Deanship of Graduate Studies

Al- Quds University

The Effect Of Anti prostaglandin COX2 on NileTilapia Fish Growth Rate and Mortality Rate

Iman Ibrahim Irhail AL-Hirsh

Master Thesis

Jerusalem- Palestine

2011/1432

The Effect Of Anti prostaglandin COX2 on NileTilapia Fish Growth Rate and Mortality Rate

Prepared By:
Iman Ibrahim Irhail AL-Hirsh

BSc of Biology -Al Quds University -Palestine

Supervisor: Dr. Mutaz Qutob

A Thesis Submitted in Partial Fulfillment of Requirements
for degree of Master of Environmental Studies.
Department of Earth and Environmental sciences
Al-Quds University

Jerusalem- Palestine

2011/1432

Al_ Quds University
Deanship of Graduate Studies
Department of Earth&Environmental Sciences
Environmental Studies

Thesis Approval

The Effect of Anti prostaglandin COX2 on NileTilapia Fish Growth Rate and Mortalitiy Rate

Prepared by: Iman Ibrahim AL-Hirsh
Student Number: 20714312

Supervisor: Dr. Mutaz Qutob

This Master thesis was submitted and accepted on 14/52011 by the following committee members:

Head of committee (supervisor): Dr. Mutaz Qutob	Signature: ……
External examiner: Dr. Ahmad Naser	Signature: …….
Internal Examiner: Dr Mutaz Akawi	Signature: …….

Jerusalem- Palestine

2011/1432

Dedication

To my lovely parents

To my sisters & brothers

To My faithful friends

& finally to all who gave me help and support throughout my life.

Iman

Declaration:

I certify that this thesis submitted for the degree of Master is the results of my own research, except where otherwise acknowledged, and that this thesis (or any part of the same) has been not submitted for a higher degree to any other university or institution.

Signature: …….

Iman AL_Hirsh

Date: ……..

Acknowledgment

This study is part of GFD project funded through the German Ministry for Education and Research.

My Great appreciation and many thanks to Dr. Mutaz Qutob, (Research supervisor), for his unlimited, absolute and comprehensive support, in science research.

Great regards and thanks to all faculty member in the department of earth and environmental science.

I would like to express my great sincere gratitude to all aquaculture lab staff Housam Malasah, Sobhi Yagi, Mustafa Amarna for their helping .also a lot of thanks to Dr .Peter Lawrence for his encouragement and my thanks extended to Mr .Olaff for his continuous advises.

My great thanks to Fida malek first for her effort to analysis my samples by using HPLC Device and secondly as a friend of mine.

The last and continuous thanks to my family and all my friends especially Tasneem AlJabarie for her encourage & kindness.

Table of contents

List of Tables:

List Of Figures

List of abbreviation:

Sympol	Description
O.niloticus	Oreochromis niloticus
ppt	part per thousand
DE	Digestible Energy
DO	Dissolved Oxygen
MT	Methyltestosterone
ET	Ethinyl-testosterone
FDA	Food and Drug Administration
USA	United State Of America
Dpf	Day post fertilization
Hpf	Hour post fertilization
Bp	Base pair
ORF	Open Reading Frame
AI	Aromatase Inhibitor
E_2	Estradiol
Shbgb	Sex hormone binding globulin
Amh	Anti Mullarian hormone
Gsdf	Gonadal soma derived growth factor
tgfb	Transforming growth factor b
fst	follistatin
PGs	Prostaglandins
NSAIDs	NonSteroidal AntiInflammatory Drugs
Cox	Cyclooxygenase
MAPEG	Membrane associated protein in eicosanoide and glutathione metabolism
MGST 1-3	Microsomal Glutathione Transferase 1-3
TPA	Tetra decanoyl phorbol acetate
HPLC	High Performance Liquid Chromatography

EMEA	European Medicines Evaluation Agency
Auc	Area Under the plasma concentration Curve time
DMSO	DiMethyl Sulfoxide
STPs	Sewage Treatment plants
GI	Gastrointestinal
CMA	Coeliaco Mesenteric Artery
CO	Cardiac Output
GBF	Gastric Blood Flow
CCK	cholecystokinin
TH	Thyroid Hormone
IRD	Inner Ring Deiodination
ORD	Outer Ring Deiodination
TSH	Thyroid Stimulating Hormone
ASA	Acetyl Salicylic Acid
FSH	Follicle Stimulating Hormone
LH	Luteinizing Hormone
GnRH	Gonadotropin Releasing Hormone
KT	Ketotestosterone
IGF-1	Insuline Like Growth Factor
GH	Growth Hormone
MHC	Major HistoCompatibility
LOEC	Lowest Observed Effective Concentration
LC_{50}	Lethal concentration for 50% of population
EC_{50}	Effective concentration for 50% of population
NAPQI	N-acetyl-p-benzoquinoneImine
PNEC	Predicted No Effect Concentration
OM	Organic Matter
GR	Growth Rate
BWG	Body Weight Gain

FCR	Food Conversion Ratio
FCE	Food conversion Efficiency
SGR	Specific Growth Rate

Abstract

Several studies have been conducted to detect the direct effect of inhibiting the aromatase activity, the rate limiting enzyme that converts androgens to estrogens needed for ovarian differentiation in fish to overcome the immediate need for a more environmentally friendly substitute of methyl testosterone. Cyclooxygenase (COX2)-inhibitors are potent and irreversible inhibitors of the COX2 pathway and since studies on human breast cancer cells shows that they decrease aromatase messenger ribonucleic acid (mRNA) expression at the transcriptional level we tested the effects of supplementation of COX2-inhibitors (Etodolac and Etoricoxib) in the diets of fry tilapia on growth rate, and mortality.On the other hand, human and veterinary pharmaceuticals have been shown to occur in considerably high amounts in sewage treatment plant (STP) effluents and surface waters, with the non-steroidal anti inflammatory drugs representing one of the most commonly detected compounds. Information concerning possible ecotoxicological risks of these substances is rather scarce. So far there are no data available on their possible effects in fish after prolonged exposure. Thus, highlight on Etoricoxib pharmacokinetics was carried out by determination of Etoricoxib in fish feces using HPLC. The study was carried out at the Aquaculture research laboratory, in AlQuds University, Jerusalem. At an age of 8 days post – hatched 30 genetically mixed population of Oreochromis. niloticus larvae were stocked in duplicate, into aquariums each with a capacity of ≈ 45L in a closed system. Treatments included 5 different experimental diets and one standard diet serving as control with two repeats for each group from 0.5 % groups of diets. The test diets were prepared by mixing 600 mg of etodolac with 20g,60 g,30 g commercial feed, to achieve (0.5% etodolac,1%etodolac, 2% etodolac)concentrations respectively, the same process was done to the etoricoxib by mixing 90 mg of etoricoxib with 18 g, 9 g, commercial feed, to achieve (0.5% etoricoxib,1% etoricoxib) concentrations respectively. Another experiment was conducted using 10 adult mixed populations at age of 2 months stocked in triplicate in a closed system and, treated as above. Feeding started on the same day of stocking and fish were fed once daily. Feed was adjusted according to fish weight, by 10 % of their weight during the first four weeks and 5% of their body weight after changes of all diets to control diet. Fish were weighted every week and counted in each aquarium to determine survival rate during 12 weeks period. Individual fish in each aquarium was weighted to the nearest 0.1 g using a

digital scale. The growth rate (GR) was determined using linear regression: yt = a + bxt, where yt is the average total weight (g) of the fishes at time t and a is the average weight (g) of fishes at the start of the experiment. Results showed that the different growth rate parameters (final body weight, weight gain and growth rate (GR) of *O niloticus* fed with selective COX_2 inhibitor Etodolac and Etoricoxib respectively were significantly affected with the highest growth rate obtained with the 0.5% etodolac followed by etoricoxib 0.5% followed by untreated sample. However, no increase or decrease of growth in mixed adult population was observed. Growth rate increased with increasing concentrations with the highest growth rate in the aquarium treated with (2% Etodolac) followed by (1% Etodolac), but (1% Etoricoxib) showed a decreased growth rate compared to standard which could indicates a toxic potential toward fish at this concentration. In addition, no peak for Etoricoxib was detected on HPLC in feces samples collected after half an hour, 1 hour, 2 hours, 3 hours and 4 hours,24 hours following treatment with 1% etoricoxib diet in 4 months age tilapia. This reflected, that Etoricoxib, was well absorbed by tilapia, extensively metabolized with no unchanged fraction excreted, or may undergo enter hepatic circulation, increasing further its toxic potential. No mortality was observed in adult mixed population. In fish fry there were no differences in mortality rates between (0.5% Etodolac), and (0.5% Etoricoxib) treatments, but survival rate (96.6% and 90%) were improved compared to control (86.6%, 83.3%) during the experimental feeding. Higher mortalities were shown as concentrations increase with (1%Etoricoxib), (1% Etodolac) and (2% Etodolac). These results indicated that the inclusion of selective COX-2 inhibitor in fish diet efficiency was maximally exerted during critical period of sexual differentiation, which occurs in tilapia fry between 2 and 6 weeks after spawning. After finishing of this period there was no effect on the growth rate. Thus, COX inhibitors could modulate aromatase activity needed for proper sexual development and reproduction during the crucial period. Furthermore, this is the first time to our knowledge we test the effects of these agents on fry teleosts during the crucial period of sexual development which points to possible alterations in reproduction following chronic exposure to these drugs at an early stage in contaminated surface water. Subsequent field investigations in normal aquaculture bonds are needed to confirm these results in larger population, using different classes of COX-inhibitors at different concentrations.

Chapter one

Introduction

1.1 Growth in NileTilapia:

The growth rate of tilapia is determined by several factors and it is important to take all these factors into consideration. The growth rate will for instance be affected by water quality, temperature, oxygen levels and the general health of your fish. The type of food you provide them with and in which quantities will naturally also be of imperative importance. Last but not least, you have to pick an optimal stocking density. In addition to this, it is important to choose a species, hybrid or strain that is fitting for particular fish farm. Many producers advertise about strains with a super-fast growth rate, but this growth rate will not be attained unless the environment is ideal for that particular strain. You must for instance take the climate in your part of the world into account and the salinity level in the water you plan on using is also important.

1.2 Growth rate in mixed-sex culture

In a mixed-sex tilapia culture, the fish is normally harvested before the fish reaches sexual maturity or soon afterwards. This restricted culture period makes it even more important than normally to make the fish grow fast, since they have to reach their proper size within a limited time frame. It is therefore common to avoid dense stocking of mixed-sex tilapia cultures. It is also important to avoid using stunted fish since such fish will reach sexual maturity while they are still too small for the food market. Blue tilapia (Oreochromis aureus), Nile tilapia (Oreochromis niloticus) and their hybrids are common in mixed-sex cultures since they will attain a marketable size before commencing spawning. Species such as Mozambique Tilapia (Oreochromis mossambicus) and Wami tilapia (Oreochromis urolepis hornorum) are normally avoided since they will be too small when they reach sexual maturity. By choosing the right species or strains and providing the fish with a suitable environment and proper nutrition, it is possible to achieve a growth rate fast enough to allow fry produced in the spring to reach a marketable size by autumn in temperate regions. For a 4-5 month long culture period it is common to stock 2,000-6,000 one month-old fry per acre in grow out ponds. With a stocking density of 4,000 fry per

acre, the average weight at harvest can then be expected to be around 0.5 pounds (220 grams), if supplemental feedings with protein rich food has been carried out.

1.3 Growth rate for all-male fingerlings:

In mono-sex cultures, it is common to opt for male fish only since the male tilapia grows faster and reaches a larger size than the female. All male batches can be obtained through hybridization, hormonal treatment or manual sexing and separation. It should be noted that none of these methods can guarantee 100% males in any batch. If you want really large tilapia, the amount of females in the growing unit should not exceed 4 percent. Many farmers therefore use more than one method to ensure a low degree of females in the growing unit. Predator fish of a suitable size can also be added to the growing unit to devour any offspring. All-male tilapia cultures are often densely stocked. This will decrease the individual growth rate of each fish, but it will normally result in a higher yield per unit area. Densely stocked cultures are more susceptible to ill-health and careful water management is recommended, since poor health can have a devastating effect on growth rate and lead to massive losses. The normal stocking rate for all-male tilapia cultures varies from 4,000 to over 20,000 fishes per acre. If you have no supplemental aeration, it is safest to stay in the lower range. In a suitable environment with an adequate supply of nutrition, it is possible for 50 gram fingerlings to become 500 gram fishes within 6 months even without supplemental aeration if the stocking rate is 4,000/acre. This means an average growth rate of 2.5 grams per day and it is possible for such a culture to yield 2.2 tons/acre. A stocking rate of 8,000/acre can yield up to 4.4 tons/ acre, but will require night time emergency aeration. You can expect the average weight gain to be 1.5-2.0 grams/day. The culture period will need to be at least 200 days, often more, if you want to produce fish that weighs almost 500 grams. Stocking rates above 12,000/acre will require extensive aeration, but can on the other hand yield up to 6-10 tons / acre. Keeping the water quality up will be difficult and you might have to resort to sub-optimal feeding rates and this will naturally affect the growth rate.

Tilapia growth can generally be broken into three distinct phases:

- Exponential or Accelerating Phase
- Linear Phase
- Decelerating or Plateau Phase

Young fry are ravenous eaters and they can consume feed that is equivalent to up to 25% of their body weight daily. As a result, the fish grow very fast when measured in percent of body weight per day. This phase is referred to as the Exponential or Accelerating Phase. However, since the fish are initially very small, the total weight gain is initially low. This rate of weight gain (in grams per day) increases until the fish weigh approximately 100 grams, at which point they enter the Linear Phase of growth. During the Linear Phase, Tilapia eats approximately the same amount each day and the growth remains fairly linear. Their feeding rate does not change drastically during this period because, although the fish are growing, they eat less feed as a percent of their body weight as they grow. The duration of the linear growth phase differs dramatically with the species and strain of tilapia. Some inferior strains may show growth deceleration at sizes of less than one pound. When fish growth begins to decelerate, they enter what is referred to at the Plateau Phase. At this point, it begins to take more food and time to achieve a given amount of growth. This can be very frustrating for the aquaculturist who is trying to get fish to market. Cheaper fingerlings often become more expensive in the long run due to poor genetics. During an in-house growth trial of AmeriCulture Tilapia, the linear growth phase of genetic males was maintained up to a size of approximately 3 pounds before decelerating. This is an important consideration for aquaculturists who want to get their fish to market size before they begin to plateau. Of course, the fish must be reared optimally from the time they arrive at your facility in order to achieve these types of results for practical reasons. (www.Americulture.com)

1.4 Sex reversal: -

Sexual behavior is one of the most profound events during the life cycle of animals that reproduce sexually. After completion of gonadal development that is mediated by various hormones, oviparous teleosts perform a suite of behaviors, often termed as spawning behavior. This is particularly important for teleosts that have their gametes fertilized externally as the behavior patterns ensures the close proximity of both sexes for gamete release, fusion and ultimately the production of offspring. As in other vertebrates, sexual behavior of fish is also under the control of hormones. Testicular androgen is a requirement for male sexual behavior to occur in most fish species that have been studied. Unlike tetrapods, however, ovarian estrogen does not appear to be essential for the occurrence of female sexual behavior for fish that have their gametes fertilized externally.

Prostaglandins produced in the ovary after ovulation act as a trigger in some teleosts to induce female sexual behavior. Potentiating effects of gonadotropin-releasing hormone in the brain on sexual behavior are reported in some species. Under endocrine regulation, male and female fish exhibit gender-typical behavior during spawning, but in some fish species there is also some plasticity in their sexual behavior. Sex changing fish can perform both male-typical and female-typical sexual behaviors during their lifetime and this sexual plasticity can also be observed in non-sex changing fish when undergoing hormonal treatment. Although the neuroanatomical basis is not clear in fish, results of field and laboratory observations suggest that some teleosts possess a sexually bipotential brain which can regulate two types of behaviors unlike most other vertebrates which have a discrete sex differentiation of their brain and can only perform gender-typical sexual behavior.

1.5 Sex inversion by different methods

1.5.1 Androgen treatment : - There are several techniques used to obtain a predominantly male population of tilapia and the most used technique in Israel is Chemical Sex Reversal : First feeding fry are fed a diet laced with methyl-testosterone (MT) or Ethinyl-testosterone (ET) for 21-30 days, at a dose of 60mg/kg feed. When done properly this technique is very effective, often resulting in populations greater than 95% male. The most commonly used drugs (MT and ET) are both not approved by the FDA, and known as carcinogenic. In many areas in USA and Europe the use of MT or ET is forbidden. Recently, it was found that it is possible to induce sex reversal of O. niloticus by egg immersion, an alternative technique from the traditional sex reversal method of feeding the fry with hormone-treated feeds. The highest average proportion of males (91%) was obtained at 800 μg/l comparable with the 88- 89% male obtained at 400μg/l and 600 μg/l at immersion time of 96-hr. These values of percent males were lower compared to the reported average masculinization rate employing the traditional sex reversal using hormone-treated feed. A hormone administered at 40 mg/kg to first feeding fry for approximately 25 days fed 4 times a day, can give an excess of 90% male, although 100% populations are seldom achieved.

1.5.2 Genetic control: - The best way to obtain all-male populations is through genetic control (Baroiller et al., 1999). Based on the first data on tilapia sex determination and differentiation, it has been possible to produce genetically “all-male populations” through

the development of YY “supermales” (Baroiller et al., 1999). Nevertheless, this approach is unreliable and hampered by the very long procedure of producing and identifying putative YY male individuals. Moreover, sex determination has been shown to be more complex than a simple agent XX / XY monofactorial system.

1.5.3 Non-steroidal: - genetically female tilapia fry that were treated with Fadrozole, a non steroidal aromatase inhibitor, in the diet during the period of sexual differentiation showed a dose- dependent increase in the percentage of males (Kwon et al., 2001). In the current work we will try to use non-steroidal agents for sex reversal.

1.5.4 Environment and sex inversion: - Environmental factors (temperature, pH, density and social interactions) could influence the sex ratio in gonochoristic species. In fish, the main environmental factor influencing sex seems to be temperature. In tilapia, as thermo sensitive species, male to female ratio increases when temperatures were higher than 32ºC (Baroiller et al., 1999).Despite the fact that the fate of the gonad is determined genetically, temperature can override it and switch the mechanism when the gonad is undifferentiated. But once the decision is established it cannot be modified anymore, being committed towards the development of one sex .The critical period of gonad differentiation in tilapia has been established from 9 to 15 days post fertilization (Ijiri et al., 2008). Temperature or hormonal treatments have to be applied from this period onwards to be efficient. Despite the strong genetic basis for determining sex in tilapia it is now clear that other factors are also acting on sex. A strong effect of temperature on sex differentiation has been demonstrated in various tilapia species and in a hybrid (Baroiller et al., 1999). It was possible to masculinise XX tilapia progenies 100% females with elevated temperatures above 32 °C, giving functional male phenotypes. The use of female monosex populations as well as the progeny testing of temperature treated males, has definitely demonstrated the existence of skewed male sex ratios corresponding to a sex-inversion of 19genetic females (XX) to functional phenotypic males. These thermo-neomales provide in their offspring all-female or almost all-female progenies depending on the breeders. High temperatures could efficiently masculinise some progenies if started around 10 days post fertilization (dpf) and if applied for at least 10 days, with longer periods being just as effective. Recently, demonstrated that even a precocious elevated temperature-treatment applied 12 h post fertilization (hpf) and kept for 52±2 h till hatching, can also induce significantly skewed sex ratios towards males (8–27%, n=4) on a true all-female progeny

100% females in the control group. Even if the high mortality associated to the precociously high temperature treatment (35–36 °C) has to be considered, this data strongly suggest that there is a thermo sensitivity window very shortly after fertilization (between 12 and 52 hpf (=4 dpf)), long before the development of the presumptive gonads.

1.6 Fish ovarian aromatase:

The aromatase enzyme is part of an enzymatic complex which includes the cytochrome P450 aromatase, the product of the cyp19a1 gene, and a NADPH-dependent cytochrome P450 reductase known as a ubiquitous flavoprotein. Aromatase is a microsomal enzyme localized in the smooth endoplasmatic reticulum of steroidogenic cells. Interestingly, it has been stressed that its high affinity for the substrates facilitates estrogen synthesis even when androgens are not synthesized in high concentrations. In fish, only a few kinetic studies have been performed, and mostly in tissues potentially expressing both aromatase genes (cyp19a1a and cyp19a1b). The enzyme has more or less similar affinities for androstenedione and testosterone, and the Michaelis–Menten affinity constant (Km) is usually in the nanomolar range (<10 to _400), whereas the catalytic maximum activity is in the order of fmol–pmol/mg protein/hour (Hinfray et al., 2006). The effects of estrogen treatment on fish sex differentiation have been investigated since the fifties, however, physiological and molecular studies on the role of estrogens in fish have initially been developed mainly in relation to vitellogenesis. Their biosynthesis potentiality was looked for in immature or developing ovaries using testosterone or androstenedione as a substrate, although androstenedione has been shown to be more efficiently metabolized in rainbow trout and goldfish (Zhao et al., 2001). These studies have mainly focused on the production of estradiol-17b (E2) and estrone, while estriol has rarely been identified in fish, but other estrogen metabolites would be worth considering. For instance, glucuronides and sulfates of estrogens are not only produced in adults but also in larvae during the sex differentiation period (Yeoh et al., 1996a,). The enzymes responsible for the hydrolysis of conjugated steroids might then also play a role in the local regulation of the concentrations of biologically active estrogen metabolites, but they are poorly known in fish. Besides, several unknown free or conjugated metabolites of estrogens have been found to be produced by whole fish embryos or their gonads during the sex differentiation period (Rowell et al., 2002) or in transitional gonads during sex change in a protandrous hermaphrodite species, but their possible biological activity is not known today. Interestingly, ovarian catecholestrogens, 2/4-hydroxyestradiol-17b and 2-

methoxyestradiol, have been found recently in stinging catfish, Heteropneustes fossilis, and these compounds have even been shown to modulate the ovarian aromatase activity.

1. 7 Aromatase enzyme and estrogens during natural sex differentiation

Estrogens have been considered as female-promoting steroids in gonochoristic fish species since the pioneering work of Yamamoto (1969). This assumption was first based on the fact that feminization was observed when estrogens were administered exogenously during early development in fish. This led to the hypothesis that estrogens were gynoinductors, triggering ovarian differentiation, and androgens were androinductors, driving (Yamamoto, 1969). These early studies were then confirmed by a large number of other experiments showing that estrogen treatments were highly effective in feminizing many fish species (Piferrer, 2001). Besides these feminizing effects of estrogens, treatments with aromatase inhibitors (AI) that blocked estrogen synthesis were found to induce masculinization when applied during the sex differentiation period in Chinook salmon, Oncorhynchus tshawytscha (Piferrer et al., 1994), Nile tilapia (Guiguen et al., 1999; Afonso et al., 2001; Kwon et al., 2002), rainbow trout (Guiguen et al., 1999), zebrafish (Fenske and Segner, 2004), Japanese flounder, golden rabbitfish, Siganus guttatus, Japanese fugu and common carp, Cyprinus carpio. These results have been crucial in the understanding of fish gonadal differentiation as they demonstrated quite unambiguously that estrogens were major endocrine factors in determining gonadal sex-fate, as their synthesis was required to trigger ovarian differentiation and their depletion was sufficient to trigger testicular development. Until recently sex differentiation was also thought to be a rather irreversible fate in gonochoristic fish. Thus, sex inversion treatments in fish were always applied during a so-called sex differentiation period with only a few reports suggesting that this sensibility to sex inversion treatments could be extended to adult fish. In post differentiated Nile tilapia females, AI treatment was able to induce a partia l, but functional, masculinization of the gonad. This may actually be a more wide spread feature than previously thought, since it was also observed recently in common carp. This sheds new light on the implication of estrogens in gonadal differentiation, as these experiments clearly demonstrated that estrogens are probably not only needed for triggering ovarian differentiation, but are also needed to maintain this ovarian differentiation. The effects of specific estrogen receptor antagonists, such as tamoxifen have also been investigated. With tamoxifen treatments, either no deviation of the sex ratio (Guiguen et al., 1999), or masculinization of genetic females (Sun et al., 2007) have been

observed, although in many cases no complete masculinization could be obtained. This, however, indicates that any impairment of the classical estrogen signaling pathway leads to some degree of masculinization.

