($P < 0.01$).

Table 2 : The effect of Estradiol-17β and testosterone hormone (mean ± STD) at three different time periods during the reproduction cycle.

Hormone	Group	24 hours after spawned	24 hours after temp. shock	24 hours before spawned
Estradiol-17β	Treated	6.4 ± 2.7	7.3 ± 3.6	7.9* ± 3.4
	Control	4.4 ± 1.6	8.4 ± 3.4	3.5* ± 1.2
Testosterone	Treated	4.8 ± 2.1	9.6 ± 5.2	12.3* ± 5.9
	Control	4.7 ± 2.5	12.1 ± 5.4	4.8* ± 3.0

* The two groups were significant different ($P < 0.01$)

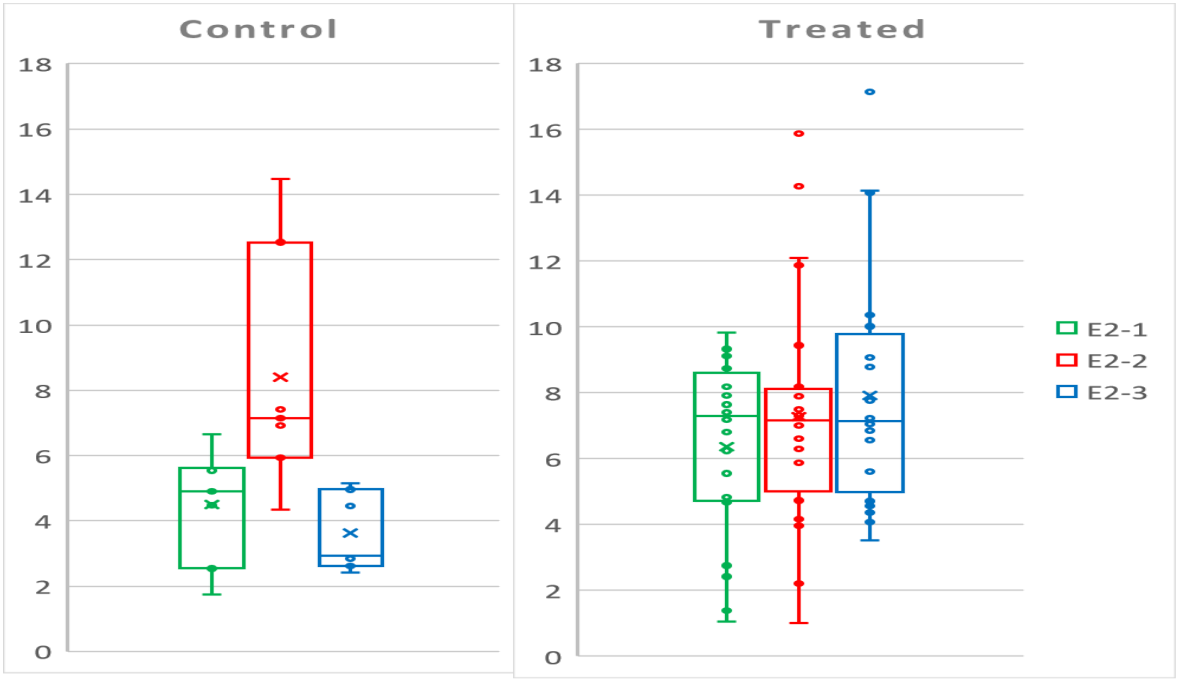

Figure 18 : Secretion of estradiol for the three periods measured, for Control and Treated group of fish.

E2-1. blood sample taken 24 hours after spawned
E2-2: blood sample taken after temperature shock
E3-3: blood sample taken before stripping
The "boxes" represent the interquartile range (IQR), the distance between the third quartile and the first quartile. Max and min values are marked by the whiskers. An outlier is defined if it is

outside max or min values, i.e. 1.5 times the IQR. X represents average, whereas the line inside the box is the median.