1. 8 Germ cell proliferation, gonadal estrogen production and ovarian differentiation

Experimental germ cell depletion during the early steps of gonadal differentiation has recently been shown to produce masculinization of gonads in Japanese medaka genetic females (Shinomiya et al., 2001) and also in zebrafish. These masculinizing effects are supposed to be in relation to the intimate cross-talk between germ cells and somatic cells during the early sex differentiation steps of the gonad (Tanaka et al., 2008). From these observations it has been suggested that somatic cells in the gonad would autonomously activate a male phenotype, and that germ cells would allow the activation of the female phenotype by sending signal(s) to both repress male predisposition and maintain feminization (Tanaka et al., 2008). This assumption fits well with the above discussion suggesting that ovarian somatic cells need to maintain their fully differentiated phenotype in order to produce estrogens that in turn will support their differentiation. Any disruption of this germ cell/somatic cell cross-talk would then disrupt ovarian somatic cell differentiation, leading to an absence of estrogen synthesis and a subsequent masculinization. But more subtle regulations involving not only the complete loss of germ cells, but instead some differential germ cell proliferation rates, could then be also suspected as important triggers of gonadal sex differentiation, These germ cell proliferation rates could eventually be affected by endogenous estrogen levels as suggested by some authors. In this respect, the bioavailability of active E2 from the hydrolysis of its sulfate counterpart would also be worth considering. In agreement with the hypothesis that germ cell proliferation could affect gonadal differentiation, point to the fact that this germinal/somatic cell cross-talk could also be involved in the natural gonadal differentiation of zebrafish as germ cell apoptosis always precedes testicular differentiation in this species (Uchida et al., 2002). In this respect, it is interesting to note that proliferation of germ cells has been often found to be more active during ovarian differentiation, than during testicular differentiation and that masculinizing high temperatures in thermo sensitive fish species have an opposite effect by stimulating germ cell apoptosis. It is also quite intriguing to see that the Japanese medaka sex determining gene, dmrt1Y (dmy), has been found to be an inhibitor of germ cell proliferation and is only expressed during testicular differentiation (Kobayashi et al., 2004). In the same

context, the Japanese medaka hotei mutation affecting the anti-Mullerian hormone receptor (amhr2) leads to an increase in germ cell number and some male to female sex reversions. However, and apparently in contradiction with these results, anti-Mullerian hormone (amh) has also been found to be required for proliferation of germ cells in the same species (Shiraishi et al., 2007). Another tgfb member, the gonadal soma-derived growth factor (gsdf), was also described in rainbow trout as a potential stimulator of primordial germ cell and spermatogonia proliferation. However, like amh, gsdf is also expressed in both sexes, albeit at much higher levels during testicular differentiation (Baron et al., 2007). Thus, any sex specific effect of these tgfb members on germ cell proliferation should either be a function of their local protein abundance or of their concerted action with additional partners. For instance, follistatin (fst) has been found to be expressed at much higher levels during ovarian differentiation in rainbow trout (Baron et al., 2005b) and is also known to maintain germ cell survival in the mouse ovary (Yao et al., 2004)

.

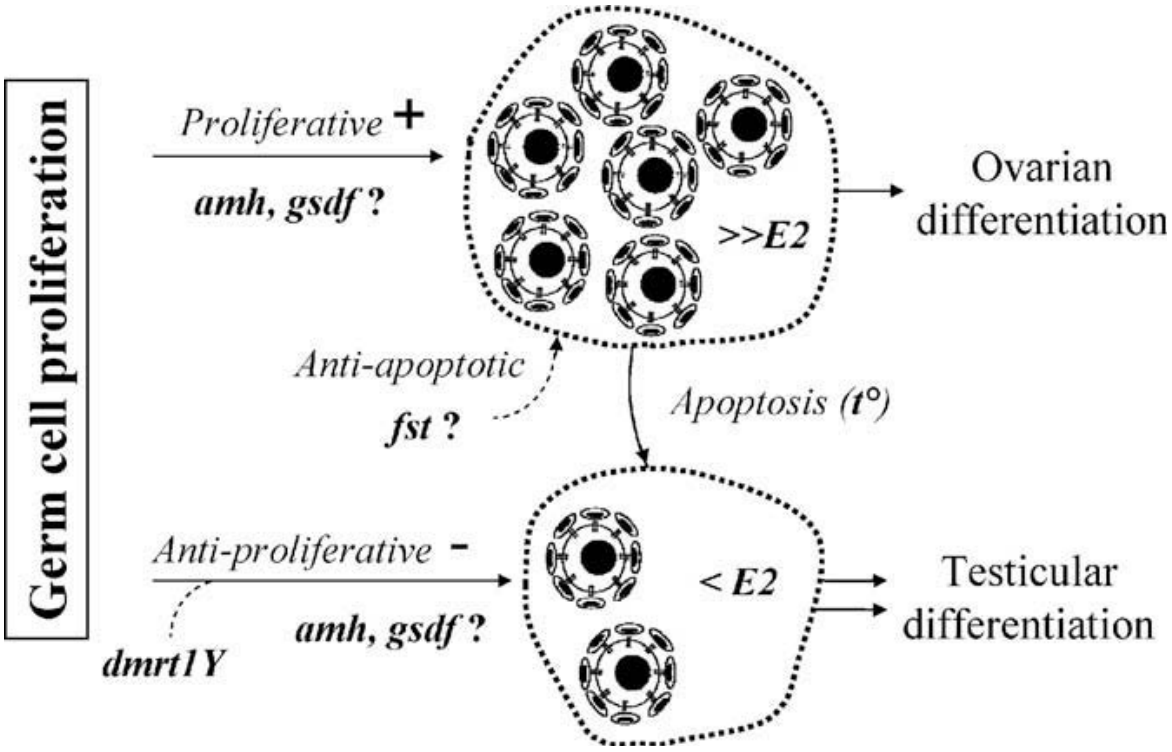


Figure (2 .1) Germ cell proliferation in mouse. (Yao et al., 2004)

1.9 Prostaglandin

The prostaglandins are biologically active derivatives of arachidonic acid and other polyunsaturated fatty acids that are released from membrane phospholipids by phospholipase A2. The initial transformation of arachidonic acid involves oxygenation and cyclization to an unstable endoperoxide intermediate, prostaglandin (PG1) G2 by cyclooxygenase (COX) enzymes. The same enzymes reduce PGG2 to PGH2 via a separate peroxidase site. Various isomerases and oxidoreductases convert PGH2 to prostaglandins and thromboxane A2. The cyclooxygenase exists in two forms, the constitutive COX-1 and the induced form, COX-2. Whereas the traditional NSAIDs inhibit both COX-1 and COX-2, the more recently developed anti-inflammatory coxibs inhibit COX-2 preferentially. ProstaglandinE2 exerts its actions via four types of receptors, (EP1, EP2, EP3, and EP4). PGE2 plays a role both in normal physiology and in pathology. The biological actions include areas such as inflammation, pain, tumorigenesis, vascular regulation, neuronal functions, female reproduction, gastric mucosal protection, and kidney function. The formation of PGE2 from PGH2, formed by COX-1 or COX-2 is catalyzed by isomerases, PGE synthases. There has been an explosive development of our understanding of these enzymes triggered by the discovery of mPGES-1, which belongs to a recently defined superfamily membrane-associated protein in eicosanoid and glutathione metabolism (MAPEG). This superfamily also contains leukotriene C4 synthase and 5-lipoxygenase-activating protein as well as three glutathione transferases/peroxidases called microsomal glutathione transferase 1–3 (MGST1–3). Microsomal glutathione transferase 1 is the best characterized for substrate specificity, kinetic mechanism, and three-dimensional structure and, being the closest relative to mPGES-1 (38% identity), serves as a good model for generating hypotheses on structure function relationships. mPGES-1 has emerged as a potential target for the development of drugs for treatment of inflammation, pain, cancer, atherosclerosis, and stroke.

1.9.1 Prostaglandin in Fish

Cyclooxygenase has been sequenced in several fish species, including the rainbow trout (Oncorhynchus mykiss) (Zou et al., 1999), Brook trout (Salvelinus fontinalis) (Roberts et al., 2000), Atlantic croaker (Micropogonias undulates), and zebrafish (Danio rerio) (Grosser et al., 2002). These species, along with genomic sequence data from the stickleback (Gasterosteus aculeatus), green spotted puffer (Tetraodon nigroviridis),

pufferfish (Takifugu rubripes), and Japanese medaka (Oryzias latipes) suggest that fishes posses both COX-1 and COX-2 forms (Jarving et al., 2004). Furthermore, COX-2 has recently been sequenced from European sea bass (Dicentrarchus labrax) (Buonocore et al., 2005) and euryhaline killifish (Fundulus heteroclitus) (Choe et al., 2006), supporting this prediction. A cyclooxygenase has also been sequenced from the spiny dogfish shark (Squalus acanthias) (Yang et al., 2002). However, this form was not designated as COX-1 or COX-2 because of its near equal identity to both forms (it is only slightly more similar to COX-1). This form may represent an evolutionarily distinct form of cyclooxygenase found in the elasmobranchs. Ishikawa and colleagues have recently further characterized COX evolution in the teleosts by demonstrating that both the zebrafish (Ishikawa et al., 2007) and rainbow trout (Ishikawa and Herschman, 2007) have two functional COX-2 genes (named COX-2a and COX-2b). In addition, by analyzing sequence databases, they concluded that not all teleosts have two COX-2 forms, but that the stickleback, green spotted puffer, pufferfish, and Japanese medaka have two COX-1 forms instead (named COX-1a and COX-1b) (Ishikawa et al., 2007). They concluded that a genome duplication before the teleosts, and a subsequent loss of either one COX-1 or COX-2 form characterizes the species mentioned (Ishikawa and Herschman 2007). This is supported by the apparent genome duplication events in teleosts.

1.10 Cyclooxygenase function in fish

Cyclooxygenase has likely been studied in the fishes more than other non-mammals due to their status as model organisms. As noted above, COX forms have been found in several species and along with genomic sequence data there is a general consensus that fishes possess both COX-1 and COX-2 forms that are homologous to those found in mammals. However, there is recent evidence that teleosts also possess an additional form of COX-1 or COX-2 (Ishikawa et al., 2007; Ishikawa and Herschman, 2007) and some functional data also exist for these forms.

1.10.1 Osmoregulation

In fishes, the gills are the dominant site of acid/base regulation, nitrogenous waste secretion, gas exchange, and ion transport (Evans et al., 2005). Several studies have investigated the osmoregulatory role of COX in the gills of the euryhaline killifish, Fundulus heteroclitus. This teleost can withstand instant salinity transfer between full strength seawater and fresh water without any apparent physiological stress (Wood and

Laurent, 2003). One study showed that short circuit current across the opercular epithelium (a tissue with known ion transport capabilities) was reduced by using a non-specific COX inhibitor (Evans et al., 2005). This suggests that COX-2 plays a role in ion transport in fish that may be similar to its function in the kidneys of mammals. Further studies supported this initial hypothesis by showing that COX-2 is expressed most abundantly in the gill, opercular epithelium, and kidney of the killifish (Choe et al., 2006), tissues that are known to be involved in ion transport in fish. This is supported in the zebrafish, which shows the highest levels of COX-2 in the gills (Grosser et al., 2002). Furthermore, COX-2 was localized in mitochondrion-rich cells in the gills of killifish, the main sites of ion uptake and secretion in the gills (Choe et al., 2006). Finally, COX-2 expression was shown to significantly increase following either hypotonic or hypertonic salinity transfers, suggesting COX-2 may play a role in maintaining cell homeostasis or promoting cell survival during periods of osmotic shock (Choe et al., 2006). Taken together, these results strongly suggest that COX-2 in the gills of teleosts acts in the same way as COX-2 in the kidneys of mammals, including regulating ion transport and promoting cell survival. Cyclooxygenase has also been shown to play an osmoregulatory role in the rectal gland of the dogfish shark, Squalus acanthias. Sharks rely on the rectal gland rather than the gills for regulation of salt secretion and a COX form (sCOX) has been cloned from the rectal gland (Yang et al., 2002). This form was expressed most abundantly in the rectal gland of the shark, where PGE2 production was also high (Yang et al., 2002). Finally, using a COX-2 specific inhibitor, vasoactive intestinal peptide mediated chloride secretion decreased in the rectal gland, but then recovered following removal of the inhibitor (Yang et al., 2002). Even though sCOX is slightly more similar to COX-1 of mammals than COX-2, this result can be explained by the presence of valine at position 523 instead of isoleucine (and thus conferring COX-2 inhibitory properties). This result suggests that sCOX plays a role in ion transport in the rectal glands of sharks that may be similar to the gills of teleosts or the kidneys of mammals. The osmoregulatory role of COX in fish needs to be examined in greater detail using other species and diverse techniques. This may be highly feasible because COX sequences exist for a wide range of teleosts.

1.10.2 Reproduction

As in mammals, COX has been shown to play a role in reproduction in fish. By using the non-specific COX inhibitor indomethacin, it was shown in the Atlantic croaker

(Micropogonias undulatus) that COX pathways may play a role in the maturation of ovarian follicles and ovulation through prostaglandin formation, although other proteins may play a more dominant role (Patino et al., 2003). Results from the European sea bass also indicate a similar role for prostaglandins in ovulation, with indomethacin inhibiting follicle maturation (Sorbera et al., 2001). However, it has been shown in the brook trout that indomethacin does not block ovulation, although it does in other fish species (Goetz et al., 1991). This apparent loss of function in the brook trout may be explained by changes in the levels of COX-1 and COX-2 during ovulation. It was shown that COX-1 levels remained constant and high during ovulation but that COX-2 levels did not increase prior to ovulation as they do in mammals (Roberts et al., 2000). This is supported by data from the zebrafish which show high levels of COX-1 but not COX-2 in the ovaries (Grosser et al., 2002). This suggests that COX-2 function in the reproduction of the brook trout is different than in other vertebrates. In the Japanese medaka, it was shown that low chronic levels (perhaps comparable to those found in waste water) (Metcalfe et al., 2003) of the non-specific COX inhibitor ibuprofen causes altered reproduction by decreasing the number of spawning events but increasing the number of eggs per spawning event (Flippin et al., 2007). This conforms to delayed pregnancies associated with NSAID use in mammals. These results indicate that COX likely plays a role in fish reproduction, although it may vary among species.

Chapter Two

Effect of pharmaceutical on the aquatic environment

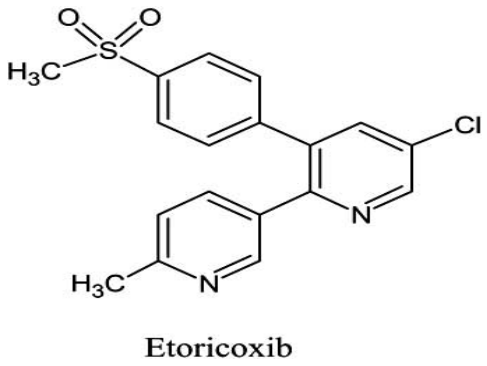
2.1 NSAIDs :

Non steroidal Antiinflammatory Drugs (NSAIDs),chemically heterogeneous large groups of drugs which suppress inflammation in a manner similar to steroids, but less side effects of sedation, respiratory depression, or addiction than steroids. They are widely used for the treatment of inflammatory disorders and painful conditions such as rheumatoid arthritis, gout, bursitis, painful menstruation, and headache. They are effective in the relief of pain and fever. NSAIDs inhibit the cyclooxygenase (COX) activity resulting in decreased synthesis of prostaglandin, leukotriene and thromboxane precursors such as the ubiquitous enzyme which catalyzes the initial step in the synthesis of prostanoids. Prostanoid is any of a group of C-20 fatty acids complex with an internal five or six carbon rings such as prostaglandins, prostanoic acid, prostacyclins, and thromboxane; derived from arachidonic acid (C-20 polyunsaturated fatty acid with four cis double bonds). The action or the synthesis of prostanoids are involved in the modulation of a variety of pathophysiologic processes including inflammation, hemostasis, thrombosis, cytoprotection, ulceration, hemodynamics and other the progression of kidney diseases. Thus, NSAIDs as non-selective inhibitors of the cyclooxygenases (both the cyclooxygenase-1 and cyclooxygenase-2 isoenzymes) may have beneficial as well as untoward effects on a variety of human diseases. Low stomach prostanoid levels caused by COX-1 inhibitors can result in ulceration and internal bleeding and perforation. The selective COX-2 inhibitors such as oxicam, meloxicam, and coxibs (celecoxib, rofecoxib, valdecoxib, parecoxib and etoricoxib) do not interfere with COX-1. The most prominent NSAID is aspirin

2.2 ETORICOXIB

Etoricoxib or 5-chloro-6’-methyl-3-[4-(methylsulphonyl) phenyl]-2, 3’-bipyridine is the newest addition to the group of non-steroidal anti-inflammatory drugs (NSAIDs) known

as selective cyclooxygenase-2 inhibitors. Its chemical structure is shown in the Fig(2:2). Being the latest molecule, very few HPLC methods are reported in the literature.



Etoricoxib

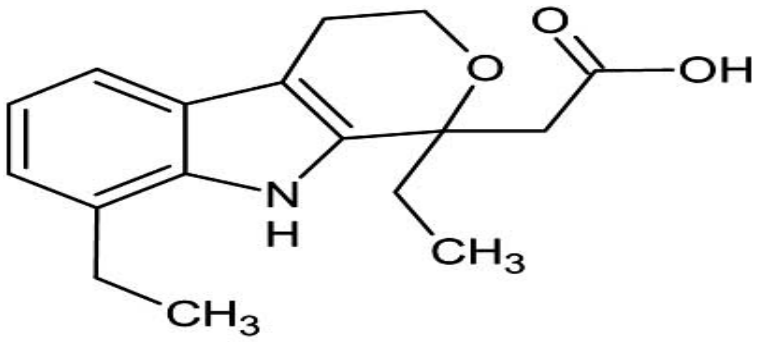
Figure (2:1) Etoricoxib chemical structure

Etoricoxib (brand name **Arcoxia** worldwide; also **Algix** and **Tauxib** in Italy) is a COX-2 selective inhibitor (approx. 106.0 times more selective for COX-2 inhibition over COX-1) from Merck & Co. Doses are 60, 90 mg/day for chronic pain and 120 mg/day for acute pain. Currently it is approved in more than 60 countries worldwide but not in the US, where the Food and Drug Administration (FDA) requires additional safety and efficacy data for etoricoxib before it will issue approval. Current therapeutic indications are : treatment of rheumatoid arthritis, psoriatic arthritis, osteoarthritis, ankylosing spondylitis, chronic low back pain, acute pain and gout. Note that approved indications differ by country. Like any other COX-2 selective inhibitor, Etoricoxib selectively inhibits isoform 2 of cyclo-oxygenase enzyme (COX-2). This reduces the generation of prostaglandins (PGs) from arachidonic acid. Among the different functions exerted by PGs, their role in the inflammation cascade should be highlighted. COX-2 selective inhibitor showed less marked activity on type 1 cyclooxygenase compared to traditional non-steroidal anti-inflammatory drugs (NSAID).This reduced activity is the cause of reduced gastrointestinal toxicity, as demonstrated in several large clinical trials performed with different. Some clinical trials and meta-analysis showed that treatment with some coxibs (in particular Vioxx, rofecoxib) led to increased incidence of adverse cardiovascular events compared to placebo. Because of these results, some molecules were withdrawn from the market (Rofecoxib, September 2004 and Valdecoxib, April 2005). In addition, the FDA and EMEA (USA and European Community Health Authorities respectively) started a revision process of the entire class of both NSAID and COX-2 inhibitors.

Etoricoxib, that is used for patients with chronic arthropathies and musculoskeletal and dental pain, is absorbed moderately when given orally. A study on its pharmacokinetics showed that the plasma peak concentration of etoricoxib occurs after approximately 1 hour. It has shown to be extensively bound to plasma albumin (about 90%), and has an apparent volume of distribution (V_D) of 120 L in humans. The area under the plasma concentration-time curve (AUC) increases in proportion to increased dosage (5-120 mg). The elimination half-life is about 20 hours in healthy individuals, and such long half-life enables the choice to have once-daily dosage. Etoricoxib, like the other coxibs, is excreted in urine and feces and also metabolized in likewise manner. CYP3A4 is mostly responsible for biotransformation of etoricoxib to carboxylic acid metabolite, but a non CYP450 metabolism pathway to glucuronide metabolite is also at hand. A very small portion of etoricoxib (<1%) is eliminated unchanged in the urine. Patients with chronic renal insufficiency do not appear to have different plasma concentration curve (AUC) compared to healthy individuals. It has though been reported that patients with moderate hepatic impairment have increased plasma concentration curve (AUC) by approximately 40%. It has been stated that further study is necessary to describe precisely the relevance of pharmacokinetic properties in terms of the clinical benefits and risks of etoricoxib compared to other clinical options. (Brautigam et al., 2003) .

2.3 ETODOLAC

Etodolac or 1, 8-diethyl-1,3,4,9-tetrahydropyano[3,4-b] indole-1-acetic acid, is a non-steroidal anti-inflammatory drug which has been shown to be effective in the treatment of rheumatoid and osteoarthritis and a selective COX-2 inhibitors in a wide range of clinical relevant assays in direct comparisons with other NSAIDs. Its chemical structure is shown in figure (2:2). Etodolac is metabolized in humans by hydroxylation and acyl glucuronidation to yield the corresponding 1-O-glucuronides. The acylglucuronides of etodolac and one of its hydroxylated metabolites were determined by. By HPLC using a RP18 column. In order to increase the lipophilicity of these metabolites the hydroxy groups were acetylated and the carbonilic functions were methylated. The S-etodolac-glucuronide is mainly excreted during the first 6-hours. (Berendes&Blaschke., 1996)



Etodolac

Figure (2:2) chemical structure of Etodolac

Etodolac, indole analog compound, is a NSAID (nonsteroidal anti-inflammatory drug) of non-selective inhibitor of COX-1 and COX-2. It is used as an analgesic and anti-inflammatory for the treatment especially arthritis. Its chemical designation is (±)-1,8-diethyl-1,3,4,9- tetrahydropyrano- [3,4-b]indole-1-acetic acid. It is a white crystalline compound insoluble in water, soluble in alcohols, chloroform, DMSO and polyethylene glycol; administered orally. (www.drugbank.com)

2.4 Cyclooxygenase Inhibitors

In 1971, Vane reported that aspirin and indomethacin inhibit the biosynthesis of prostanoids and shortly after it was established that these inhibitors block the oxygenation of arachidonic acid. COX-1 and 2 are the major targets of NSAIDs including aspirin, ibuprofen, and the new COX-2 inhibitors. Even though cyclooxygenases have both cyclooxygenase and peroxidase activities, NSAIDs only block the cyclooxygenase part. NSAIDs can be grouped into three classes based on their modes of COX inhibition: (a) Class I – simple, competitive, reversible inhibitors that compete with arachidonic acid for binding to the COX active site (e.g. ibuprofen) (b) Class II - competitive time dependent, reversible inhibitors that bind to the COX active site in a first phase, to form reversible enzyme inhibitor complexes, that if retained for a sufficient time, cause a non covalent conformational change in the protein, associated with tighter binding (e.g. indomethacin) or (c) Class III – competitive, time dependent, irreversible inhibitors that form an enzyme inhibitor

Complex after a covalent conformational change in the protein (e.g. aspirin). The discovery of COX-2 in 1991, the key enzyme involved in inflammation, set a new goal for

researchers. The new goal was to develop COX-2 selective inhibitors to avoid the numerous side effects associated with COX-1 inhibition. The new concept brought another classification for NSAIDs as selective or non-selective depending on their specificity at inhibiting one of the isoforms or both. These efforts have culminated with the introduction of successful drugs like celecoxib as a potent anti-inflammatory agent for the treatment of arthritis.

2.5 Association between Non-steroidal Anti-inflammatory Drugs (NSAIDs) and breast Cancer

Nonsteroidal anti-inflammatory drugs (NSAIDs) are used to decrease inflammation by inhibiting cyclooxygenases. Even though the Nurses' Health Study found no difference in aspirin intake among women who developed breast cancer and those who did not, a growing body of experimental and epidemiological evidence suggests that the use of NSAIDs may decrease the risk of breast cancer. In a case-control study, a 40% reduction in breast cancer incidence was observed in women who reported daily intake of aspirin, ibuprofen, or other NSAIDs for at least 5 years. Independent studies found that both indomethacin and ibuprofen reduced the incidence of dimethylbenz[a]anthracene-induced mammary tumors in rats. Celecoxib and ibuprofen produced striking reductions in the incidence of mammary cancer, tumor burden, and tumor volume compared to those seen in the control group in animal models. Aspirin and flurbiprofen were shown to reduce mammary tumorigenesis. Genetic and pharmacological evidence has shown that specific COX-2 inhibition is more effective than traditional NSAIDs in suppressing polyposis in the mouse. SC-560, celecoxib, and indomethacin treatment resulted in statistically significant inhibition of tumor size in comparison with vehicle-treated control animals in a murine model of breast cancer. Regular intake of NSAIDs for 2-5 years was associated with a 24% reduction in invasive breast cancer. All these findings suggest that cyclooxygenases are involved in the promotion of this type of cancer. The relationship between cyclooxygenases and aromatase was examined in a preliminary study of CYP19 gene expression with COX-1 and COX-2 gene expression in breast cancer patient specimens. This study showed that the levels of CYP19 gene expression remained relatively constant in breast cancer tissue with an increased expression in tissue showing signs of invasion. While COX-1 was present in both normal and cancerous tissue, COX-2

was only present in cancerous tissue with a significant linear association between tumor cell density and COX-2 gene expression.

Regression analysis using a bivariate model showed a strong linear association between the sum of COX-1 and COX-2 expression and CYP19 expression. Another study using immunohistochemistry staining for aromatase and COX-2 in tumor samples revealed a marked correlation between COX-2 and aromatase expression in tumor samples. These data confirm the previous reports showing a positive correlation for CYP19 and cyclooxygenases. These observations suggest that both autocrine and paracrine mechanisms may be involved in growth and progression of human breast cancer via stimulation of estrogen production regulated by a high biosynthesis of prostaglandins. Intratumoral aromatase may be important as a source of estrogens for tumor growth and intratumoral COX-2 may be important in the production of prostaglandins (such as PGE2) to direct aromatase gene expression through promoter II. PGE2, mainly produced by the epithelial cancer cells, up regulates aromatase expression and activity in the stromal neighboring cells through binding to the EP1 and EP2 receptors. A higher expression of aromatase could result in higher levels of estrogens. The resulting increased estrogen biosynthesis in local sites in turn may result in increased growth and development of the tumor by both paracrine and autocrine actions. (Brueggemeier et al., 1999) pointed to linear dependence between aromatase gene (CYP 19) expression and expression of COX-2 in mammary carcinoma. The above mentioned facts predetermine the use of NSAIDs in mammary carcinogenesis prevention.Cyclooxygenase inhibitors suppress aromatase expression and activity in breast cancer cells (Diaz-Cruz et al ., 2005).

2.6 Impact of NSAIDs in fish

Information concerning possible sublethal effects of NSAIDs on aquatic organisms is scarce. In most cases, studies have been restricted to short-term acute lethality tests in algae, invertebrates and fish (Webb, 2001). However, chronic exposure to diclofenac in rainbow trout revealed histopathological alterations and cytological effects in liver, kidney, gills and intestine that were correlated with a dose-related tissue accumulation of the drug (Schwaiger et al., 2004). These studies are consistent with the well known gastrointestinal toxicity by NSAIDs in mammals. histological analysis in fish revealed an increase in granulocyte numbers in primary gill filaments, as well as granulocyte accumulation and enhanced major histocompatibility complex (MHC) II expression in

kidney, suggesting an inflammatory response in these organs (Hoeger et al., 2005). In addition, diclofenac, at sublethal but environmentally relevant concentrations, inhibited the EROD activity in primary cultures of trout hepatocytes, suggesting potential impact on biotransformation of xenobiotics in fish. NSAIDs are widely used for their analgesic and anti-inflammatory properties. The therapeutic effects are based on the fact that their amphiphilic acid structure binds to the lipid-water interphase of cell membrane proteins such as the prostaglandin-G/H synthase and inhibit their function. This enzyme exhibits two catalytic activities, hydroperoxidase and cycloooxygenase, which together catalyze the transformation of arachidonic acid to prostaglandins (PGs) and thromboxanes. Most NSAIDs are known inhibitors of cyclooxygenase activity. For instance, ibuprofen, indomethacin, diclofenac and acetylsalicylic acid (ASA) are commonly used in mammalian studies as well as in fish as inhibitors of cyclooxygenase-2 (COX-2) activity. Consequently, NSAIDs will inhibit the synthesis and release of PGs, which are involved in the inflammatory response and also in modulating the functioning of the pituitary-adrenal axis in humans and fish. To this end, a recent study demonstrated that ASA administered *in vivo* to Mozambique tilapia (*Oreochromis mossambicus*) disturbed the endocrine axes, including attenuated plasma cortisol response to a stressor, leading to the proposal that PGs are involved in the stress response process in fish (van Anholt et al., Non-steroidal anti-inflammatory drugs (NSAIDS) have been detected in the aquatic environment, but little is known about either their impact or mode of action in aquatic organisms. We tested the hypothesis that NSAIDs disrupt the evolutionarily conserved heat shock response, critical for defense against stressor-mediated proteotoxicity, in rainbow trout (*Oncorhynchus mykiss*). Trout fry were exposed by immersion to a range of salicylate or ibuprofen concentrations (1, 10, 100 or 1000 g/L) for 4 days. Ibuprofen, but not salicylate, at all concentrations induced heat shock protein 70 (hsp70) in trout liver. We used the highest concentration of the drugs to investigate their mode of action on the heat shock response.Fry were subjected to a standardized heat shock, 10◦C above ambient (13◦C) for 1 hour, and the temporal changes in liver hsp70 mRNA and protein content as well as glucose dynamics during recovery from the heat stress or assessed. Ibuprofen exposure did not modify hsp70 mRNA abundance, but significantly depressed the heat shock-induced hsp70 protein expression in the liver and gill of trout. Salicylate exposure elevated hsp70 mRNA abundance and delayed the hsp70 expression after a heat shock. Liver glucose levels and the activities of hexokinase, pyruvate kinase and lactate dehydrogenase, were elevated by NSAIDs suggesting enhanced tissue glycolytic capacity.

Effects on whole body glucose dynamics, induced by the heat shock, were either absent with ibuprofen or completely modified by salicylate. Overall, NSAIDs disrupt the heat shock response in rainbow trout, while the mode of action of salicylate and ibuprofen in impacting the cellular stress response appears distinct.

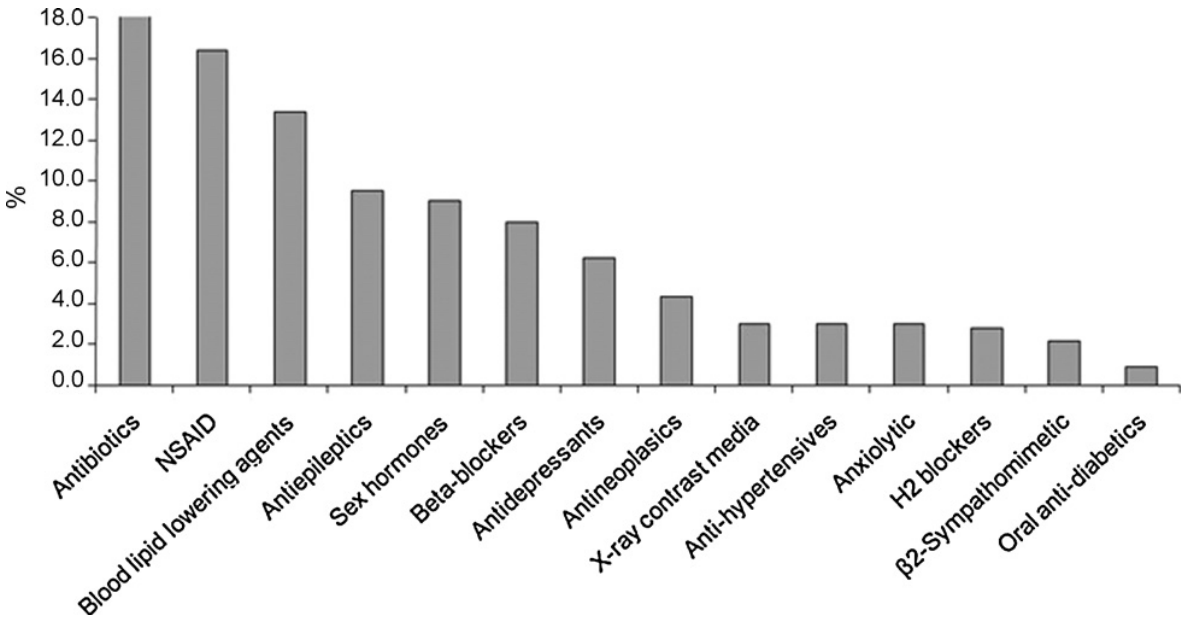


Figure (2:3) Percentage of published studies on different therapeutic classes, expressed in relative percentage, described on 183 articles published between 1996 and 2009.(Sanota et al ., 2010)

Non-steroidal anti-inflammatory drugs are weak acids acting by reversible or irreversible inhibition of one or both isoforms of the cyclooxygenase enzymes, COX-1 and COX-2, involved in the synthesis of different prostaglandins from arachidonic acid. A cyclooxygenase enzyme similar to human COX-2 has been found in fish thereby making them a potential target for aquatic contamination. Prostaglandins also play an important role in the synthesis of bird eggshells and from inhibiting its synthesis, shell thinning has been observed. Among the NSAID, diclofenac showed the most acute toxic nature with effects being observed at concentrations below $100mgL^{-1}$. Chronic toxicity trials performed on rainbow trout (Oncorhynchus mykiss) evidenced cytological changes in the liver, kidneys and gills after 28 days of exposure to just 1 g L^{-1} of diclofenac. For a concentration of 5 g L^{-1} renal lesions were evident as well as drug bioaccumulation in the liver, kidneys, gills and muscle. Brown trout (Salmo trutta f. fario) showed similar cytological damage and a reduction of haematocrit values after 21 days of exposure to 0.5 g L^{-1} of this active substance. Schmitt-Jansen et al. Evaluated both diclofenac

phytotoxicity and its photochemical products on the unicellular chlorophyte Scenedesmus vacuolatus. Inhibition of algal reproduction by the parent compound only occurred at a concentration of 23 mgL^{-1}, hence indicating no specific toxicity. However, the threat significantly increased when metabolites were produced from 53 h of exposure to daylight.

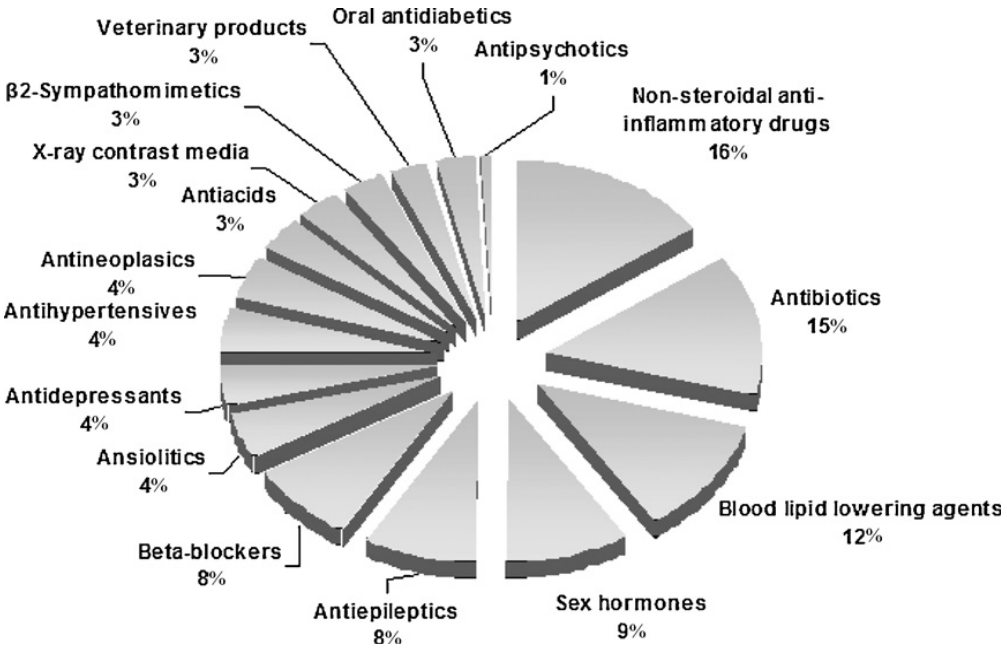


Fig (2:4) Therapeutic classes detected in the environment, expressed in relative percentage. Data collected from 134 articles published between 1997 and 2009.(Sanota et al ., 2010)

Diclofenac also inhibited the growth of marine phytoplankton Dunaliella tertiolecta for concentrations of 25mgL^{-1} and above. For this organism, 96 h EC50 of 185.69mg L^{-1} was found. Diclofenac was detected in STP effluents at maximum concentrations of 2.4 and 1.42 g L^{-1} in Switzerland and Belgium respectively which highlighted that the effects cited are of sufficient magnitude to suspect chronic toxicity in aquatic organisms. Diclofenac has also been found in rivers groundwater, hospital effluents and drinking water but at concentrations in the order of ng L^{-1}. Ibuprofen is another NSAID with documented chronic toxicity. Female Japanese medaka (the Japanese killifish, Oryzias latipes) exposed to different concentrations of the drug over six weeks, showed a sharp rise in liver weight together with enhanced egg production, yet with a reduction in the number of weekly spawning events. Authors associated these phenomena with changes in the spawning process and vitellogenin production, a glycoprotein precursor in yolk formation. With the water flea Daphnia magna population growth rate was significantly

reduced for concentrations ranging from 0 to 80mg L^{-1} Reproduction was affected at all concentrations and completely inhibited at the highest pharmaceutical levels. An activity decrease of the freshwater amphipod Gammarus pulex was noticed when in contact with ibuprofen concentrations of 1 and 10 ngL^{-1}, the latter value corresponding to the LOEC obtained for behaviour change. Regarding aquatic photosynthetic organisms, specific effects have been noticed. A 5-day exposure to concentrations in the 1–1000 gL^{-1} range stimulated the growth of the cyanobacterium Synechocystis sp. while inhibiting that of the duckweed plant Lemna minor after 7 days. Ibuprofen has been detected in STP effluents at concentrations that can reach 28 g L^{-1}. Two metabolites of ibuprofen (carboxyl-ibuprofen and hydroxyl-ibuprofen) were also found in surface waters and in a Swedish STP (influent and effluent). Due to demonstrable chronic toxicity, this may represent a real threat to non-target organisms, even at those lower concentrations. Ibuprofen was also found in rivers and drinking water which may broaden the scope of the problem to public health. However, effects in humans caused by chronic exposure to this active substance still remain unknown. Acute toxicity tests performed on the rotifer Brachionus calyciflorus, the water flea Ceriodaphnia dubia and the fairy shrimp Thamnocephalus platyurus, showed that naproxen had LC_{50} and EC_{50} values within the 1–100mgL^{-1} range, with the photolysis products being significantly more toxic. Highly chronic toxic properties were equally noticed with algae being the less sensitive organisms. Yet again, degradation products were shown to be more toxic with EC_{50} values of 26 and 62g L^{-1} for C. dubia, relative to growth inhibition. Naproxen had been found in STP effluents in a concentration range between 31 ngL^{-1} and 7.96 gL^{-1} and in surface waters, at concentration levels that can reach 250 ngL^{-1}. This active substance was also detected in drinking water. The highly prescribed paracetamol (or acetaminophen) is a weak inhibitor of the cyclooxygenase enzyme, whose side effects are mainly associated with the formation of hepatotoxic metabolites, such as N-acetyl-p-benzoquinone imine (NAPQI) when the levels of liver glutathione are low. Tests were carried out on algae, water fleas, fish embryos, luminescent bacteria and ciliates. The most sensitive species was shown to be D.magna for which EC_{50} values of 30.1 or 50mgL^{-1} were reported. Some authors reported the presence of paracetamol in STP effluents at concentrations below to 20 ngL^{-1} to 4.3 gL^{-1}, and in surface waters, values can reach 78.17 gL−1, which are values higher than the predicted no-effect concentration (PNEC) of 9.2g L^{-1}. Hence, paracetamol might represent a threat for non-target organisms.

2.7 Study hypothesis:

We hypothesize as a result of these previous studies that the use of cox inhibitors during crucial period could modulate aromatase activity and will affect reproduction in Niletilapia. The use of the NSAID cox inhibitor (non selective cox_1, cox_2 Etodolac) and (selective cox_2 inhibitor Etoricoxib) would produce an effect on growth rate, mortality rate and sex reversal. Also we hypothesized that there is an environmental effect for using antiprostaglandin (non selective cox1, cox2 and the selective cox2), especially on fishes.

2.8 Study objectives

The main goal of our study was to predict the effect of Etodolac and Etoricoxib during the crucial period of sexual differentiation in Niletilapia where sufficient amount of estrogen is needed for proper ovarian development and the suppression of aromatase activity the rate limiting enzyme in aromatization of androgens to estrogens leads to sex reversal and the arise of male population (Kown et al., 2001). Cyclooxygenase inhibitors suppress aromatase expression and activity in breast cancer cells (Diaz-Cruz et al., 2005) and since fish posses both COX-1 and COX-2 forms and both are widely expressed during development with both isoforms genetically and functionally homologous to their mammalian orthologs (Grosser et al., 2002), human and veterinary pharmaceuticals have been shown to occur in considerably high amounts in sewage treatment plant (STP) effluents and surface waters, with the non steroidal anti inflammatory drugs representing one of the most commonly detected compounds Fig(2:4). Information concerning possible ecotoxicological risks of these substances is rather scarce. So far there are no data available on their possible effects in fish after prolonged exposure. Thus, highlight on Etoricoxib pharmacokinetics was carried out by determination of Etoricoxib in fish feces using HPLC.

Specific objectives:

1- Effect of 0.5% Etodolac and 0.5% Etoricoxib on growth rate of mixed population fry tilapia during the crucial period of sexual differentiation.
2- Effect of 0.5% Etodolac and 0.5% Etoricoxib on growth rate of adult tilapia mixed population.

3- Effect of increasing concentrations on growth rate.
4- Mortality and toxicity at different concentrations.

Chapter Three

Materials and methods

3.1Experimental system

The study was carried out at the Aquaculture research laboratory, in AL-Quds University, Jerusalem. A warm water recirculation system consisting of a 24 aquaria, with capacity ≈ 45 L each one was used for the study. Tap water from municipality was used for fish rearing. The experimental aquaria were housed indoor. Daily, quarter of the water was renewed from each aquarium in order to maintain optimum water quality and better hygiene. The flow rate of water was maintained at 0.5 L l min. All the aquaria were kept on 80 –cm high platform to facilitate better observation and easy maintenance. Water temperature was maintained at 28 ± 1 Cº. A constant photoperiod of 12 h light and 12 h dark was maintained throughout the experimental period. The water in the header tank was aerated by an air stone and the rates of water flow were adjusted to maintain oxygen saturation above 60%. Total nitrogen ammonia was always less than $3mgL^{-1}$.The water quality parameters in the system were monitored by doing water analysis. Genetically mixed population of tilapia fry aged 8 days post- hatched ≈ 400 fry were used in the experiment; Commercial feed was used as the control diet. Feed compositions are shown in table (3.1). Etodolac and Etoricoxib were purchased from Sigma and Alderich.

Table (3:1) Feed Composition and treatment concentrations

Treatment	Composition	Concentration
Control	91.8%DryMatter (OM) 43.5% Crude protein 10.9% Crude lipid 13.0% Crude Ash 18.7Kj Gross energy	
Etodolac		0.5 %, 1 %, 2%
Etoricoxib		0.5%, 1%

At an age of 8 days post – hatched 30 genetically mixed population of Oreochromis. niloticus larvae were stocked in duplicate, into aquariums each with a capacity of ≈ 45L at aquatic and aquaculture lab, where the study was conducted. Treatments included 5 different experimental diet and one standard diet serving as control with two repeats for each group from 0.5 % groups of diets. All aquariums in the experiment were disconnected from the recirculation system in order to avoid cross- contamination. The test diets were prepared by mixing 600 mg of etodolac with 120g, 60 g, 30 g commercial feed, to achieve (0.5% etodolac, 1% etodolac, 2% etodolac) concentration respectively, the same process done to the etoricoxib by mixing 90 mg of etoricoxib with 18 g, 9 g, commercial feed, also to achieve (0.5% etoricoxib, 1% etoricoxib) concentration respectively.Feeding started on the same day of stocking and fish were fed once daily. Feed was made into pellets crushed into smaller sizes and spread on the water surface slowly by hand. Feed was adjusted according to fish weight during the 8th week of the experiment when I gave the feed with etodolac and etoricoxib I fed the fish 10 % of their weight after this period gives them 5% of their body weight and all diets were changed to control diet.

Fish were weighted every week and counted in each aquarium to determine survival rate during 12 weeks period. Individual fish in each aquarium was weighted to the nearest 0.1 g using a digital scale. The growth rate (GR) was determined using linear regression: $yt = a + bxt$, where yt is the average total weight (g) of the fishes at time t and a is the average weight (g) of fishes at the start of the experiment.