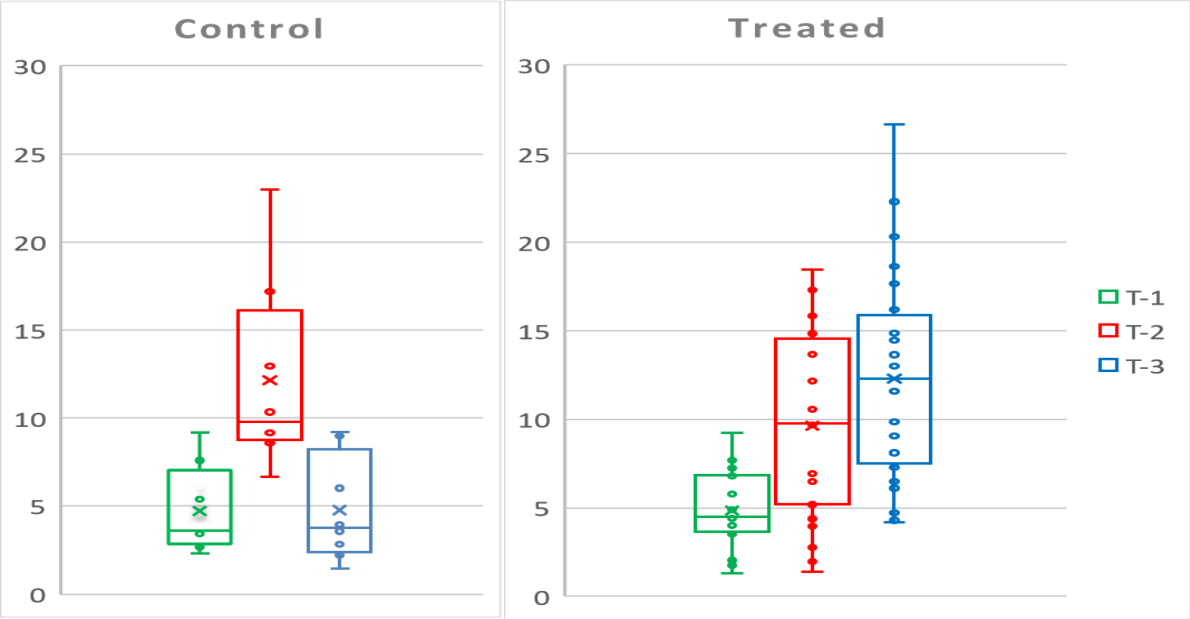

Figure 19 : Secretion of testosterone for the three periods measured, for Control and Treated group of fish.

E2-1. blood sample taken 24 hours after spawned.
E2-2: blood sample taken after temperature shock.
E3-3: blood sample taken before stripping.
The "boxes" represent the interquartile range (IQR), the distance between the third quartile and the first quartile. Max and min values are marked by the whiskers. Max or min values are defined as 1.5 times the IQR. X represents average, whereas the line inside the box is the median.

In general, the graphs show different trends of hormone level for the two treated and untreated groups: For both hormones, the Control group show first an upward and then downward trend, whereas the Treated group shows an overall upward trend, particularly for Testosterone.

Fecundity

The short-term temperature effect of various fecundity parameters in Nile tilapia in this experiment are indicating that the treatment group has improved performance compared with the control group (table 3). All parameters were better in the treatment group (with temperature manipulation) than in the control group (no temperature manipulation), except for egg width and egg length which were similar in both groups. The improvements were particularly strong and significant ($P<0.05$) for the parameters deformities and egg weight, which showed ca 40% and 30% improvement, respectively.

Table 3: Comparative study of the short-term temperature effect of various fecundity parameters in Nile tilapia in this experiment.

Group	Egg weight (g)	Number of eggs	Dead eggs	Length of eggs	Egg width (mm)	Number of Fry	Deformities
Treated	8.9*±2.1	520 ±126	45±38	2.2±0.1	2.0±0.1	367±90	7*±6
Control	6.9* ±2.7	476±75	55±37	2.2±0.1	2.0±0.1	354±76	12*±4

* The two groups were significant different ($P < 0.05$)

Discussion

Hormone levels

The results of the present study indicate that temperature has the ability to affect the hormonal level of Nile tilapia, as also shown by others (El-Gamal & El-Greisy, 2005). Temperature has for long been known to be important in regulating gonadal maturation in tilapias (Brummett, 1995), and is considered in many fish species as an essential signal in gonadal development and in seasonal induced spawning (Trudeau, 1997). Temperature is thus generally regarded as an important cue in gonadal activity in fish (El-Gamal & El-Greisy, 2005). Specifically, sudden environmental changes are believed to influence spawning synchrony in tilapia (Pradeep et al., 2014)).

As shown for the Control fish in this study, steroid levels are normally low during the non-reproductive stage, but rise slowly during gametogenesis and go down abruptly thereafter (Brummett, 1995), and temperature treatment has shown to influence temporal changes in plasma testosterone and 17β-estradiol in common wolffish (Tveiten & Johnsen, 2001). The reason why this does not happen as pronounced in the treated fish is uncertain, but the result show that the average value of estradiol-17β level in all the three time periods for treatment is higher than the control. The results show that treatment had significant effect on the hormonal levels in the last period, 24 hours before spawning. However, as can be seen from both the figures and the table, the variance among observations was generally high and did not ideally follow a normal distribution, which may indicate that this assumption associated with the t-test is not fully met.