3.2 Experiments done for fish growth rate:

1) Experiment number (1): Mixed population of Niletilapia treated with (Etodolac 0.5%) and (Etoricoxib 0.5%) at age more than 2 month

There were nine aquarium chosen for the experiment from the recirculatory system after separated them from the system to prevent cross contamination. These aquarium were divided to three different group, three for standard population without treatment, three with (Etodolac0.5%) treatment, and finally the last three for(Etoricoxib 0.5%) treatment. these aquarium were distributed randomly in all side of the system to achieve the same condition for each group. 10 fish were stocked in each aquarium, the diet given to fish in each aquarium once daily , amount of diet was 10 % of the fish weight as table (3.2) show,every week each aquarium fish were weighed and calculate the 10% amount of diet should given to them during the new week.

2) Experiment number (2) : Mixed population of Niletilapia treated with (Etodolac 0.5%) and (Etoricoxib 0.5%) at age of 8 days.

The same procedure done as in experiment one but with duplicate aquarium for each type of (etodolac 0.5 %), (etoricoxib 0.5%), and standard.

30 fry fish were stocked in each aquarium, the fry fish were given 0.1- 0.3 during 8 weeks, after this period the fish were weighed and given 10% of their total weight in each aquarium as table (3.3) show, three weeks were weight with NSAIDs treatment and another three weeks they were weighed without treatment to record the result of growth rate if it continuous after stop treatment .

3) Experiment number (3) : mixed population fry of Niletilapia with concentrations of 1% Etodolac, 1%Etoricoxib and 2% Etoricoxib

In small aquarium 30 fish at age of 8 days post hatch were stocked in 1% concentration of etodolac, etoricoxib respectively and standard, 20 fish were stocked for 2% etodolac, only one aquarium used for each concentration and they were treated in the same way as the previous experiments (1, 2).

Table (3:2) weight of food given to adult fish every week about 5% of their body weight in grams.

	Std1	Std 6	Std 9	Etd 2	Etd 5	Etd 8	Eto 3	Eto 4	Eto 7
weeks 1	2.9	2.7	2.9	3	2.9	3.3	3	3	3.8
weeks 2	3.6	2.9	3.1	3.3	3.4	4	3.5	3.3	4.4
weeks 3	4.6	3.6	3.8	4	4.2	4.6	4.2	4	5.3
weeks 4	5.8	4.8	5.1	5.1	5.1	5.7	5.1	4.4	7
week 5	6.6	5.8	6.1	5.8	6	6.4	5.2	4.2	7.5
week 6	7.4	6.8	7.1	7.1	7	7.1	6.2	4.4	7.6
week 7	7.6	8.4	8.5	8.4	8.5	8.1	7.4	5.6	8.6
week 8	6.3	8.5	11.5	8.	9	8.	8.4	6.8	11.5

** The numbers from (1_9) represent the number given to the aquarium in the recirculating system

Table (3:3) weight of food given to small fry fish as 10% of their weight in grams

Week	Std 0.5% 1	Std 0.5% 2	Eto 0.5% 1	Eto 0.5% 2	Etd 0.5% 1	Etd 0.5% 2
Week 1	1.5	1.3	1.6	1.9	1.7	1.5
Week 2	2.3	1.9	2.7	2.9	2.8	2.4
Week 3	3	2.6	4.2	3.1	5	4
Week 4	4.5	4.3	7.1	5	9	8.3
Week 5	8.1	7.7	12.5	9.3	16	14
Week 6	10.5	10.4	15.6	12.1	19.1	17

*std : abbreviation for standard

*etd : abbreviation for etodolac

*eto : abbreviation for etoricoxib

* (1,2) : aquarium given number

Table (3:4) weight of food given to fry fish as 10 % of their weight for different concentration in grams .

	Standard	Etoricoxib 1%	Etodolac 1 %	Etodolac 2%
Week 1	1.1	1	0.7	0.8
Week2	1.6	1.4	1.0	1.0
Week3	1.7	1.7	1.3	1.4
Week 4	2.2	2	1.9	1.9
Week 5	3.9	2.8	3.7	3.8
Week 6	5.4	3.7	5.6	5.4

Chapter 4

Results

Table (4:1) Weight of mixed population fish more than 2 month treated with 0.5% Etodolac or 0.5% Etoricoxib in grams.

	Std 1	Std 6	Std 9	Etd2	Etd 5	Etd 8	Eto 3	Eto 4	Eto 7
weeks 1	58	54	58	60	59	67	61	59	76
weeks 2	72	58	62	65	68	78	70	65	89
weeks 3	91	72	77	79	84	92	84	78	107
weeks 4	116	96	102	102	103	115	102	88	141
week 5	132	116	122	116	120	128	105	85	150
week 6	148	137	142	143	140	143	125	89	153
week 7	152	169	170	169	170	162	149	113	172
week 8	127	170	231	168	181	165	168	137	230

* The above values represent the weight of 10 fish in each aquarium .

As the table (4:1) above show there were no differences in the weight of fish treated with Etodolac, Etoricoxib in concentration 0.5% respectively if compare with standard aquarium. This result indicate that there is no clear effect for the use of 0.5% NSAIDs concentration for adult fish given food represent 5% of their weight in the aquariums.

Also about the mortality rate during the experimental period there was no mortality shown during all experimental period the rate was 0 %, this mean that the survival rate was 100 % during the 8 weeks of experiment.

Table (4:2) Weight of mixed fry population during experiment period treated with 0.5% Etodolac or 0.5% Etoricoxib in grams.

Days	**std 1**	**std 2**	**etd 1**	**etd 2**	**eto 1**	**eto 2**
1	15	13	17	15	16	19
7	23	19	28	24	27	29
14	30	26	45	39	42	31
21	45	43	88	83	71	40
35	81	77	160	140	124	93
49	105	103	191	170	156	121

Table (4:3) Average weight of mixed fry fish treated with0.5% Etodolac or 0.5% Etoricoxib during experiment period in grams.

Days	std 1 av	std 2 av	etd 1	etd 2	eto1 av	eto 2 av
1	0.55	0.41	0.51	0.56	0.51	0.61
7	0.85	0.67	0.82	0.93	0.87	0.93
14	1.11	0.92	1.44	1.5	1.4	1.06
21	1.73	1.53	3.07	2.93	2.36	1.37
35	3	2.75	5.18	5.33	4.13	3.2
49	3.88	3.96	6.29	6.58	5.2	4.32

As the table (4:2) above shows that the growth rate of fish was higher in the treated aquarium in comparison with standard aquarium.

The growth rate was higher in the aquarium treated with Etodolac, then it followed by aquarium treated with etoricoxib in comparison with standard.

This result show that 0.5% of etodolac or etoricoxib mixed with food have an effect on growth rate, and this concentration of NSAIDs increase the rate of growth in compared with standard one.

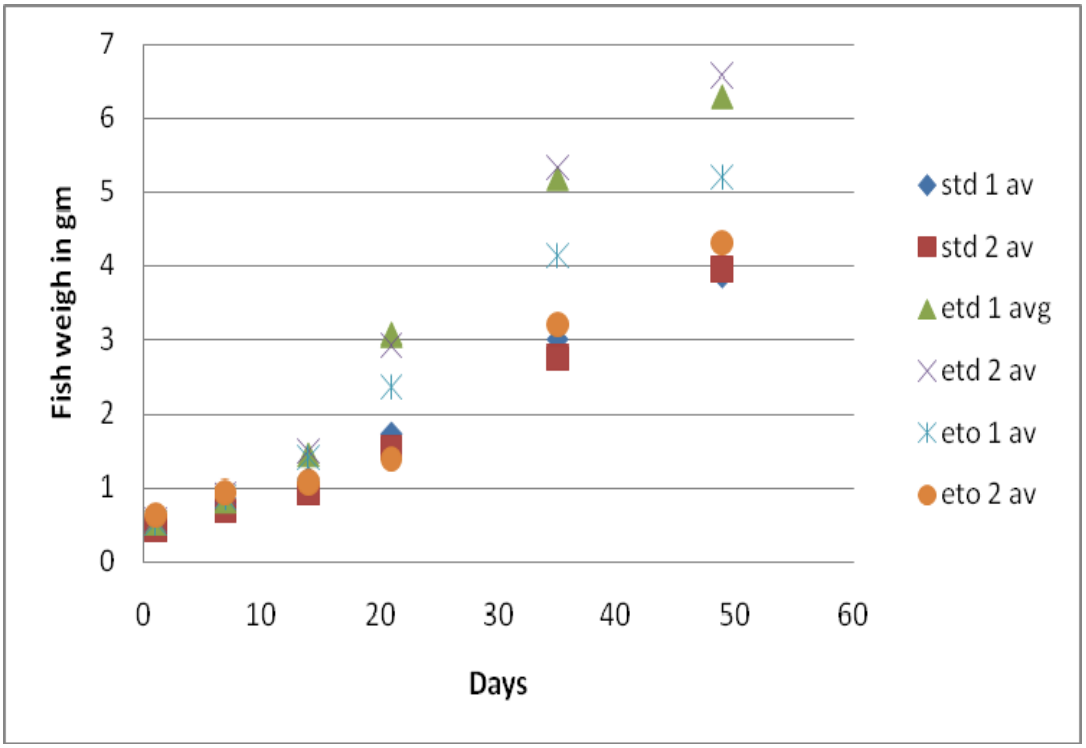


Figure (4: 1) growth rate of mixed population of Nile tilapia fry during experiment period treatment with 0.5% etodolac or 0.5% etoricoxib mixed with food

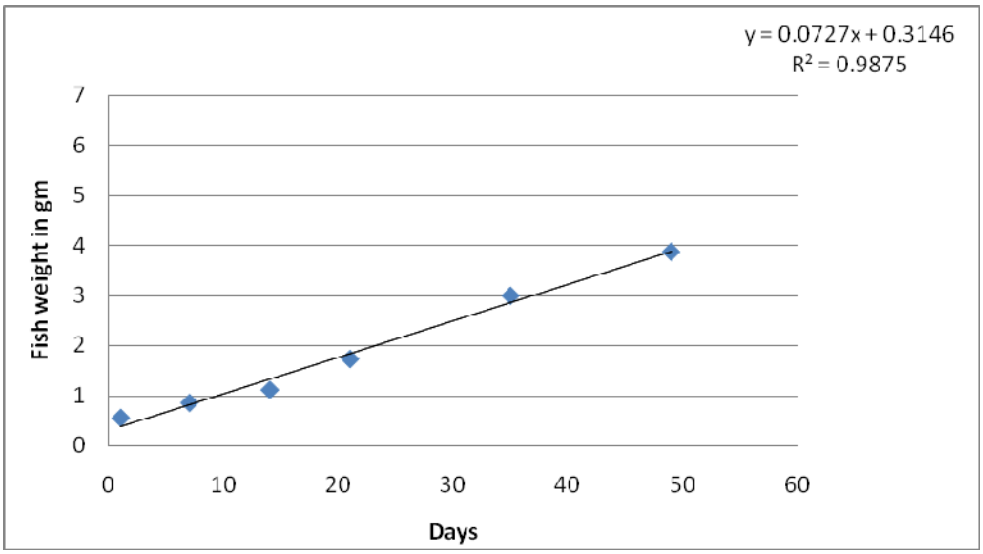


Figure (4: 2) average growth rate of Niletilapia fry during experiment period in standard aquarium1.

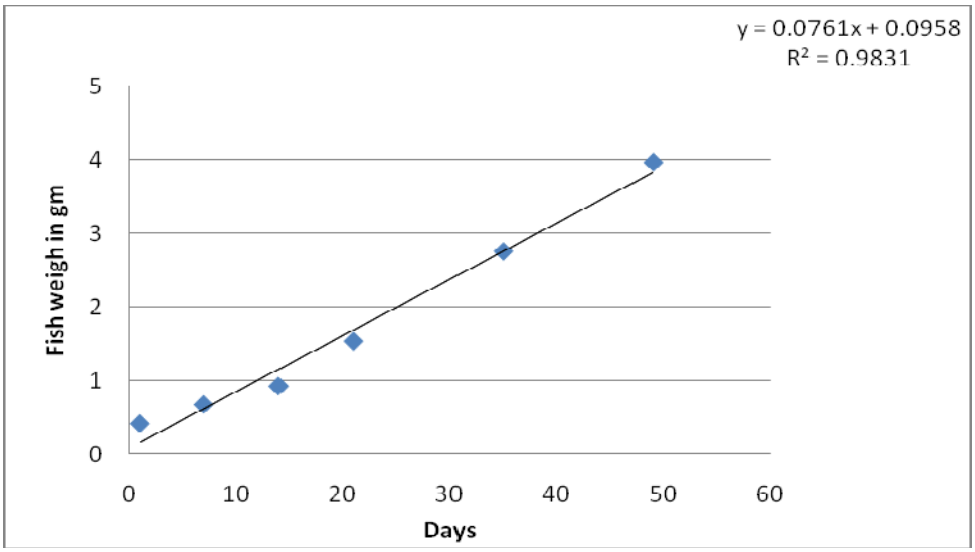


Figure (4: 3) average growth rate of Niletilapia fry during experiment period in standard aquariums2**.**

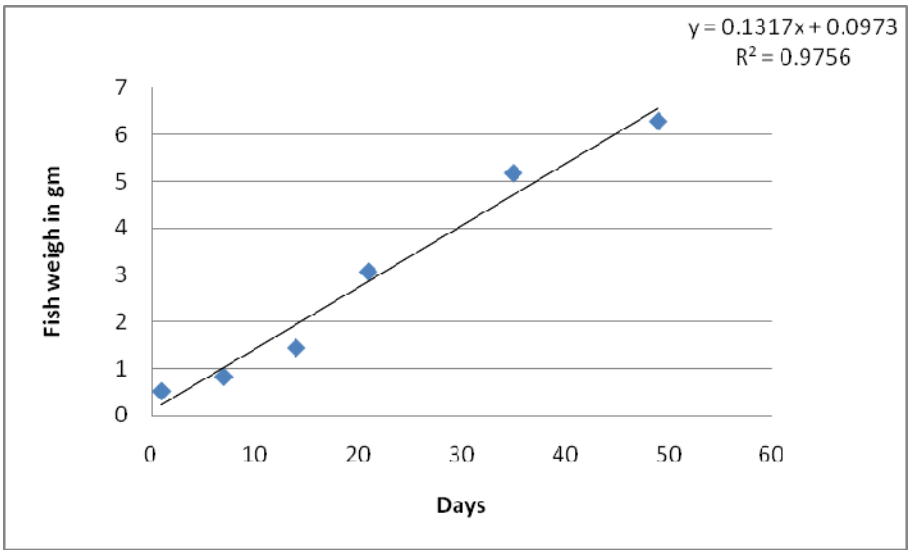


Figure (4: 4) average growth rate of Niletilapia fry during experiment period in 0.5% Etodolac aquarium1.

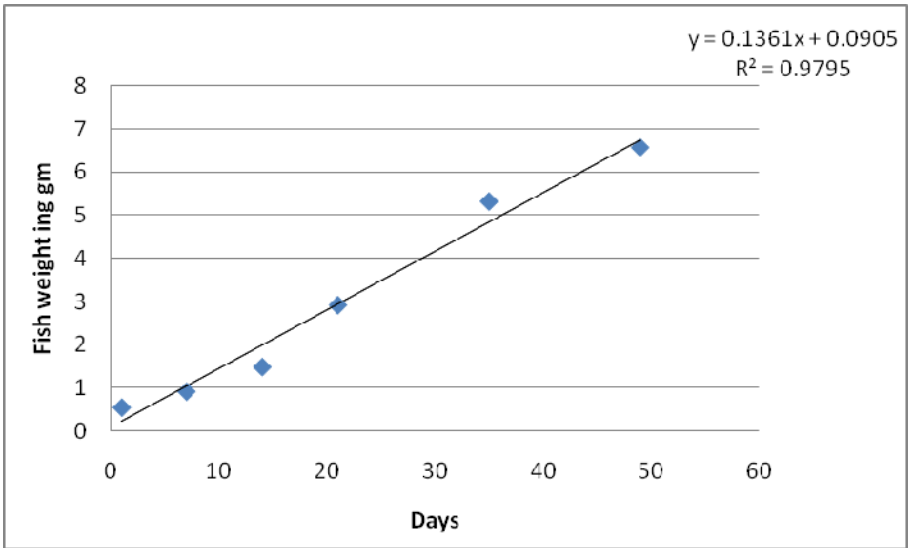


Figure (4: 5) average growth rate of Niletilapia fry during experiment period in 0.5% Etodolac aquarium2.

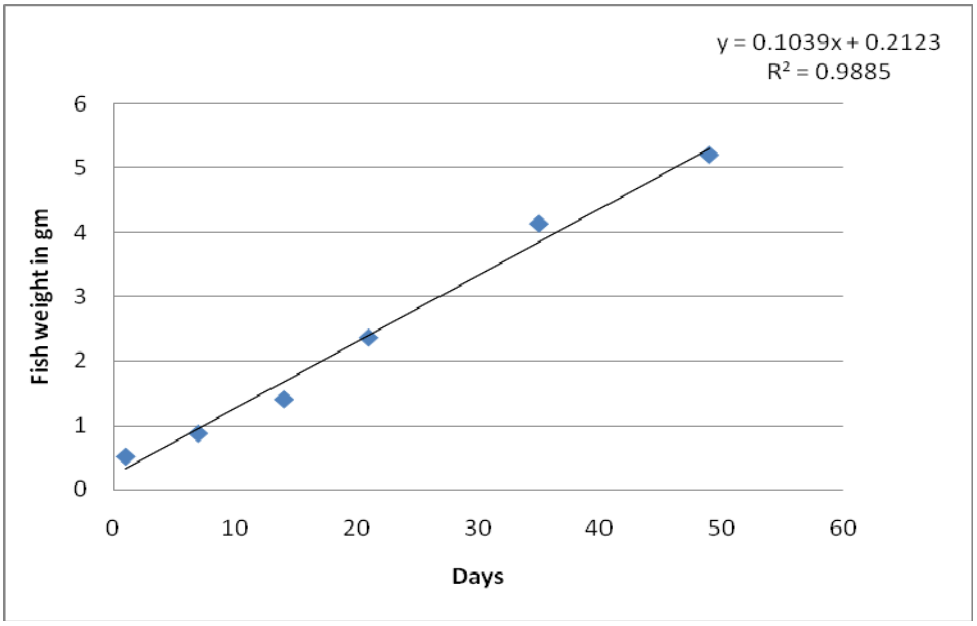


Figure (4: 6) average growth rate of Niletilapia fry during experiment period in 0.5%Etoricoxib aquarium1**.**

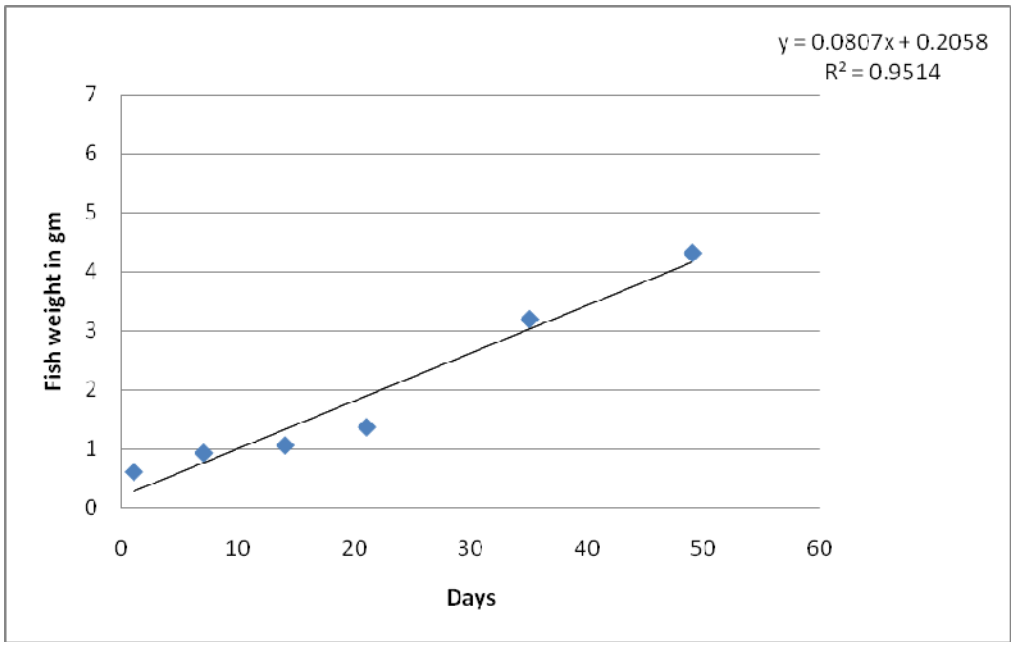


Figure (4:7) average growth rate of Nile tilapia fry during experiment period in 0.5% Etoricoxib aquarium2**.**

Growth performance and diet nutrient utilization were analysed in term of percent body weight gain (BWG), growth rate, feed conversion ratio, feed conversion efficiency, specific growth rate

BWG = 100* (final body weight –initial body weight)/ initial body weight.

FCR = the weight of the feed fed to the fish along the study period / live body weight gain.

FCE = fresh body mass gain/the weight of the feed fed to the fish along the study period.

SGR (%) = 100* [ln (final body weight)-ln (initial body weight)]/ no. of days.

Table (4:4) Body weight gain percentage and other important growth parameters

	BWG (in gram)	FCR	FCE	SGR %
Standard 1	600	0.333	3.00	3.97
Standard 2	692	0.312	3.20	4.22
Etodolac 1	1023	0.304	3.28	4.93
Etodolac 2	1033	0.303	3.29	4.95
Etoricoxib 1	875	0.311	3.211	4.64
Etoricoxib 2	536	0.326	3.06	3.77

Result for Experiment (3)

Table (4:5) Mixed population fish fry weight in small tanks by different concentrations of 1%Etodolac, 1%Etoricoxib, and 2% Etodolac in grams.

days	Std	Eto 1%	Etd 1%	Etd 2%
1	11	9	7	7
7	16	14	10	10
14	17	17	13	14
21	22	20	19	19
35	39	28	37	38
49	54	37	56	54

Table (4:6) Average Weight of fish fry treated with different concentrations of 1%Etodolac, 1%Etoricoxib, and 2% Etodolac in grams.

days	Std	Eto 1%	Etd 1 %	Etd 2%
1	0.36	0.3	0.25	0.43
7	0.53	0.46	0.4	0.62
14	0.56	0.6	0.54	0.87
21	0.75	0.76	0.86	1.35
35	1.39	1.33	1.68	3.45
49	2	1.7	2.66	5.4

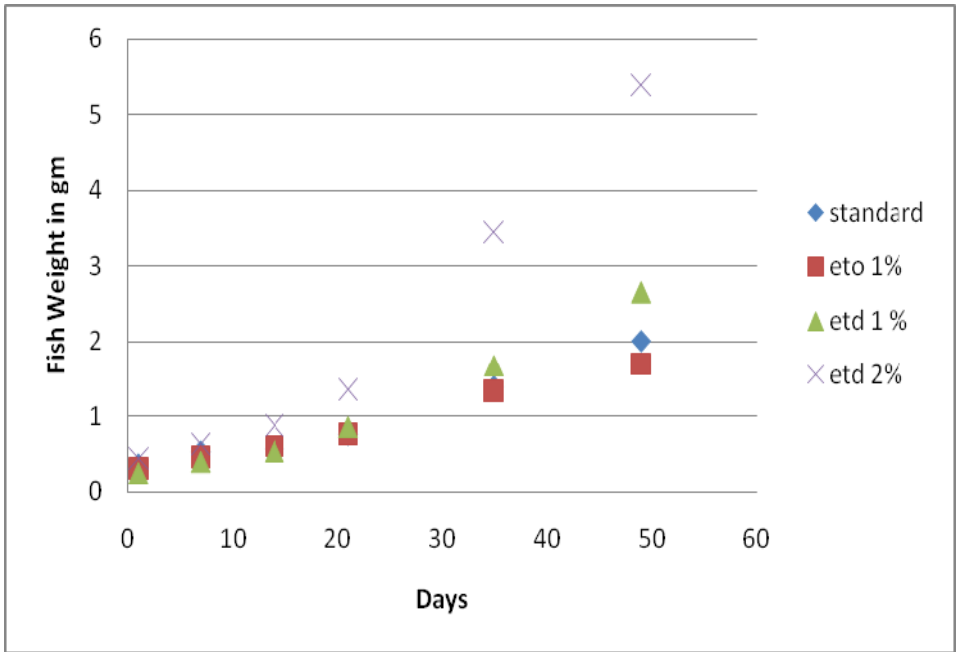


Figure (4: 8) growth rate of mixed population Niletilapia fry during experiment period treatment with 1%Etodolac,1%Etoricoxib , 2% Etodolac mixed with food

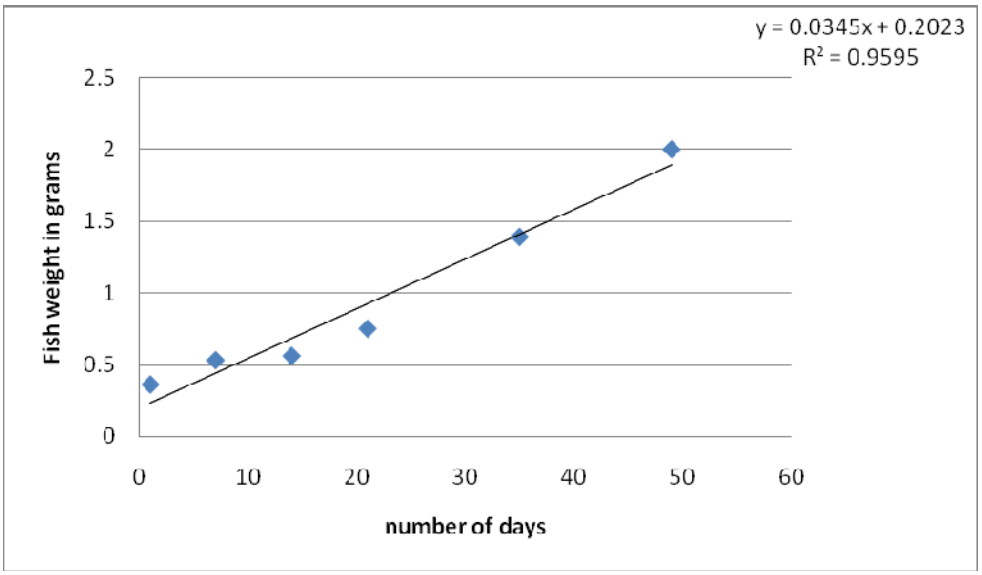


Figure (4: 9) average growth rate of Niletilapia fry during experiment period in standard aquarium

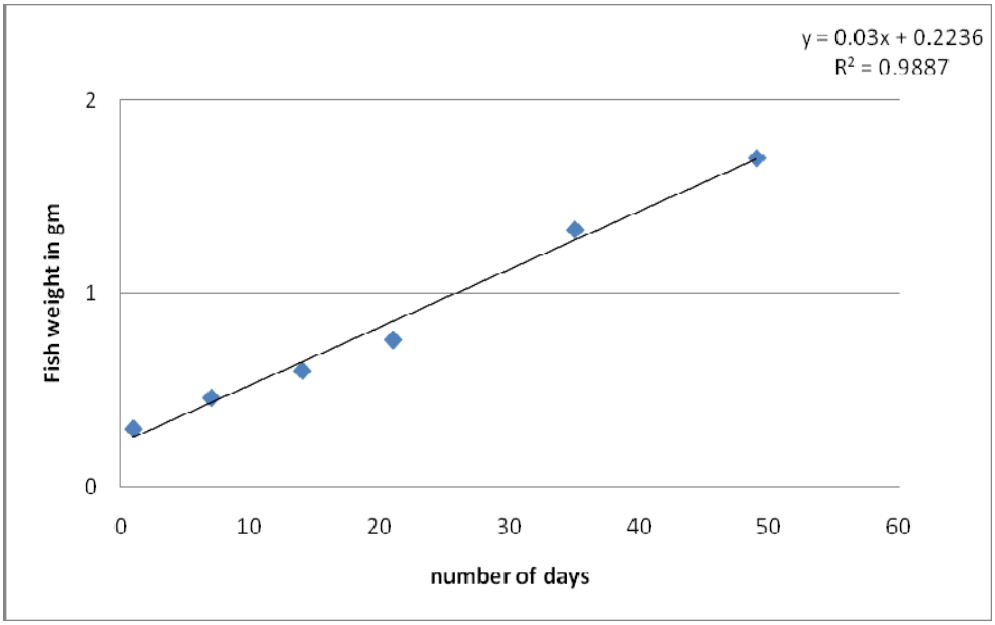


Figure (4:10) average growth rate of Niletilapia fry during experiment period in 1% Etoricoxib aquarium

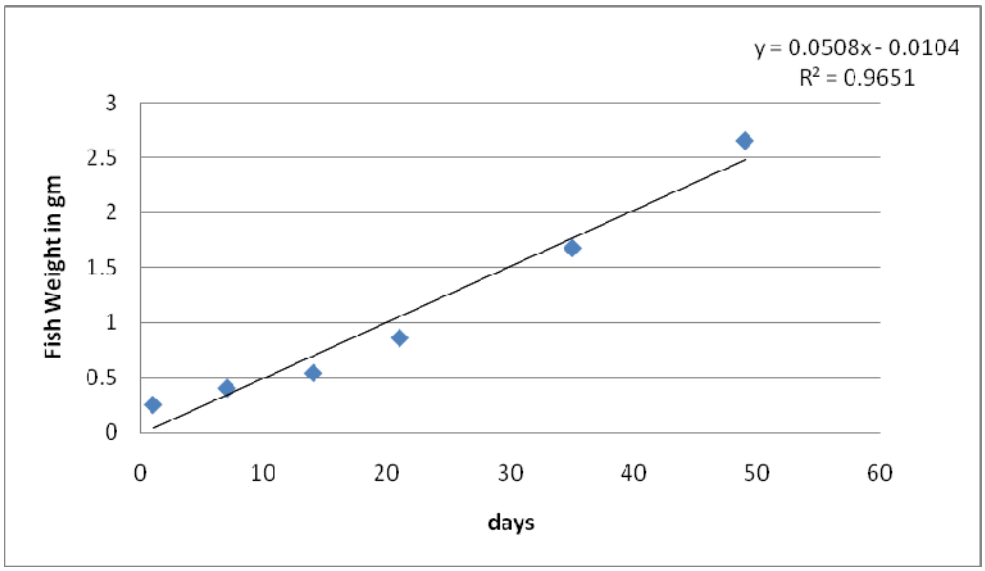


Figure (4:11) average growth rate of Niletilapia fry during experiment period in 1% Etodolac aquarium.

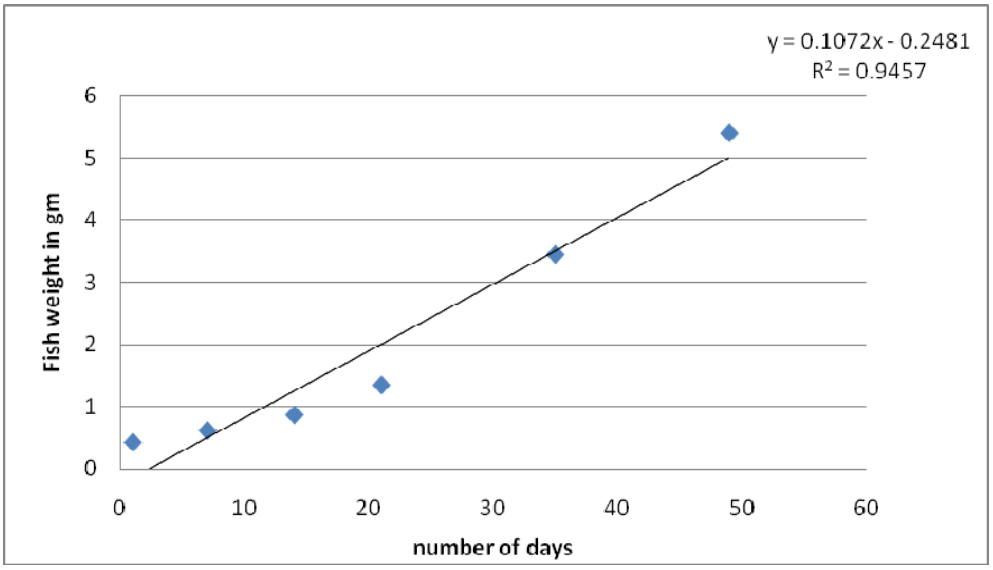


Figure (4:12) average growth rate of Nile tilapia fry during experiment period in 2% Etodolac aquarium.

The different concentration affect the growth rate in a dose- dependent manner.This appears with the weight of fish treated with Etodolac in compared with Etoricoxib or Standard. The highest growth rate was in the aquarium treated with Etodolac at 2% concentration followed by Etodolac 1% concentration followed by standard. Etoricoxib at 1% was toxic with decreased growth rate compared to standard.

Table (4:7) percentage of survival rate and mortality rate during the second experiment.

Week	Standard - 1	Standard - 2	Etoricoxib 0.5% 1	Etoricoxib 0.5% 2	Etodolac 0.5% 1	Etodolac 0.5% 2
Week 1	27	30	30	30	30	29
Week 2	27	28	30	30	30	29
Week 3	27	27	29	28	30	27
Week 4	26	27	29	28	30	27
Week 5	26	27	29	28	30	27
Week 6	26	25	29	27	29	27
Survival rate	86.6%	83.3%	96.6%	90%	96.6%	90%
Mortality rate	13.4%	16.7%	3.4%	10%	3.4%	10%

Table (4:8) percentage of survival rate and mortality rate for treatment with different concentrations of Etoricoxib 1%, Etodolac1%, and Etodolac2%.

Week	standard	Etoricoxib 1%	Etodolac 1%	Etodolac 2%
Week 1	30	30	27	16
Week 2	30	30	25	16
Week 3	30	28	24	16
Week 4	29	26	22	14
Week 5	28	21	22	11
Week 6	27	19	21	10
Survival rate	90%	63%	70%	50%
Mortality rate	10%	37%	30%	50%

Chapter Five

Discussion

5.1 Growth Performance Parameters

The different growth rate parameters (final body weight, weight gain and growth rate (GR) of *O niloticus* fed with Etodolac (non-selective COX-1 inhibitor with preferential COX-2 selectivity) and Etoricoxib (selective COX-2 inhibitor) respectively shown in table (4:2,4:3) Figs (4.1__4.7) were significantly affected by NSAIDs administration with Nile tilapia diet, with the highest growth (final weight, weight gain and GR) table(4:3) obtained with the 0.5% Etodolac followed by Etoricoxib 0.5% followed by untreated sample. These results indicate that the inclusion of selective COX-2 inhibitors in fish diet may be beneficial for fish growth. Etodolac, in humans, is reported to cause a gradual weight gain attributed to the effect of medication itself or due to its effect on other factors (Boullata and Armenti., 2004). The increase in fish growth may be because of that NSAIDs modulated gastrointestinal blood flow and increased the proteolytic activity of the gut inducing the feed digestion and absorption rate causing increase in body weight (Rust., 2002). The gastrointestinal (GI) tract of most teleost species studied to date is, in contrast to most mammalian species, supplied mainly via one major vessel, the coeliacomesenteric artery (CMA), which branches off the dorsal aorta. The CMA then divides into two major arteries, the larger intestinal artery (also often referred to as the mesenteric artery) and the smaller gastric artery (coeliac artery), about 10-40% of CO reaches the GI tract in unfed fish, (Axelsson *et al*., 1989; Dupont-Prinet et al., 2009). However, after feeding over 50% of CO reaches the GI circulation (Axelsson and Fritsche, 1991). For example the increase in GBF after the ingestion of a normal sized meal ranges from around 70% in the sea bass (Dicentrarchus labrax) (Axelsson et al.,2002) to over 150% in the sea bass and the rainbow trout (Dupont-Prinet et al., 2009; Grans et al., 2009). An increase in the blood available to the GI tract after feeding can be achieved either through an increase in the blood volume pumped by the heart per time unit (i.e. cardiac output; CO) and/or a redistribution of blood to the GI circulation from other systemic vascular beds without a concomitant increase in CO. The relative contribution of each factor is strongly influenced by the physical status of the animal (i.e. exercise or other stressors) as well as the environmental factors such as oxygen availability. Consequently, the postprandial increase in Gastric blood flow will depend on both

the decrease in resistance of the GI vasculature, i.e. the hyperemia, and how much blood that is available to the GI tract, either by an increase in Cardiac output or a redistribution of blood. In contrast to what happens postprandially in mammals such as dogs, where there is a limited increase in the CO after feeding in a stationary dog, thus, the increase in GBF is therefore almost entirely due to a redistribution of blood. There is a shift in the amount of CO reaching the GI vasculature in fish, which is mediated via a decrease in the resistance of the GI vasculature and a maintained or increased resistance of other systemic vascular beds. However, if the animal goes from being stationary to being more active there is in general also an increase in cardiac output in order to maintain the postprandial increase in GBF in dogs (Gallavan *et al.*, 1980). On the other hand, the vascular endothelial cells play a central role in synthesizing and releasing prostaglandins, which may have constricting as well as relaxing effects on blood vessels. Following their intracellular synthesis, prostaglandins probably exit the cell by facilitated diffusion and affect the parent cell and/or neighboring cells in an autocrine /paracrine manner. They act through specific G-protein-linked prostanoid receptors, altering levels of different second messengers (Smith., 1992). Prostaglandins have diverse effects in fish circulation, and are likely to serve a multitude of functions (Janvier1997., Brown and and Bucknall1985; Sundin and Nilsson., 1998). PGF2 is involved in a haemostatic vasoconstriction and may take part in the control of brachial circulation, by constricting filament arteries. In contrast, PGE2 has most of its effect on the heart. PGE2 causes a reduction in cardiac output with a decrease in systemic resistance and increase in gills vascular resistance. It also reduces dorsal aortic blood pressure (Stenslokken et al.,2002).

After the meal had been digested i.e. Enzymatically broken down into smaller components by carbohydrases, lipases and proteases (Kitamikado and Tachino., 1960a, b, c). It is likely that it is these hydrolyzed products that induce the subsequent GI hyperemia and mechanical distension of the stomach elicits an increased adrenergic tone on the systemic vasculature, producing a vasoconstriction in the systemic circulation including the GI portion, and a subsequent increase in dorsal aortic pressure. This leads to either a redistribution of blood flow from the systemic circulation or a larger fraction of the cardiac output reaching the GI tract in the event that cardiac output increases. This pressor response increases the driving force for the perfusion of the GI vasculature when chemical stimuli (hyperemia) induce a decrease in the resistance of the GI vasculature mediated via hormones like cholecystokinin (CCK),(Seth et al., 2009,2010). The circulating level of CCK is also influenced by the diet (Jonsson *et al.*, 2006). NSAIDs may enhance the proteolytic activity of the gut by increasing proteases activity

(Rust., 2002), thus accelerating the hyperemia event (Seth., 2010). In addition, blocking prostaglandins synthesis abolishes there effect previously mentioned on the heart. Further more acidic water from, accumulation of these drugs in a closed system could act as a stressor that activate catecholamine release which have additional stimulatory effects on cardiac output (Randall and Perry.,1992).Growth is under multiple endocrine control, with growth hormone as the pivotal factor. Insulin, thyroid hormone, and gonadal steroids, in particular during the initial phases of sexual maturation, also have growth-promoting capacities in teleost fish (Pickering.,1990). The maintenance of adequate thyroid status in fish is a prerequisite for normal growth. Thyroid hormones are thought to play a permissive role in the growth process, potentiating the effects of other anabolic hormones, most notably growth hormone. The main actions of TH in fish appear to involve regulation of growth, early development, metamorphosis and aspects of reproduction (Brown et al., 2004). The thyroid tissue in most teleosts is scattered diffusely in the basibranchial region but, as in other vertebrates, consists of follicles formed of a single layer of epithelial cell surrounding an extracellular lumen containing proteinaceous colloid. Unlike mammals, the fish thyroid cascade can be broken down into three elements. First is the centrally controlled brain-pituitary-thyroid axis, responsible for synthesis, storage, and secretion of T4 and maintenance of T4 levels for a given physiological state (central control). The second element is the peripherally controlled (e.g., in liver tissue) availability of the active hormone T3 via outer-ring monodeiodination of T4, which occurs in peripheral tissues (e.g., liver tissue). The third phase is the receptor-mediated effects of T3 on target cells to regulate development, growth, and aspects of reproduction (Eales and Brown., 1993). While similarities exist between mammalian and fish thyroidal systems, the fish thyroidal system does not appear to be centrally driven (i.e., via brain-pituitary-.thyroid axis) but instead is under peripheral control. The type I deiodinase (D1), which is capable of both activation (outer-ring deiodination (ORD) and inactivation (inner-ring deiodination (IRD), (Sanders *et al*., 1997) . However, the type II deiodinase (D2) only has ORD activity and deiodinase (D3) capable only of IRD (Sanders et al., 1999;Orozco et al., 2003).Thyroid status itself is one of the most potent regulators of iodothyronine deiodinase expression. The study by (Molka et al.,1999) showed that in the Nile tilapia, induction of hyperthyroidism by feeding the animals with T3-supplemented food (12 ppm) for 11 days resulted in a prominent decrease in hepatic D2 activity, where as hepatic D3 activity increased. However, neither brain nor gill D3 activity was affected, nor was kidney D1 activity. On the other hand induction of hypothyroidism by providing fish food containing 0.2% methimazole for 11 days increased liver type II deiodinase (D2) with no changes in kidney

type I deiodinase(D1), and resulted in a significant decrease in brain, gill and liver type III deiodinase (D3). Other studies demonstrated that the hepatic D1activity, as well as D1 mRNA expression, is up regulated in hypothyroid tilapia, (VanderGeyten et al., 2001). Numerous chemicals can modify vertebrate thyroid function. They range from complex synthetic halogenated or phosphorylated hydrocarbons to simple cations or anions (Brown et al, 2004). Several pharmaceuticals, discharged mainly in untreated or treated sewage, have the potential to influence the fish thyroid. Iodinated radiologic contrast agents that contain a triiodo- and a monoamino-benzene ring with a propionic acid chain (e.g., iopanoic acid, iopodate used for oral cholecystography) are potent antagonists of peripheral deiodinating activity in mammals, Iodinated diagnostic contrast agents like iopromide and diatrizoate are resistant to transformation during sewage treatment and can be found in effluent-dominated rivers at mg/l levels. Though these agents have a low acute toxicity to fish, their chronic impacts have not been investigated. In rainbow trout, i.p.-injected ipodate at 5 mg/100 g depressed plasma T3 levels, T4 monodeiodination and plasma clearance of both T4 and T3, and reduced the hepatic nuclear T3 receptor maximum binding capacity. Iopanoic acid also has been used to induce a hypothyroid state in brown trout and *C. batrachus.* Several other pharmaceuticals influence the following steps in the thyroid cascade : Active transport of I2 (complex anions—perchlorate, fluoroborate, pertechnetate), iodination of TG (thionamides—propylthiouracil, methimazole, carbimazole; aniline derivatives; sulfonamides), DIT coupling reaction (thionamides; sulfonamides); TH release (lithium salts) ;peripheral deiodination (thiouracil, amiodarone, hormone excretion and inactivation through competition for TH binding sites to plasma proteins or induction of hepatic drug-metabolizing enzymes (salicylates, clofibrate, methadone, 5-fluorouracil, mefenamic acid, furosemide, Phenobarbital, rifampin, carbamazepine, phenytoin, hormone action (TH analogs, amiodarone and phenytoin) and TH binding in the gut lumen (cholestyramine, Cyclooxygenase (COX) inhibitors, such as the methylated indole and indomethacin, block the synthesis of prostaglandins that contribute to regulation of cell growth in the mammalian thyroid gland, and indomethacin can alter cell growth in thyroid cancers (Frenkian, *et al*, 2001). Numerous types of other anti-inflammatory COX inhibitors (e.g., ibuprofenb, naproxen, rofecoxib, and celcoxib, are now prescribed but no reports found for their effects on thyroid function in any vertebrate. In humans, salicylates inhibit the binding of T4 to all plasma transport proteins, resulting in a decrease in T4 and a transient increase in fT4. With sustained use, these agents cause a 20% to 40% decrease in T4 concentrations but do not alter fT4 .Other studies of various NSAIDs (e.g., oxaprozin, ketoprofen, etodolac) given at therapeutic doses for more than 3 weeks demonstrated no change in T4 concentrations, but

ketoprofen and nabumetone caused a decrease in serum T3 concentration(Carlson.,1999). Data on NSAIDs are limited in dogs, but carprofen (2.2 to 3.3 mg/kg PO q12h) given for 5 weeks resulted in a small but statistically significant decrease in T4 (20.8 to 17.0 nmol/l) and TSH (0.16 to 0.12 ng/ml) concentrations without altering serum fT4 (Ferguson et al., 1999).Also, Serum total T4 concentrations decreased significantly within 24 hours of receiving aspirin with no significant changes in thyroid function associated with ketoprofen administration (Daminet et al., 2003). In another study thirty eight dogs that were treated, for orthopedic disorders were given etodolac (10 to 13.3 mg/kg, orally, once daily) for14 days, serum T4 concentration decreased significantly after etodolac administration with increased cTSH. Serum free T4 concentration was not significantly affected (Ness et al., 2003). PGs are known to influence a wide range of physiological processes by enhancing the release of hormones and altering the sensitivity of target organs (Lands., 1991; Mustafa and Srivastava, 1989). (Langer et al., 1978) found that ASA treatment reduced plasma levels of TSH, T3, and T4 in humans, which indicated the involvement of PGs in the release of thyroid hormones. Studies on mammals further showed that PGs enhanced the response of thyroid tissue to TSH, resulting in higher T3 and T4 levels (Lands., 1991). Basal levels of T3 were also significantly reduced in tilapia after ASA treatment, suggesting that PGs have a similar regulatory function (van Anholt et al., 2003). According to our literature review we propose that Etodolac and Etoricoxib could induce a hypothyroid state in tilapia. Since the thyroid cascade may respond indirectly and it has considerable capacity to compensate for abuses that otherwise would disrupt thyroid hormone homeostasis, it up regulates peripheral deiodinase enzymes as mentioned previously. In addition inhibition of plasma protein binding has increased f T4 and f T3. Less than 1% of plasma total T4 (TT4) is free (FT4) with 99% reversibly bound to plasma proteins. Plasma FT4 has a strong negative feedback action on the brain–pituitary–thyroid axis aggravating the hypothyroid state. Although the proportion of total T3 (TT3) that is free in plasma (FT3) is usually less than that for T4, the T3 binds also to some plasma proteins that bind T4.Thus further investigations by conducting a deiodination assay along with the measurement of plasma T4 and T3 levels should provide a good indication of the peripheral thyroid system status.