The results may thus indicate that treatment stress was higher in treated group than in the control group. For instance has capture, handling and incarceration has been shown to induce a stress response in fish that can elevate plasma level of estradiol-17β (Pickering et al.,1987).

Fecundity

The present results show that the fecundity parameters, number of fry, weight of egg and reduction in number of dead or unfertilised eggs, were improved in treated group of fish compared to the control group, and the results were significantly higher for number of eggs produced and reduction in number of deformities. Similarly, a short term manipulation of temperature resulted in an increase in the percentage of mature eggs and gave significant effect on the numbers of hatched fry in Nile tilapia (Srisakultiew & Wee, 1988), and another study showed that exposing tilapia to water 22^0C for a period preserved their spawning to a great amount (El-Sayed et al.,2005). Sudden environmental changes may also perk up spawning synchrony in tilapias, as shown in the study of (Srisakultiew, 1988).

(Wang & Tsai, 2000) also showed in their study that deformities in tilapia were influenced by water temperature.

Conclusions

The focus of this study was to study the change of levels in two important reproduction hormones by manipulating temperature for a short period of time and thus synchronise the artificial spawning of Nile tilapia.

It is clear from this study that short time temperature shock has an effect on hormonal levels during different stages of reproduction compared to the control (no temperature shock). The result also show that short time temperature shock on tilapias has a positive effects on reduction in deformities, egg weight, number of eggs, reduction in dead eggs, and number of fry produced. In this study it was not found any significant differences among the temperature shocked and not treated tilapia in terms of observed egg length and width. It is proven that the sudden change of temperature in a short time act as a trigger on the tilapia reproduction system. The association of changes in gonad condition with plasma levels of gonadal steroids has proven to be a valuable tool in the development of a better understanding of endocrine control of reproduction in tilapia.

The results observed in the experiment show that the manipulation of an environmental factor (temperature) could be significant for greater control on fry production of Nile tilapia. Thus, it is suggested that more experiments should be conducted in order to acquire further knowledge of the reproduction control in this species.

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Appendix

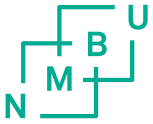
Table 4 : The initial weight and length of experimental fish

Number of fish	Initial weight and length		Final weight and length (after striping)	
	B.W (g)	Length (cm)	B.W (g)	Length (cm)
Fish 1	835	35.1	838	35.2
Fish 2	758	33.4	761	33.5
Fish 3	768	34.1	770	34.2
Fish 4	751	33.1	759	33.1
Fish 5	823	33.6	829	33.6
Fish 6	724	32.5	730	32.6
Fish 7	835	32.8	840	32.8
Fish 8	765	33.5	770	33.6
Fish 9	863	35.9	872	36.0
Fish 10	829	32.0	835	32.1
Fish 11	957	33.1	961	33.2
Fish 12	838	33.7	843	33.7
Fish 13	731	34.1	733	34.1
Fish 14	757	33.2	761	33.2
Fish 15	723	33.8	732	33.8
Fish 16	742	33.1	748	33.2
Fish 17	818	33.5	822	33.6
Fish 18	735	32.1	730	32.2
Fish 19	818	32.9	832	32.9
Fish 20	769	33.2	770	33.3
Fish 21	864	35.5	872	35.6
Fish 22	836	32.5	838	32.8
Fish 23	912	33.1	916	33.3
Fish 24	839	33.5	843	33.7
Control Fish 1	893	35.3	896	35.4
Control Fish 2	729	33.6	739	33.7
Control Fish 3	766	33.4	773	33.4
Control Fish 5	751	33.1	761	33.2
Control Fish 6	831	35.1	841	35.3
Control Fish 7	742	33.4	739	33.6
Control Fish 8	741	33.2	758	33.4
Control Fish 4	756	33.3	761	33.4

Table 5 : Chemical composition of feed used during the experiments

Chemical composition of feed used	Percent
Protein	23.00
Fat	11.00
Fiber	1.50
Water	8.00
Ash	7.00
Calcium	1.50
Phosphorus	1.20
Extra added per kg feed	
Iron as Fe_2SO_4	12 mg
Zinc	125 mg
Selenium	0.1 mg

Metabolisable energy per. kg: ca.14.500kJ / 3,470 kcal



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