Results showed no increase or decrease of growth in mixed population adult Nile tilapia treated with 0.5% Etodolac and 0.5% Etoricoxib (Table 4:1). This result reflects the fact that growth is regulated by several controls including differences in levels of both sex- related hormones and metabolic hormones, with growth hormone having a pivotal role. Thyroid hormones and

gonadal steroids are important In particular during the initial phase of sexual maturation and development (Pickering., 1990). Thyroid hormones assist in control of osmoregulation, metabolism, somatic growth, development, and posthatching metamorphosis (Janz., 2000). Metamorphosis in flounder and flatfish eye, mouth, and neural structures is associated with a dramatic spike in thyroxine (T4) concentrations (Janz., 2000). These results support our hypothesis that COX-inhibitors could modulate aromatase enzyme during the crucial period of sexual differentiation as we will discuss later. In addition deiodination activity in fish is responsive to many environmental and physiological conditions (e.g., food quantity, food quality, pH, salinity, turbidity) (Eales et al., 1999). Tilapia kept in partially closed systems and fed artificial diets have been reported to have a decreased growth after a certain period of time. This may be due to the fact that tilapia needs a continuous supply of their natural food or because of a build-up of growth inhibiting factors in the system (Jackson et al., 1982).

The main goal of our study was to predict the effect of etodolac and Etoricoxib during the crucial period of sexual differentiation where sufficient amount of estrogen is needed for proper ovarian development and the suppression of aromatase activity the rate limiting enzyme in aromatization of androgens to estrogens leads to sex reversal and the arise of male population (Kown et al., 2001). It is well known that there are differences in male vs female growth rates in Oreochromis species.Monosex production or even incomplete female prevents reproduction and therefore feed energy could be diverted into growth instead of production of unwanted juvenile fish. Brueggemeier *et al.* (1999) pointed to linear dependence between aromatase gene (CYP 19) expression and expression of COX-2 in mammary carcinoma. The above mentioned facts predetermine the use of NSAIDs in mammary carcinogenesis prevention. Cyclooxygenase inhibitors suppress aromatase expression and activity in breast cancer cells (Diaz-Cruz et al 2005) and since fish posses both COX-1 and COX-2 forms and both are widely expressed during development with both isoforms genetically and functionally homologous to their mammalian orthologs (Grosser et al., 2002), we tested the hypothesis of possible cyclooxygenase inhibition during the crucial period that may modulate aromatase activity, thus altering sexual differentiation. Our observation of significant differences in growth rates of mixed fry Nile tilapia (table 4:2,4:3) compared to adult mixed population table(4:1) following exposure to 0.5% Etodolac and0.5% Etoricoxib at this early stage indicates that: Cox inhibitors could alter aromatase activity needed for proper sexual development and reproduction as mentioned earlier. An earlier study conducted in our lab showed no significant differences in growth rate (GR) of 20 genetically females (XX) (O.

niloticus) fry following treatment with 1%diclofenac, 5%diclofenac, and 5%ibuprofen. The results were 36%, 17% and 22% of the fish never produced egg in the 1% diclofenac group, 0.5% ibuprofen group, and 0.5% diclofenac group respectively during the entire experimental period. Macroscopically all the non-spawning fish in the experimental groups were females with apparently larger ovaries and full of eggs compared to control. Microscopically they were full of apparently normal eggs with morphology similar to those of control (Al-Qutob2009). Previous studies also showed that Ibuprofen in the ranges of (1-100 ugL-1) decreased the number of spawning events and increased the number of eggs per reproductive cycle in adult Japanese medaka (Flippin et al., 2007).

We propose that alteration of aromatase activity at the transcriptional level by the COX-inhibitors decreases the rate of aromatization which alters E2 (estrogen) levels. Low (E2) release the feedback inhibition on gonadotropins producing high FSH levels that inhibits ovulation, since it is present in the blood of immature fish and levels increase during the vitellogenic phase but should decline towards follicular maturation and spawning (Kawauchi et al., 1989). FSH is aheterodimeric glycoprotein synthesized and secreted by the anterior pituitary gland. This hormone is involved in the regulation of essential vertebrate reproductive processes such as gametogenesis and follicular growth. They are composed of a common a subunit and a unique b subunit that confer biological specificity to each hormone (Pierce et al., 1981). These glycoprotein hormones are structurally and functionally conserved in various vertebrates and have been identified in most lineages of action pterygian (bony) fish (Park et al., 2005). cDNA sequences encoding gonadotropin subunits have been isolated and characterized from more than 19 fish species, including tilapia (Yaron et al., 2001). The two gonadotropins, LH and FSH, are synthesized by two different cell types in the pituitary of tilapia, and have distinct temporal expression and release profiles (Yaron et al., 2001). In most fish, FSH is generally prevalent during early gonadal development and the vitellogenic phase, while LH secretion remains at low levels during these phases and exhibits a sharp peak around final oocyte maturation and ovulation or during spermiation (Yaron et al., 2003). FSH release in female tilapia is under the control of the hypothalamic decapeptide GnRH and the feedback inhibition loop at the gonadal-pituitary-axis (Aizen et al., 2007; Yaron et al., 2003). This contrast with the situation in mammals in which most of the FSH secretion occurs is dependent of signals arising from the GnRH receptor to the release mechanisms (Pawson and McNeilly., 2005). It was found that recombinant tFSH significantly enhanced 11-ketotestoster-one (11-KT) and estradiol secretion from tilapia testes and ovaries, respectively, in a dose-dependent

manner (similar to tilapia pituitary extract, affinity-purified pituitary FSH, and porcine FSH (Aizen et al., 2007) The somatic growth of teleosts is controlled by the growth axis consisted of hypothalamus–pituitary–liver, i.e.The GH/IGF-I axis. GH released from the pituitary gland binds to its receptor and stimulates insulin-like growth factor-I (IGF-I) synthesis and secretion from the liver and other sites, evoking biological actions through IGF receptors (Butler and LeRoith., 2001). However, the modulation patterns in the axis are not a basic point to point linear regulation and feedback, but rather are a kind of multifactorial and multiregulational manner, which makes up a regulation network of GH synthesis and secretion in fish. In the hypothalamus, a number of neuro endocrine factors directly act on somatotropes, including pituitary adenylate cyclase-activating peptide (PACAP), GH-releasing hormone (GHRH), gonadotropin-releasing hormone (GnRH), Neuropeptide Y (NPY), somatostatin (SS). In addition, these neuroendocrine factors have interactions controlling GH secretion and are also affected by some of the peripheral factors. Gonadotropin (GtH) released from the anterior pituitary regulating reproductive functions, interacts with GH at multiple levels to respectively modulate the functions of the gonadotrophic and somatotrophic axes. In mammals, at the pituitary level, transcripts of GH receptors and GH-binding sites are observed to appear in gonadotrophs, and the stimulatory actions of GnRH on LH and FSH release are inhibited by GH immune neutralization, suggesting that endogenous GH may act in a para-crine manner regulating gonadotroph functions. Similarily, GH release is also under the influence of the gonadotrophic axis, especially via the release of sex steroids.In fact according to our hypothesis which is previously stated the high FSH could increase growth hormone secretion by somatotrophs. (Zhou et al.,2004) proposed a novel mechanism regulating GH release and synthesis in fish where by the local interactions between gonadotrophs and somatotrophs may form an intrapituitary feedback loop for regulating GH release and synthesis. In this model, GTH released from gonadotrophs induces GH release and GH production in neighboring somatotrophs. GH secretion maintains somatotroph sensitivity to GtH stimulation, and simultaneously, inhibits basal GtH release in gonadotrophs. In tilapia, the somatotrophs are located in the proximal pars distalis (PPD) forming a palisade around the nerve ramifications; the gonadotropin (GtH) I (FSH-like) gonadotrophs are adjacent but slightly peripheral to them, where as the GtH II(LH-like) gonadotrophs outlay these cells (Melamed et al.,1998). Paracrine interactions also exist between the gonadotrophs and somatotrophs in the tilapia pituitary (Melamed et al., 1999). No direct effects of gonadal steroids on expression of the tilapia GH gene were reported. Although they did appear to increase the sensitivity of the somatotrophs to some of the hypothalamic GH-releasing hormones. The effects of testosterone could be

mimicked by estradiol (E2), but not the non aromatizable 11-ketotesoterone, as the testosterone is aroma-tized before eliciting these effects (Melamed et al., 1995; Melamed et al., 1997). It is also worth to mention that the thyroid axis (TRH, TSH, and T3) stimulates both synthesis and release of GH in tilapia (Melamed et al., 1995), thus interfering with this axis could indirectly alter GH secretion.

Studies showed that many endocrine disruptive chemicals could alter aromatase Cyp19 expression or activity at both transcriptional and post transcriptional levels and this is evident by the presence of multiple transcriptional regulatory elements which include cyclic adenosine monophosphate (cAMP) responsive elements, a steroidogentic factor 1/adrenal 4 binding protein site, an estrogen responsive elements (ERE), half-ERE'S, dioxin-responsive element and elements related to diverse other nuclear receptors and the cAMP responsive elements (CREs) were predicted in the 5-flanking regions of both cyp19a and cyp19b genes in teleosts (Cheshenko et al., 2008). cAMP responsive element binding protein (CREB) regulates the transcription upon activation by phosphorylation with protein kinase A upon elevation of intracellular cAMP levels. Around 300 different stimuli can provoke CREB phosohorylation (Johannessen et al 2004). The use of NSAIDs like Etodolac(non-selective COX-1 inhibitor with preferential COX-2 selectivity) and Etoricoxib(COX-2 inhibitor) inhibit the production of prostaglandins (PGs) and lowers intracellular, cAMP, which could result in decreased aromatase expression via affecting promoter through cAMP responsive elements (CRE) at the transcriptional level. In addition, prostaglandin metabolites have also been shown to activate transcriptional targets directly in mammals, by binding to peroxisome proliferative-activated receptors (PPARs) which are members of orphan nuclear superfamily, that constitutively bind to the promoter elements of their target genes (Forman et al., 1997). PPARs super family was identified in tilapia (Chang et al., 2005). Other possible postulated mechanisms could be through free ArA (arachidonic acid) generated by blocking the cyclooxygenase pathway (COX-1 and COX-2), which can modulate mRNA cyp19 expression by altering the binding of estrogen to its estrogen receptor elements (ERE) thus down regulating cyp19 expression, indeed estrogen will not be synthesized in sufficient amount, or it can modulate cyp19 activity at the post transcriptional levels by competing with the substrate for binding to active site of the enzyme. Endogenous long chain fatty acids are known to be regulators of cell signaling pathways and to affect either positively or negatively the binding of steroid hormones to their specific plasma proteins and their specific intracellular receptors and have also shown to co-regulate glucocorticoid-dependent gene expression (Sumida ., 1995).

PPARs family members exhibit also a strong binding affinity for both saturated and unsaturated fatty acids which may indicate that the increase in free arachidonic (ArA) from cyclooxygenase inhibition could have an inhibitory effect on aromatase though binding to PPARs receptors (Mu et al., 2001).

Ovarian follicular development lies with the interaction between pituitary gonadotropins, follicle stimulating hormone and luteinizing hormone (and glucocorticoid hormone and prolactin in some species), and intraovarian factors such as steroid, cytokines and other growth factors. Among these, prostaglandins (PGs) possess vasoactive, mitogenic, and differentiating properties and are implicated in various female reproductive functions. In particular, PGs have been shown to play key roles in ovarian physiology, the periovulatory period, and female reproduction. Several studies have suggested a role for PGs in the maintenance and function of the cumulus–oocyte complex (COC). PGE2 was one of the earliest substances shown to induce cumulus expansion *in vitro.* Additionally, COCs obtained from rats and mice after superovulation synthesize PGE1, PGE2 and PGF2α. Therefore, any drug that blocks synthesis of PGs would affect female reproductive function. In fact, it is reported that high doses of indomethacin (an inhibitor of both cyclooxygenase-1 (COX-1) and -2 (COX-2), one of the nonsteroidal anti-inflammatory drugs NSAIDs or a "specific" COX-2 inhibitor (NS-398) can block ovulation in rats. In addition, treatment of COCs with indomethacin greatly reduces the *in vitro* fertilization rate of oocytes, and this effect is reversed if PGE1 and PGE2 are added to the media in the presence of indomethacin. These data suggest that PGs may be critical for maintaining an optimal microenvironment for oocyte survival and fertilization. However, although use of low-dose indomethacin can abolish ovarian PGE2 synthesis, low-dose indomethacin failed to affect ovulation. In contrast, high-dose indomethacin resulted in significant inhibition of ovulation. It was also noted that absence of COX-2 in mice led to defects in ovulation, fertilization, and implantation, suggesting that COX-2 enzyme may play an important role in female reproduction. Selective COX-2 inhibitors are commonly used in clinical practice, including for the management of dysmenorrheal, menorrhagia or other rheumatism, all of which often attack women in reproductive age. In this study, a low dose of the selective NSAID COX-2 inhibitor, celecoxib, was used to evaluate its effects on the reproductive function of female mice. COX-2 inhibitor may be safe if the dose is low because we clearly demonstrated that low-dose COX-2 inhibitor of $\leq$ 5 mg/kg celecoxib per day did not seem to affect the normal fertility of female mice, suggesting that implantation may not be impaired when using a low dose of COX-2 inhibitor. Since differences exist between primates

and other species in the sites and timing of COX-2 expression in the ovary, COX-2 inhibitors may produce different results in these species. In addition, emerging evidence shows that COX-2 inhibitors may impair fertilization, implantation, and maintenance of pregnancy. From this study, we found that low-dose COX-2 inhibitor did not affect fertilization, implantation and maintenance of pregnancy in a mouse model. Overall, the therapeutic window between pain relief and fertility impairment in using COX-2 inhibitors in clinical practice is worthy of further investigation (Elvin et al., 2000; Findlay et al., 2002; Dey et al., 2004).

5.2 Mortality vs Toxicity:

In reference to tables (4:5,4:6), there were no differences in mortality rates between (0.5%Etodolac),and (0.5%Etoricoxib) treatments, but survival rate (96.6% and 90%) was improved compared to control (86.6%,83.3%) during the experimental feeding. Higher mortalities were shown as concentrations increase with (1%Etoricoxib), (1% Etodolac) and (2% Etodolac) table (4:6) treatments. Improved survival could reflect the anti inflammatory effect of COXs inhibitors. One could expect that use of NSAIDs is associated with lower levels of inflammatory markers. NSAIDs are documented to be used by athletes as an ergogenic aid to enhance performance especially before and during long endurance races and longer training sessions. The theory behind this practice is that the prophylactic inhibition of the production of inflammatory mediators will lead to decreased muscle soreness, fatigue, and ultimately shorter recovery times and improved performance. However, currently there is no convincing evidence that NSAIDs enhance performance or recovery time (Nieman et al., 2006; Donnelly et al., 1990). (McAnulty et al., 2007) studied the effects of inflammatory markers in a group of ultramarathon ibuprofen “users” versus “non-users”. He found that some inflammatory markers were actually increased in the ibuprofen user vs. the non-user group. In another study, the use of NSAIDs was associated with lower levels of IL-6 and higher levels of TNF-a but was not associated with CRP levels (Il’yasova et al., 2005).Mortality rates for (0.5%Etodolac), and (0.5%Etoricoxib) were comparable to (0.5% Ibuprofen, and significantly lower than (0.5% diclofenac) reported in a previous study (Al Qutob.,2009). So far, there are no data available on the chronic toxicity of Etodolac, and Etoricoxib to fish. Previous studies with Ibuprofen showed its toxicity on aquatic organism Daphnia magna, and the mollusk Planorbis carinatis (Pounds et al.,2008) which had been reported to be in mgL-1 range.Other study carried on adult Japanese Medaka, Oryzias latipes showing that exposure to nominal concentrations of ibuprofen in the range of (1-100ugL^{-1}) for 6 weeks altered reproduction with no evident

pathological damage in the gills, liver and kidneys of fish from the highest exposure group (Flippin et al.,2007). In contrast, investigations of (Schwaiger et al., 2004) of diclofenac toxicity on rainbow trout revealed that the lowest observed effect concentration (LOEC) at which both renal lesions and alteration of gills occurs is 5 $ugL^{-1}$with concentration related accumulation of the drug in all organ examined, and the highest amount detected in liver, followed by kidney, gills and muscle tissue.

Although increasing drugs concentrations increased mortality table (4:6),the different concentrations as shown in table(4:5,4.6) and figures (4:8__4:12),affected the growth rate in a concentration dependent manner. This appears with the weight of fish treated with Etodolac and Etoricoxib compared to standard. The highest growth rate was in the aquarium treated with (2% Etodolac) followed by (1% Etodolac). (1% Etoricoxib) showed a decreased growth rate compared to standard which could indicates a toxic potential of Etoricoxib toward fish at this concentration. In addition growth rate with 0.5% Etodolac(0.131 and 0.136) was higher than with increasing concentrations of Etodolac that show growth rate of (0.05) with 1% Etodolac and (0.107) with 2% Etodolac respectively. These facts could be considered as sign of toxicity.

Under controlled experimental factors we should take into consideration the altered physiological effects mediated by the inhibition of prostaglandins (PG) synthesis. Gills alterations may interfere with normal respiratory functions and renal changes might lead to impaired osmoregulation (Evans et al 2004). Furthermore prostaglandins are involved in many physiological functions eghomeostasis and nervous system function (Mustafa & Srivastava., 1989) of which blocking could enhances the non-specific toxic effects. In the kidneys of mammals, cyclooxygenase type 2 (COX-2) is expressed in medullary interstitial cells, the macula densa and epithelial cells of the cortical thick ascending limb where it generates prostaglandins that regulate hormone secretion, inhibit ion transport, and support cell survival during salt loading and dehydration (Harris and Breyer., 2001). Gills are primary site of osmoregulation in fishes and contains specialized cells that secrete NaCl (chloride cells) via a mechanism that is similar to the NaCl transport mechanism of shark rectal glands and the mammalian cortical thick ascending limb of the kidney (Evans et al., 2005). Prostaglandins inhibit short circuit currents (Isc) that are a result of active Cl secretion (Evans *et al.*, 2005). Using specific pharmacological inhibitors, like cox-2 inhibitor NS-398 which was much more effective than the COX-1 inhibitorSC560, basal Isc may be moderately inhibited byCOX-2-mediated prostaglandin synthesis and that COX-2 mediated a large fraction of endothelin-

induced inhibition of Isc (Evans *et al.*, 2005).. COX-1 and COX-2 cDNAs have been cloned from rainbow trout (Onchorynchus mykiss) (Zou *et al.*, 1999), brook trout (Salvelinus fontinalis) (Roberts *et al.*, 2000) and zebrafish (Danio rerio) (Grosser *et al.*, 2002), and genome sequences predict the presence of both isoforms in puffer fish (Fugu rubripes), demonstrating that teleosts have both isoforms (Jarving *et al.*, 2004). constitutive branchial COX-2 expression is also enhanced in seawater, where prostaglandins can regulate NaCl secretion in chloride cells in gills of the euryhaline killifish (Fundulus heteroclitus)(Choe et al., 2006).Since is selective cox-2 inhibitor more pronounced effect is expected to be seen on osmoregulation in tilapia,but, for most studies, fish from domesticated stocks have been used, and such fish may have a blunted stress response when compared with wild-type strains of the same species (Woodward & Strange., 1987). Tilapia also can maintain internal osmotic balance without any change in drinking rate or branchial Na^+, K^+-ATPase activity when exposed to salinities up to 65 g/l for five days. At 65 g/l salinity or greater, there are changes in chloride cell turnover, drinking rate, and Na^+, K^+-ATPase activity; followed by increases in plasma osmolality and ion levels (Sardella et al., 2004).

The feces sample injected to HPLC system, showed no peak for Etoricoxib (see appendix). As a result all Etoricoxib is metabolized and the metabolites are excreted with no unchanged fraction of the drug. This is in contrast to humans,where minor fraction of the dose (<1%) is excreted unchanged (Rodrigues et al., 2003).In healthy male subjects, Etoricoxib is well absorbed, with an absolute oral bioavailability of 83%. and a low clearance with a t1/2 of 24.8h. In addition, Etoricoxib is metabolized extensively (more than 98%) via6-methyl hydroxylation (major) and1-N-oxidation. These metabolites are either excreted directly, or are metabolized further to secondary metabolites that are also excreted largely by urine(70%) and 20% by feces with less than 2% recovered unchanged as(Etoricoxib). 6-Carboxylic acid derivative of Etoricoxib is the major metabolite observed the oxidative metabolism is catalyzed by multiple P450s in the presence of NADPH-fortified human liver microsomes, with CYP3A4 playing an Important role (60%), and the remainder of the activity shared more or less equally among other P450s (e.g., CYP2C9, 2D6, 1A2, and 2C19) (Kassahun et al., 2001).Because of the low levels in plasma and weak COX-2 activity, the metabolites of Etoricoxib are unlikely to contribute to the inhibition of COX-2 in vivo (Rodrigues et al., 2003). Moreover, the involvement of multipleP450s, and the low first pass effect, as the hepatic extraction is calculated to be 0.04, with negligible gut first pass metabolism, effectively minimizes the potential for significant drug interactions with potent P450 inhibitors (Rodrigues

et al., 2003). We do not know if it's, low first pass effect, with high bioavailability and low hepatic extraction ratio, contributes to its long t1/2=28.6h, renders it available to systemic circulation and increase its toxicity towards fish. Unrecovered of unchanged drug from feces as shown in appendix, could indicate that etoricoxib was either extensively metabolized by liver or some of the drug have undergone enterohepatic circulation. While renal excretion is the most important route for final elimination of NSAIDs, nearly all undergo varying degrees of biliary excretion and reabsorption (enterohepatic circulation). In fact, the degree of lower gastrointestinal tract irritation correlates with the amount of enterohepatic circulation. Far, there is no report on etoricoxib and enterohepatic circulation (Brune et al., 2010). However, enterohepatic recirculation is unique to rofecoxib and has been reported in both rat and human studies, with resultant biphasic serum peaks of drug (Huntjens et al., 2008). On the other hand,Diclofenac undergo enterohepatic recirculation in brown trout and tend to form protein adducts which are responsible for kidney and liver pathology (Schwaiger et al.,2004).Prolonged availability of Diclofenac in the brown trout due to enterohepatic cycling promotes its accumulation, despite its basically low tendency for bioaccumulation as judged by its octanol/water partition coefficient.(Hoeger et al., 2008).The uptake and metabolism of anti-inflammatory drug diclofenac (DCF) was studied by exposing rainbow trout (*Oncorhynchus mykiss*) to DCF intraperitoneally, and via water at concentration of 1.7 μg L^{-1}. Unmetabolized DCF was detected in the bile with bioconcentration factors ($BCF_{total\text{-}bile}$ for DCF and its metabolites) which, varied between individuals ranging from 320 to 950(Kallio et al., 2010). This finding suggest that fish living downstream the wastewater treatment plants (WWTPs) and which are chronically exposed to the drug may accumulate the drug and its metabolites in bile.Although most of these pharmaceuticals undergo extensive dilution and degradation during sewage treatment, the potential effects of COX inhibitors on physiologic functions in fish will require attention, particularly as human longevity increases requiring greater use of anti-inflammatory drugs. Thus it should be investigated further for their ability to alter fish thyroidal status, and reproduction chronically.

Chapter Six

6.1 Conclusions

Based upon my work and the experiments done, The effect of COXs-2 inhibitors treatment on NileTilapia is maximally exerted during a critical period of sex differentiation, which occurs in tilapia fry between 2 and 6 weeks after spawning. After the end of this period there was no effect on the growth rate. Thus, COX-2 inhibitors could modulate aromatase activity needed for proper sexual development and reproduction during the crucial period. The highest growth rate was with (2% Etodolac) followed by (1% Etodolac), but optimum dose with lower mortality(3.4% and10%) was with (0.5%Etodolac),and (0.5%Etoricoxib) which show SGR(%) of 4.93 and 4.64 respectively.(1% Etoricoxib) showed a decreased growth rate compared to standard which could indicates a toxic potential of Etoricoxib toward fish at this concentration. (0.5%Etodolac),and (0.5%Etoricoxib) could be the optimum dose for improving growth rate with lower mortality. Etoricoxib have been metabolized in the fish body with no change in the movement, appetite for food or any other differences in compared with the fish treated with both drugs compared to control during the experimental period.Since Etodolac is non-selective COX-1 inhibitor with preferential COX-2 selectivity and Etoricoxib is selective COX-2 inhibitor,COX- 2 may be constantly expressed as COX-1 in fish,as in contrast to mammalian red blood cells,the nucleated erythrocytes of fishes posses a significant ability to synthesize prostaglandins,resulting in its availability in any well perfused organ. This study further highlights the potential endocrine-disrupting impact of environmental chemicals, which interfere with steroidogenesis and the P450arom system. Furthermore, this is the first time to our knowledge we test the effects of these agents on fry teleosts during the crucial period of sexual development which points to possible alterations in reproduction following chronic exposure to these drugs at an early stage in contaminated surface water. Subsequent field investigations in normal aquaculture bonds are needed to confirm these results in larger population, using different classes of COXs-inhibitors at different concentrations.

6.2 Recommendations:

- First of all the work in any part of aquaculture experiment need a lot of patience, accuracy, sincerely and commitment during every step in the experiments because you were dealing with living organisms.

- To achieve super-fast growth rate, and to get a marketable fish size the environment should be ideal for particular strain, water quality, salinity, climate and stock density.

- Further detail research in histopathology about the effect of NSAIDs on the internal organs should be done to know more about the effect of Etodolac and Etoricoxib drugs on cells physiology.

- The wastewater which was used during the experiment should be used for other efficient activities such as agricultural; the wastewater from fish aquaculture was very works as a fertilizer if we use suitable crops that tolerate the component of this type of water .

- Further studies should be done on the same subject by increasing the feeding allowance daily three times of both drugs.in two side by increase the dose to know the $\mathbf{LC_{50}}$, and decrease the dose to know the $\mathbf{EC_{50}}$.

- Other studies should be done by mixing many type of pharmaceutical together because the pollution inside aquatic environment contain mixed of different pharmaceutical product not only one type.

- ALQuds University wastewater treatment plant should provide other techniques and more interested about the method used to remove NSAIDs from wastewater.

References:

Afonso, L.O., Wassermann, G.J., De Oliveira, R.T., 2001. Sex reversal in Nile tilapia (Oreochromis niloticus) using a nonsteroidal aromatase inhibitor. J. Exp. Zool. 290, 177–181.

Aizen J., Kasuto H., Golan.M., Zakay H. and Levavi-Sivan. B., 2007. Tilapia Follicle-Stimulating Hormone FSH: Immunochemistry, Stimulation by Gonadotropin - Releasing Hormone, and Effect of Biologically Active Recombinant FSH on Steroid Secretion. Biology of Reproduction 76, 692–700.

Axelsson, M., Altimiras J. and Claireaux G., 2002. Post-prandial blood flow to the gastrointestinal tract is not compromised during hypoxia in the sea bass Dicentrarchus labrax. J Exp Biol 205, 2891-2896 .

Axelsson, M. and Fritsche, R., 1991. Effects of exercise, hypoxia and feeding on the gastrointestinal blood flow in the Atlantic cod Gadus morhua. J Exp Biol 158, 181-198.

Axelsson, M., Driedzic W. R., Farrell A. P. and Nilsson S., 1989. Regulation of cardiac output and gut blood flow in the searaven, Hemitripterus americanus. Fish Physiol. Biochem326-315 ,6 .

Baroiller, J.F., Guiguen, Y., Fostier, A., 1999. Endocrine and environmental aspects of sex differentiation in fish. Cell. Mol. Life Sci. 55, 910–931.

Baron, D., Houlgatte, R., Fostier, A., Guiguen, Y., 2005b. Large-scale temporal gene expression profiling during gonadal differentiation and early gametogenesis in rainbow trout. Biol. Reprod. 73, 959–966.

Baron, D., Montfort, J., Houlgatte, R., Fostier, A., Guiguen, Y., 2007. Androgen induced masculinization in rainbow trout results in a marked dysregulation of early gonadal gene expression profiles. BMC Genomics 8, 357.

Berendes, U.; Blaschke, G. Simultaneous determination of the phase II metabolites of the non steroidal anti-inflammatory drug etodolac in human urine. Enantiomer., 1996, 1, 415-22.

Boullata. Joseph. I. and Armenti Vincent T., 2004. Handbook of Drug–Nutrient Interactions. Adrianne Bendich, Series Editor.Humana Press.

Brautigam, L.; Nefflen, J.U.; Geisslinger, G. Determination of etoricoxib in human plasma by liquid chromatography–tandem mass spectrometry with electrospray ionisation. J. Chromatogr. B, 2003, 788, 309-15

Brown B. S., Adams A. B., Cyr, G. D., and Eales, G. J., 2004. Review: Contaminant effects on the teleost fish thyroid. Environmental Toxicology and Chemistry 23 7 :1680-1701. Published by Alliance Communication Group.

Brown J.A., Bucknall R.M., 1985. Antidiuretic and cardiovascular actions of prostaglandin E2 in the rain bow trout Salmogaird-neri. GenCompEndocrino l61:330–337.

Brueggemeier,R.W.,A.L.Quinn,M.L. Parret, 1999. Correlation of aromatase and cyclooxygenase gene expression in human breast cancer specimens. Cancer Lett. 140:27–35.

Brune K, Bertold R, and Burkhard H., 2010. Using pharmacokinetic principles to optimize pain therapy. *Nature Reviews Rheumatology* **6**, 589-598.

Buonocore, F., Forlenza, M., Randelli, E., Benedetti, S., Bossù, P., Meloni, S., Secombes, C. J., Mazzini, M. and Scapigliati, G. (2005). Biological activity of sea bass (Dicentrarchus labrax L.) recombinant interleukin-1beta. Mar. Biotechnol. (NY). 7, 609-617.

Butler A A, LeRoith D L., 2001. Control of growth by the somatotrophic axis: Growth hormone and the insulin-like growth factors have re-lated and independent roles. Ann Rev Physiol, 63: 141–164.

Carlson HE., Kaell AT., Schulman PE. 1999. Effects of several non steroidal anti inflammatory drugs on thyroid function tests. J Rheumatol 26:1855–1856,.

Chang, X.T., Kobayashi, T., Senthilkumaran, B., Kobayashi-Kajura, H., Sudhakumari, C.C., Nagahama, Y., 2005. Two types of distribution aromatase with different encoding genes, tissue and developmental expression in Nile tilapia (Oreochromis niloticus). Gen. Comp. Endocrinol. 141, 101–115.

Cheshenko, K., Pakdel, F., Segner, H., Kah, O., Eggen, R.I., 2008. Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. Gen. Comp. Endocrinol. 155, 31–62.

Choe, K. P., Havird, J., Rose, R., Hyndman, K., Piermarini, P. and Evans, D. H. (2006). COX2 in a euryhaline teleost, Fundulus heteroclitus: primary sequence, distribution, localization, and potential function in gills during salinity acclimation. J. Exp. Biol. 209, 1696-1708.

Daminet S., Croubels S., Duchateau L., 2003. Influence of acetylsalicylic acid and ketoprofen on canine thyroid function tests. Vet J 166,224-232.

Dey SK, Lim H, Das SK, Reese J, Paria BC, Daikoku T, Wang H. Molecular cues to implantation. *Endocr Rev* 2004; 25:341–73.

Diaz-Cruz E., S. Shapiro, C. L. Brueggemeter R. W., 2005, Cyclooxygenase inhibitors suppress aromatase expression and activity in breast cancer cells. J Clin Endocrinol Metab 90,2563-2570.

Donnelly AE, Maughan RJ, Whiting PH. 1990. Effects of ibuprofen on exercise-induced muscle soreness and indices of muscle damage. *British Journal of Sports Medicine.* 24, 3 : 191-5.

Dupont-Prinet A., Claireaux G. and McKenzie D. J., 2009. Effects of feeding and hypoxia on cardiac performance and gastrointestinal blood flow during critical speed swimming in the sea bass Dicentrarchus labrax. Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 154, 233-240.

Eales, J. G., Brown, S. B., Cyr, D. G., Adams, B. A., and Finnson, K. R. 1999. Deiodination as an index of chemical disruption of thyroid hormone homeostasis and thyroidal status in fish, Environmental Toxicology and Risk Assessment: Standardization of Biomarkers for Endocrine Disruption and Environmental Assessment: 8th,in ASTM STP 1364, D. S. Henshel, M. C. Black, and MC. Harrass eds. American Society for Testing and Materials, West Conshohocken, PA, 1999.

Eales, J. G., and Brown, S. B., 1993. Measurement and regulation of thyroidal status in teleost fish.Reviews in Fish Biology and Fisheries 3 4 299-347.

Elvin JA, Yan C, Matzuk MM. Growth differentiation factor-9 stimulates progesterone synthesis in granulosa cells via aprostaglandin E2/EP2 receptor pathway. *Proc Natl* Acad Sc i*USA* 2000;97:10288–93.

Evans, D. H., Piermarini, P. M. and Choe, K. P., 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol. Rev. 85, 97-177.

.

Fenske, M., Segner, H., 2004. Aromatase modulation alters gonadal differentiation in developing zebrafish (Danio rerio). Aquat. Toxicol. 67, 105–126.3527.

Ferguson D. C., Moore G. E., Hoenig M., 1999. Carprofen lowers total T4and TSH, but not free T4 concentrations in dogs abstract. J VetIntern Med 13:243.

Findlay JK, Drummond AE, Dyson ML, Baillie AJ, Robertson DM, Ethier JF. Recruitment and development of the follicle; the roles of the transforming growth factor-beta superfamily. Mol Cell Endocrinol 2002;191:35–43.

Flippin, J. L., Huggett, D., and Foran, C. M. (2007). Changes in the timing of reproduction following chronic exposure to ibuprofen in Japanese medaka, Oryzias latipes. Aquat. Toxicol. 81, 73-78.

Forman B. M., Chen J., Evans R. M., 1997. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator activated receptors alpha and delta. Proc Natl Acad Sci U S A 94:4312–4317.

Frenkian M., Segond N., Pidoux E.,Cohen R,, Julienne A., 2001. Indomethacin, acoxinhibitor, enhances 15-PGDH and decreases human tumoral C cell sproliferation. Prostaglandins Other Lipid Mediat 65:11–20.

Gallavan, R. H., Jr., Chou, C. C., Kvietys, P. R. and Sit, S. P., 1980. Regional blood flow during digestion in the conscious dog. Am J Physiol 238, H220-225.

Goetz, F. W., Hsu, S.-Y. and Selover, A. (1991). Stimulation of prostaglandin synthesis in fish follicles by a phorbol ester and calcium ionophore. J. Exp. Zool. 259, 355-364.

Gräns A., Albertsson F., Axelsson M. and Olsson C., 2009. Postprandial changes in enteric electrical activity and gut blood flow in rainbow trout Oncorhynchus mykiss acclimated to different temperatures. J Exp Biol 212, 2550-2557.

Grosser, T., Yusuff, S., Cheskis, E., Pack, M. A. and FitzGerald, G. A. (2002). Developmental expression of functional cyclooxygenases in zebrafish. Proc. Natl. Acad. Sci. USA 99, 8418-8423.

Guiguen, Y., Baroiller, J.F., Ricordel, M.J., Iseki, K., Mcmeel, O.M., Martin, S.A., Fostier, A., 1999. Involvement of estrogens in the process of sex differentiation in two fish

species: the rainbow trout (Oncorhynchus mykiss) and a tilapia (Oreochromis niloticus). Mol. Reprod. Dev. 54, 154–162.
Harris R. C. and Breyer M. D., 2001. Physiological regulation ofcyclooxygenase-2 in the kidney. Am. J. Physiol. 281, F1-F11.

Hinfray, N., Porcher, J.M., Brion, F., 2006. Inhibition of rainbow trout (Oncorhynchus mykiss) P450 aromatase activities in brain and ovarian microsomes by various environmental substances. Comp. Biochem. Physiol. C 144, 252–262.

Hoeger B., Dietrich D. R., Schmida. D, Hartmann. A, Hitzfeld, B.C., 2008. Distribution of in traperitoneally injected diclofenac in brown trout Salmotrutta f. fario.Ecotoxicology and Environmental Safety 71 4,412-418.

Hoeger B, Kollner B, Dietrich DR, Hitzfeld B. 2005. Water-borne diclofenac affects kidney and gill integrity and selected immune parameters in brown trout (Salmo trutta f. fario). Aquat Toxicol. 75(1):53-64. 24)

Huntjens DR H, Strougo A., Chain A., Metcalf A., Summerfield S., Spalding DJM, Danhofand D., DellaPasqua.O., 2008. Population pharmacokinetic modelling of the enterohepatic recirculation of diclofenac and rofecoxib in rats. British Journal of Pharmacology 153, 1072–1084.

Ijiri, S., Kaneko, H., Kobayashi, T., Wang, D.S., Sakai, F., Paul-Prasanth, B., Nakamura,M., Nagahama, Y., 2008. Sexual dimorphic expression of genes in gonads during early differentiation of a teleost fish, the Nile tilapia Oreochromis niloticus. Biol. Reprod. 78, 333–341.
Il'yasova. D, Colbert.L,Harris.B.T,Newman.B.A, Bauer.C.D,Satterfield.S and Kritchevsky.B.S. 2005. Circulating Levels of Inflammatory Markers and Cancer Risk In the Health Aging and Body Composition Cohort. CancerEpidemiolBiomarkersPrev;14 10 :2413–8.

Ishikawa, T. O., Griffin, K. J., Banerjee, U. and Herschman, H. R. (2007). The zebrafish genome contains two inducible, functional cyclooxygenase-2 genes. Biochem. Biophys. Res. Commun. 352, 181-187.
Ishikawa, T. O. and Herschman, H. R. (2007). Two inducible, functional cyclooxygenase-2 genes are present in the rainbow trout genome. J. Cell. Biochem. 102, 1486-1492.

Jackson A. J., Capper B. S., Matty A. J., 1982. Evaluationof some plant proteins in complete diets for the tilapia, Sarotherodon mossambicus. Aquaculture 27:97-109.

Janvier J. J., 1997. Cardio-vascular and ventilatory effects of pro-staglandin E2 in the Europeaneel Anguillaanguilla. J Comp Physiol B 167,517–526

Janz, D. M. 2000. Endocrine system. Chapter 25 In G. K. Ostrander ed. The Laboratory Fish Academic Press, San Diego, CA.

Jarving, R., Jarving, I., Kurg, R., Brash, A. R. and Samel, N. (2004). On the evolutionary origin of cyclooxygenase (COX) isozymes: characterization of marine invertebrate COX genes points to independent duplication events in vertebrate and invertebrate lineages. J. Biol. Chem. 279, 13624-13633.

Johannessen M., Delghandi M. P., Moens U., 2004. What turns CREB on? Cell Signal 16:1211–1227.

Jönsson E., Forsman A., Einarsdottir I. E., Egner, B., Ruohonen, K. and Bjornsson, B. T. 2006. Circulating levels of cholecystokinin and gastrin-releasing peptide in rainbow trout fed different diets. Gen Comp Endocrinol 148, 187-194.

Kallio J, Lahti.M,Oikari.A, and Kronberg.L. 2010. Metabolites of the Aquatic Pollutant Diclofenac in Fish Bile. *Environ. Sci. Technol.44 19, 7213–7219.*

KassahunK,McIntoshI,ShouM,WalshDJ,RodehefferC,SlaughterDE,GeerLA,HalpinRAAg rawalN,andRodriguesAD 2001 Role of human liver cytochrome P4503A in the metabolism of etoricoxib,a novel cyclooxygenase-2 selective inhibitor. Drug Metab Dispos29.813-820.

Kawauchi H., Suzuki K., Itoh H., Swanson P., Naito N., Nagahama Y., Nozaki M., Nakai Y., Itoh S., 1989 The duality of teleost gonadotropins. Fish Physiol Biochem 7:29-38.

Kitamikado, M. and Tachino, S. 1960a. Studies on the digestive enzymes of rainbow troue - I, Carbohydrates. Bull Jap Soc of Sci Fish 26, 679-684.

Kitamikado, M. and Tachino, S. 1960b. Studies on the digestive enzymes of rainbow troue - II, Proteases. Bull Jap Soc of Sci Fish 26, 685-690.

Kitamikado, M. and Tachino, S. 1960c. Studies on the digestive enzymes of rainbow troue - III, Esterases. Bull Jap Soc of Sci Fish 26, 691-694.

Kobayashi, T., Matsuda, M., Kajiura-Kobayashi, H., Suzuki, A., Saito, N., Nakamoto,M., Shibata, N., Nagahama, Y., 2004. Two DM domain genes, DMY and DMRT1, involved in testicular differentiation and development in the medaka, Oryzias latipes. Dev. Dyn. 231, 518–526.

Kulmacz, R. J., van der Donk, W. A., and Tsai, A. L. (2003). Comparison of the properties of prostaglandin H synthase-1 and -2. Prog. Lipid Res. 42, 377-404.

Kwon, J.Y., McAndrew, B.J., Penman, D.J., 2001. Cloning of brain aromatase gene and expression of brain and ovarian aromatase genes during sexual differentiation in genetic male and female Nile tilapia Oreochromis niloticus. Mol. Reprod. Dev. 59, 359–370.

Kwon, J.Y., McAndrew, B.J., Penman, D.J., 2002. Treatment with an aromatase inhibitor suppresses high-temperature feminization of genetic male (YY) Nile tilapia. J. Fish Biol. 60, 625–636.

LandsWem. 1991 Biosynthesis of prostaglandins. AnnuRevNutr11:41–60.

LangerP,Fo ¨ldesO,MichajlovskijN,Jezova ´D,Klimes ˇI,MichalkoJ,andZa ´vadaM. 1978. Short-term effect of acetylsalicylic acid analogue on pituitary-thyroid axis and plasma cortisol level in healthy human volunteers. Acta Endocrinol 88:698–702.

Larsen PR. 1972 : Salicylate-induced increases in free tri iodothyronine in human serum.

Li W S, Lin H R. The endocrine regulation network of growth hormone synthesis and secretion in fish: Emphasis on the signal integration insomatotropes. Sci China Life Sci, 2010, 53 4 : 462–470, doi: 10.1007/s11427-010-0084-6.

McAnulty SR, Owens JT, McAnulty LS, et al. 2007 Ibuprofen use during extreme exercise. *Medicine and Science in Sports and Exercise. ;39 7 :1075-9.*

MelamedP,EliahuN,OfirM,Levavi-SivanB,SmalJ,Rentier-DelrueF, YaronZ 1995 The effects of gonadal development and sex steroids on GH secretion in the male tilapia hybrid. Fish Physiol Biochem14:267–277.

Melamed,.P,Gur.G,Rosenfeld.H,Elizur.A,andYaron.Z. 1999. Possible Interactions between Gonadotrophs and Somatotrophs in the Pituitary of Tilapia :Apparent Roles for Insulin-Like Growth Factor I and Estradiol. Endocrinology 140: 1183–1191.

MelamedP,RosenfeldH,ElizurA,YaronZ 1998Endocrine regulation of gonadotropin and growth hormone gene transcription in fish. Comp Biochem Physiol C,119:325–338.

MelamedP,RosenfeldH,ElizurA,YaronZ 1997The mRNA levels of GtH Ib,GtH IIb and GH in relation to testicular development and testosterone treatment in pituitary cells of male tilapia.FishPhysiolBiochem17:93–95.

Metcalfe, C. D., Koenig, B. G., Bennie, D. T., Servos, M., Ternes, T. A., and Hirsch, R. (2003). Occurrence of neutral and acidic drugs in the effluents of Canadian sewage treatment plants. Environ. Toxicol. Chem. 22, 2872-2880.

Mingarro, M., Vega-Rubin de Celis, S., Astola, A., Pendon, C., Valdivia, M.M.,Perez-Sanchez, J., 2002. Endocrine mediators of seasonal growth in giltheadsea bream (Sparus aurata): the growth hormone and somatolactin paradigm.Gen. Comp. Endocrinol. 128, 102–111.

Molka,Vander GeytenS,KühnER &DarrasVM.1999.Effects of experimental hypo-and hyperthyroidism on iodothyronine deiodinases in Niletilapia,Oreochromis niloticus. Fish Physiology and Biochemistry 20 201–207.

Mu Y. M., Yanase T., Nishi Y., Takayanagi R., Goto K., Nawata H., 2001 Combined treatment with specific ligands for PPAR [gamma]: RXR nuclear receptor system markedly inhibits the expression of cytochrome P450arom in human granulose cancer cells. Mol Cell Endocrinol 181:239–248.

Mustafa T and SrivastavaK C. 1989.Prostaglandins eicosanoids and their role in ectothermic organisms. AdvCompEnvironPhysiol 5:157–207.

Ness TA, Torres SMF, Kramek EA, et al.2003.Effect of dosing and sampling time on serum thyroxine, free thyroxine, and thyrotropin concentrations in dogs following multidose etodolac administration. Vet Therap.;4:340-349.

Nieman DC, Henson DA, Dumke CL, et al. Nieman DC, Henson DA, Dumke CL, et al. 2006. Ibuprofen use, endotoxemia, inflammation, and plasma cytokines during ultramarathon competition. *Brain Behaviour and Immunity*.20 6 :578-84.

Orlando, E.F., Guillette, L.J., 2007. Sexual dimorphic responses in wildlife exposed to endocrine disrupting chemicals. Environ. Res. 104, 163–173.

OrozcoA,VillalobosP,JeziorskiMC&Valverde-RC. 2003.The liver of Fundulus heteroclitus expresses deiodinase type1 mRNA General and Comparative Endocrinology 130 84–91.

ParkJI,SemyonovJ,ChangCL,HsuSY. 2005.Conservation of the heterodimeric glycoprotein hormone subunit family proteins and the LGR signaling system from nematodes to humans.Endocrine26:267–276.

Patiño, R., Yoshizaki, G., Bolamba, D., and Thomas P. (2003). Role of arachidonic acid and protein kinase C during maturation-inducing hormone-dependent meiotic resumption and ovulation in ovarian follicles of Atlantic croaker. Biol. Reprod. 68, 516-523.
PawsonAJ,McNeillyAS. 2005.The pituitary effects of GnRH.Anim Reprod Sci;88:75–94.

Pickering,A.D. 1990. Stress and the suppression of somatic growth in teloest fish.In:progress in comparative Endocrinology,edited by A.Epple,C.G.Scanes,and M.H.Stetson.New York:Wiley-liss,P.473-479.

Pierce JG, Parsons TF. 1981.Glycoprotein hormones:structure and function. AnnRev Biochem;50:465–495.

Piferrer, F., Zanuy, S., Carrillo, M., Solar, I.I., Devlin, R.H., Donaldson, E.M., 1994. Brief treatment with an aromatase inhibitor during sex-differentiation causes chromosomally female salmon to develop as normal, functional males. J. Exp. Zool. 270, 255–262.

Piferrer, F., 2001. Endocrine sex control strategies for the feminization of teleost fish. Aquaculture 197, 229–281.

Pounds N., Maclean S., Webley M., Pascoe D., Hutchinson T., 2008 Acute and chronic effects of ibuprofen in the mollusc Planorbis carinatus Gastropoda: Planorbidae. Ecotoxicology and Environmental Safety 701:47-52.

Qutob M. and Nashashibi T., 2009. The effects of COX-Inhibitors Diclofenac and Ibuprofen on growth rate, mortality and sex reversal in Nile Tilapia Oreochromis niloticus. AACL Bioflux; 24:381-390.

Randall D. J., and S. F. Perry. Catecholamines. In:Fish Physiology, edited by W.S.Hoar, D.J.Randall and A. P. Farrell. San Diego,CA:Academic,1992,Vol.xii B,P.255-300.

Roberts, S. B., Langenau, D. M. and Goetz, F. W. (2000). Cloning and characterization of prostaglandin endoperoxide synthase-1 and -2 from the brook trout ovary. Mol. Cell. Endocrinol. 160, 89-97.

Rodrigues.A.D,Halpin.R,Geer.L,Cui.D,Woolf.E.J,Matthews.C,GottesDiener.K,Larson.P.J, Lasseter.K.C,andAgrawal.N.G.B.2003.Absorption,Metabolism,And Excretion Of Etoricoxib,A Potent And Selective Cyclooxygenase-Inhibitor,In Healthy MaleVolunteers. DMD31:224–232.

Rowell, C.B., Watts, S.A., Wibbels, T., Hines, G.A., Mair, G., 2002. Androgen and estrogen metabolism during sex differentiation in mono-sex populations of the Nile tilapia, Oreochromis niloticus. Gen. Comp. Endocrinol. 125, 151–162.

Rust,M.B.,NutritionalPhysiology,inFishNutrition,3rdEdition,Halver,J.E.and Hardy,R.W.,Eds.,Academic Press,Amsterdam,2002,pp.367-452.

SandersJP,VanderGeytenS,KapteinE,DarrasVM,KühnERLeonardJL&VisserTJ.1999.Clon ing and characterization of typeIII iodothyronine deiodinase from the fish Oreochromis niloticus Endocrinology 140 3666–3673.

SandersJP,VanderGeytenS,KapteinE,DarrasVM,KühnERLeonardJL&VisserTJ1997Chara cterization of a propylthiouracil-insensitive typeI iodothyronine deiodinase Endocrinology 138 5153–5160.

Sanota, L., Lucia, H., Araujoa,A.,Montenegroa,M.,2010. Journal of Hazardous Materials 175 (2010) 45–95

Sardella B, Matey V, Cooper J, Gonzalez RJ, Brauner CJ. 2004. Physiological, biochemical, and morphological indicators of osmoregulatory stress in California Mozambique tilapia Oreochromis mossambicus x O. urolepis hornorum exposed to hypersaline water. J. Exp. Biol. 207: 1399-413.

Schwaiger J, Ferling H, Mallow U, Wintermayr H, Negele RD. 2004. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac; Part I: histopathological alterations and bioaccumulation in rainbow trout. Aquat Toxicol. 68(2):141-50.

Seth H, Gräns A, Axelsson M. 2010.Cholecystokinin CCK as a potential regulator of cardiac function and postprandial gut blood flow in rainbowtrout Oncorhynchus mykiss. Am J Physiol Regul Integr Comp Physiol. 298, R1240-1248.

Seth H, Sandblom E, Axelsson M. 2009.Nutrient-induced gastrointestinal hyperemia and specific dynamic action SDA in rainbow trout Oncorhynchus mykiss- Importance of proteins and lipids. Am J Physiol Regul Integr Comp Physiol. 296, R345-352.

Shinomiya, A., Hamaguchi, S., Shibata, N., 2001. Sexual differentiation of germ cell deficient gonads in the medaka, Oryzias latipes. J. Exp. Zool. 290, 402–410.
Shiraishi, E., Yoshinaga, N., Miura, T., Yokoi, H., Wakamatsu, Y., Abe, S.I., Kitano, T., 2007. Mullerian inhibiting substance is required for germ cell proliferation during early gonadal differentiation in medaka (Oryzias latipes). Endocrinology 149, 1813–1819.
SmithWL1992Prostanoid biosynthesis and mechanisms of ac-tion.AmJPhysiol263:F181–F191.

Sorbera, L. A., Asturiano, J. F., Carrillo, M., and Zanuy, S. (2001). Effects of polyunsaturated fatty acids and prostaglandins on oocyte maturation in a marine teleost, the European sea bass (Dicentrarchus labrax). Biol. Reprod. 64, 382-389.
Stensløkken K.-O., Sundin L., Nilsson G.E. 2002. Cardiovascular effects of prostaglandinF2a and prostaglandin E2 in Atlantic cod Gadusmorhua. JComp Physiol B 172:363–369.

Sumida C., 1995 Fatty acids: ancestral ligands and modern co-regulators of the steroid hormone receptor cell signalling pathway. Prostag Leukotr Ess 52:137-144.

Sun, L.W., Zha, J.M., Spear, P.A., Wang, Z.J., 2007. Tamoxifen effects on the early life stages and reproduction of Japanese medaka (Oryzias latipes). Environ. Toxicol. Phar. 24, 23–29.
SundinL,NilssonGE 1998 Acute defence mechanisms against haemorrhage from mechanical gill injury in rain bow trout.AmJPhysiol275:R460–R465.

Tanaka, M., Saito, D., Morinaga, C., Kurokawa, H., 2008. Cross talk between germ cells and gonadal somatic cells is critical for sex differentiation of the gonads in the teleost fish, medaka (Oryzias latipes). Dev. Growth Differ. 50, 273–278.
Uchida, D., Yamashita, M., Kitano, T., Iguchi, T., 2002. Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish. J. Exp. Biol. 205, 711–718.
Van Anholt RD, Spanings T, Koven W, Wendelaar Bonga SE. 2003. Effects of acetylsalicylic acid treatment on thyroid hormones, prolactins, and the stress response of tilapia (Oreochromis mossambicus). Am J Physiol Regul Integr Comp Physiol. 285(5):R1098-106.

VanderGeytenS,ToguyeniA,BaroillerJF,FauconneauB,FostierA,SandersJP,VisserTJ,Kühn ER&DarrasVM2001Hypothyroidism induces typeI iodothyronine deiodinase expression in tilapia liver. GeneralandComparativeEndocrinology 124 333-342.

Webb SF. 2001. A data based perspective on the environmental risk assessment of human pharmaceuticals II –aquatic risk characterization. In. Kummerer, K. (Ed.), Pharmaceuticals in the environment. Sources, fate, effects and risks. Springer-Verlag Berlin, Heidelberg, New York, pp.203-219.

Wood, C. M. and Laurent, P. (2003). Na+ versus Cl- transport in the intact killifish after rapid salinity transfer. Biochim. Biophys. Acta. 1618, 106-119.

WoodWard,C.C.,And R. J. Strange. 1987. Physiological stress re-sponses in wild and hatchery-reared rainbow trout. Trans. Am.Fish. Sot. 116: 574-579.

Yamamoto, T., 1969. Sex differentiation. In: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology, vol. 3. Academic Press, London, pp. 117–175.

Yang, T. (2003). Regulation of cyclooxygenase-2 in renal medulla. Acta Physiol. Scand. 177, 417-421.

Yang, T., Forrest, S. J., Stine, N., Endo, Y., Pasumarthy, A., Castrop, H., Aller, S., Forrest, J. N., Jr, Schnermann, J. and Briggs, J. (2002). Cyclooxygenase cloning in dogfish shark, Squalus acanthias, and its role in rectal gland Cl secretion. Am. J. Physiol. 283, R631-R637.

Yao, H.H., Matzuk, M.M., Jorgez, C.J., Menke, D.B., Page, D.C., Swain, A., Capel, B., 2004. Follistatin operates downstream of Wnt4 in mammalian ovary organogenesis. Dev. Dyn. 230, 210–215.

Yaron Z, Gu rG, Melamed P, Rosenfeld H, Elizur A, Levavi-Sivan B., 2003. Regulation of fish gonadotropins. Int Rev Cytol;225:131–185.

Yaron Z, Gu r G, Melamed P Rosenfeld H, Levavi-Sivan B, ElizurA. 2001.Regulation of gonadotropin subunit genes in tilapia.Comp Biochem Physiol B BiochemMolBiol;129:489–502.

Yeoh, C.G., Schreck, C.B., Feist, G.W., Fitzpatrick, M.S., 1996a. Endogenous steroid metabolism is indicated by fluctuations of endogenous steroid and steroid glucuronide levels in early development of the steelhead trout (Oncorhynchus mykiss). Gen. Comp. Endocrinol. 103, 107–114.

Zhao, J., Mak, P., Tchoudakova, A., Callard, G., Chen, S., 2001. Different catalytic properties and inhibitor responses of the goldfish brain and ovary aromatase isozymes. Gen. Comp. Endocrinol. 123, 180–191.

Zhou H, Ko W K, Stojilkovic S S.,. 2004. Novel aspects of growth hormone GH autoregulation: GH-induced GH gene expression in grass carp pituitary cells through autocrine/paracrine mechanisms.Endocrinology, 145: 4615–4628.

Zou, J., Neumann, N. F., Holland, J. W., Belosevic, M., Cunningham, C., Secombes, C. J. and Rowley, A. F. (1999). Fish macrophages express a cyclo-oxygenase-2 homologue after activation. Biochem. J. 340, 153-159.

Internet References

WWW.DrugBank.Com

WWW.Americulture.com

Appendix

Experiment number (4): Niletilapia feces analysis for etoricoxib

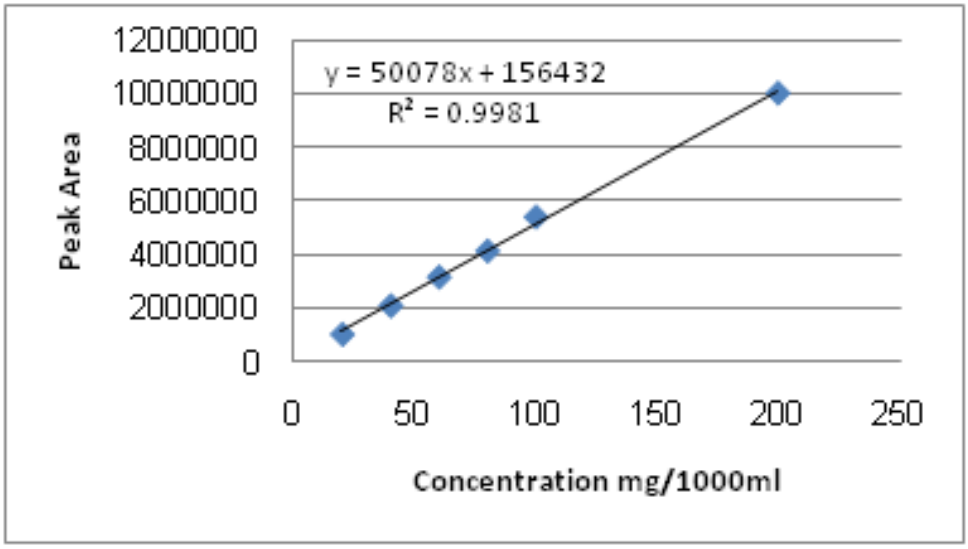
4 months age niletilapia were separated in one aquarium from the recirculating system and given diet with 1% etoricoxib and after half an hour collect its feces, and collect a second sample after 1 hour then after 2hour, after 3 hour, after 4 houres ,24 houres each sample with triplicate and then the analysis done by using HPLC device in the analysis center lab in ALQuds University.

Determination of Etoricoxib in fish feces by HPLC:

A simple reverse phase HPLC method has developed for estimation of Etoricoxib in fish feces.the method was carried out on a Water X bridge 5 Mm, C8 column with a mobile phase consisting of methanol: Acetonitrile: phosphate buffer 3.5(40: 20: 40 V\ V) at flow rate of 1ml/min, detection was carried out at 210nm and the retention time of Etoricoxib was 4.18 min.

Calibration Curve:

Linearity of a system was investigated by serially diluting the stock solution to give concentration in the range 20-100 mg/ 1000ml(ppm). an aliquot (10 ml) was injected using a mixture methanol: acetonitrile: phosphate buffer pH 3.5 (40:20:40) V/V as mobile phase.Calibration curve were obtained by plotting the peak area versus concentration the calibration curve is as shown



Treatment & preparation of feces sample to determined the presence of Etoricoxib

Four sample of fish feces taken in each 2 hours after feeding of the fish, each sample obtained by three –collection, and then each sample that is come from collection of three time homogenate and mixed at room temperature then 5ml were transferred to glass centrifuge tube &then 15ml added from phosphoric acid to acidify the sample to approximately PH=3 &then 10ml of acetonitrile / methanol (2:1) and after centrifuge the supernate were transferred to clean tube.and the sample transferred to vial and injected.

HPLC RESULTS

HPLC Peaks for the feces injected sample in Nile Tilapia Results show that : there was no any peak for Etoricoxib in the sample injected to HPLC system.

so as a result all etoricoxib is metabolized with no unchanged fraction excreted.

الملخص

كثير من الدراسات أجريت للتنبؤ بالتأثير المباشر على تثبيط عمل ونشاط إنزيم الأروماتيزaromatase enzyme وهو الإنزيم المحدد لمعدل تغيير هرمون الأندروجين وتحويله إلى أستروجين وهو الهرمون الضروري لتمايز خلايا المبيض في الأسماك. وكونها أقل ضرراً على البيئة مقارنة بمادة الميثيل تستوستيرون التي تستخدم حالياً لتغيير جنس الأسماك وهي مادة ضارة جدأبالبيئة. مثبطات الإنزيم الإختيارية لCOX-2هي أدوية فعالة وغير منعكسة باتجاه COX-2Pathway.

كثير من الدراسات التي أجريت على خلايا الثدي السرطانية أظهرت نتائجها أن استخدام هذه الأدويةعلى الخلايا السرطانية يؤدي الى تقليل معدل النسخ للحمض النووي الرايبوزي الناقل mRNA لهذا الإنزيم.

في تجربتنا هذه التي أجريت، اختبرنا تأثير تزويد صغار الأسماك بغذاء يحتوي على هذه الأدوية الاختيارية المثبطة لعمل إنزيم COX-2 وهي مادتي (Etodolac و Etoricoxib) خلال المرحلة الأولى من حياة الأسماك لندرس تأثيرها على معدل النمو والوفاة. من ناحية أخرى يزداد الكشف عن هذه الأدوية البشرية والحيوانية بكميات كبيرة في المياه الصادرة من محطات المعالجة والمياه السطحية وبالذات تشكل الأدوية غير الستيرويدية المضادة للإلتهاب واحدة من معظم الأدوية والمركبات الشائعة التي توجد في المياه .

المعلومات التي تهتم بخطر هذه الأدوية وسميتها على البيئة نادرة وقليلة جداً، ولا توجد معلومات متوفرة عن تأثير هذه الأدوية على الأسماك بعد فترة طويلة من التعرض لها.

تسليط الضوء على مادة (Etoricoxib) الدوائية تم عن طريق تحديد وجود مادةEtoricoxib في فضلات الأسماك عن طريق التحليل بواسطة جهاز HPLC .

أجريت هذه الدراسة في مختبر الزراعة السمكية للأبحاث في جامعة القدس، حيث قمت بتوزيع الأسماك الصغيرة (عمرها 8 أيام بعد الفقس)على الأحواض التي تم تجهيزها مسبقاً لاحتضان هذه الأسماك.

تم وضع 30 سمكة خليط (ذكور وإناث) من نوع (oreochromis.niloticus) في كل حوض الذي تبلغ سعته تقريباً 45 لتراً من الماء في نظام مغلق(Recirculating System) . المعالجة تمت باستخدام تراكيز مختلفة مع الغذاء .وهي كما يلي تركيز 0.5% من مادة Etoricoxib+

الغذاء،0.5% من Etodolac +الغذاء، 1% من مـادة Etoricoxib+الغـذاء، 1%مـن مـادة Etodolac+الغذاء،2% Etodolac+الغذاء كل واحدة من هذه التراكيز يتم تطبيقها في حوضين بالإضافة إلى حوضين إضافيين يتم إعطاء الأسماك غذاء عادي بدون وجود أدويـة حتـى تكـون مرجع للتجربة . تم تحضير هذه التراكيز عن طريق خلط 600مغم من Etodolac مع 120 غـم من الغذاء العادي ، 60غم ، 30غم من غذاء الأسماك لتحقيق التراكيز 0.5%، 1%، 2% علـى التوالي . والعملية نفسها تم عملها لمادة Etoricoxibعن طريق خلط 90 مغم ل Etoricoxibمع 18غم ، 9غم من غذاء الأسماك لتحقيق تراكيز 0.5% ، 1% على التوالي .

في تجربة أخرى :- تم وضع 10 أسماك خليط (ذكور وإناث) في عمر أكثر من شهرين في نظام مغلق وكل نوع يتم تطبيقه في ثلاثة أحواض وتم معاملتها كما في التجربة السابقة لتركيز 0.5% Etoricoxibو0.5 % Etodolac.

التغذية والمعالجة بدأت في نفس اليوم الذي وضعت فيه الأسماك في هذا النظـام ، ويـتم إطعـام الأسماك مرة واحدة يومياً .كمية الغذاء التي كانت تعطى تمثل 10% من وزن السمك الكلي في كل حوض .خلال فترة المعالجة وعندما يتم إيقاف المعالجة بالأدوية، يصـبح وزن الغـذاء المعطـى 5%من وزن السمك الكلي في الحوض .

تم توزين الأسماك في كل أسبوع وتم عد الأسماك في كـل حـوض لمعرفـة وحسـاب معـدل Survival rate خلال أسابيع التجربة .تم توزين الأسماك في كل حوض لأقرب0.1غم باستخدام ال digital scale .

معدل النمو تم حسابه عن طريق معادلة ميل الخط المستقيم Yt=a+bXt

Yt:تمثل معدل الوزن الكلي

t:تمثل الوقت

a:تمثل معدل الوزن للأسماك عند بدء التجربة .

أظهرت النتائج أن الاختلاف في معدل النمو (الوزن النهائي للجسم ، الوزن المكتسـب ،ومعـدل النمو) لسمك o.niloticus تم تغذيته بغذاء مخلوط مع الأدوية المثبطة لعمـل إنـزيم COX-2 لمادتي (Etoricoxib،Etodolac) .كان أعلى معدل للنمو حسب التجربة التي أجريت في تركيـز Etodolac%0.5 يليه تركيز Etoricoxib %0.5 يليه الأسماك في الأحواض العادية التي تتغذى بغذاء عادي . هذه النتائج تتعلق بالتجربة التي بدأنا بإعطاء الأدوية مع الغذاء مـن عمـر 8 أيـام

للأسماك. أما التجربة الثانية التي كان عمر الأسماك فيها أكثر من شهرين فلم تظهـر النتـائج أي زيادة أو نقصان في معدل النمو.

معدل الزيادة في التجربة الأولى كـان أعلـى مـا يمكـن فـي تركيـز 2% Etodolac يليهـا 1% Etodolac ولكن الحوض بتركيز 1% Etoricoxib أظهر نقصان في معدل النمو بالمقارنـة مع الأحواض العادية والتي يمكن أن تظهر أن هذا التركيز ممكن أن يكون سام بالنسبة للأسـماك .بالإضافة إلى ذلك لم يتم الكشف عن وجود مادة Etoricoxib في فضلات الأسـماك التـي تـم أخذها بعد نصف ساعة ،ساعة ،3 ساعات ،4ساعات وبعد 24 ساعة من سمكة عمرهـا 4أشـهر وتحليل هذه العينات بواسطة جهاز HPLC، وهذه النتـائج تظهـر أن مـادة Etoricoxib تـم إمتصاصها من قبل الأسماك ولم يتم العثور على أي جزء من مادة Etoricoxib لم يتم الكشـف عن وجوده مما يعني أن الجسم قد قام بامتصاصه وتحويله إلى مركبات أخرى داخل الجسم ومـن الممكن أنه دخل الدورة الدموية للكبد وهذا يؤدي إلى زيادة سميته للجسم و في الأسماك الكبيرة لـم يكن هناك أي حالة وفاة خلال فترة التجربة ، أما في الأسماك الصغيرة لم يكن هناك اختلاف فـي معدل الوفاة بين 0.5%Etodolacو 0.5%Etoricoxib ولكن معدل البقاء على قيد الحيـاة كـان 96.6% و90% وهو أحسن بالمقارنة مع الأحواض العادية (دون معالجة) التـي كانـت نسـبتها 86.6%-83.3% خلال فترة التجربة .معدل وفاة عالي ظهر مع زيادة التراكيز 1%Etoricoxib و 1% Etodolacو2%Etodolac .

هذه النتائج تظهر أن إضافة الأدوية المثبطة لإنزيم COX-2 في غـذاء الأسـماك مفيـدة لنمـو الأسماك وهذا التأثير يظهر واضحاً خلال الفترة الحرجة في فترة التمايز الجنسي للأسماك وهـذه الفترة تكون ما بين 2اسبوع-6 أسابيع ما بعد الفقس وبعد إنتهاء هذه الفترة لا يوجد أي تأثير لهـذه الأدوية على معدل النمو وهذا يعني أن استخدام الأدوية المثبطة لإنزيم COX-2خلال هذه الفتـرة الحرجة من الممكن أن يغير من نشاط إنزيم ال aromatase الضروري للتطور الجنسي المناسب والتكاثر خلال هذه الفترة الحرجة(الحساسة) بالنسبة للتطور الجنسي في الأسماك ، وهـذه المـرة الأولى بالنسبة لمعرفتنا التي يتم فيها اختبار تأثير هذه المواد على صغار الأسماك خـلال الفتـرة الحساسة من التطور الجنسي والتي تشير إلى احتمالية تغيير التكاثر بعد التعـرض الطويـل لهـذه الأدوية في المرحلة المبكرة من حياتها في المياه السطحية الملوثة . وحتى نثبت ذلك فعليـاً نحـن بحاجة إلى سلسلة متواصلة من البحث في البرك الزراعية لتأكيد هذه النتائج على مجتمعات أكبـر وأوسع وباستخدام أنواع مختلفة من مثبطات COX-2وبتراكيز مختلفة.