AACL BIOFLUX
Aquaculture, Aquarium, Conservation & Legislation
International Journal of the Bioflux Society

The effects of COX2-inhibitors (etoricoxib and etodolac) on growth rate and mortality in Nile tilapia (*Oreochromis niloticus*)

[1,2]Mutaz A. Al-Qutob, [2]Iman Al-Hirsh, [2]Tharwat S. Nashashibi

[1] Department of Earth and Environmental Studies, Faculty of Science and Technology, Al Quds University, East Jerusalem, Palestinian Authority. [2] Aquatic and Aquaculture Research Laboratory, Al-Quds University, East Jerusalem, Palestinian Authority.
Corresponding author: M. A. Al-Qutob, qutob@planet.edu

Abstract. The non-steroidal anti inflammatory drugs represent one of the most commonly detected compounds in sewage treatment plant (STP) effluent and surface water with scarce information concerning possible ecotoxicological risks. As in mammals, COX has been shown to play a role in reproduction in fish. Since studies on human breast cancer cells showed that COXs-inhibitors decreased aromatase messenger ribonucleic acid (mRNA) expression at the transcriptional level we tested the effects of supplementation of COX2-inhibitors (etodolac and etoricoxib) in the diet of fry tilapia on growth rate and mortality during the crucial period of sexual differentiation. Highlight on etoricoxib pharmacokinetics was carried out by determination of etoricoxib in fish feces using reversed-phase High Performance Liquid Chromatography (RF-HPLC) with Evaporative Light Scattering and Photo Diode array detector (ELSD-PDA system). At an age of 8 days post-hatched, 30 genetically mixed population of *Oreochromis niloticus* larvae were stocked in duplicate, into 45 L aquariums in a closed system for six months. Treatments included 5 different experimental diets including, respectively, 0.5% etodolac, 1% etodolac, 2% etodolac, 0.5% etoricoxib, and 1% etoricoxib concentrations and one standard diet serving as control with two repeats for each group from 0.5% groups of diets. Fish were fed experimental diets for 8 weeks and were changed to control diet after. Another experiment was conducted using 10 adults mixed population stocked in triplicate in a closed system and treated as above. Growth rates (GR) were significantly affected with the highest growth rate obtained with the 0.5% etodolac. However, no increase or decrease in growth was observed in mixed adults population. GR increased with increasing concentrations with the highest GR in the aquarium treated with 2% etodolac, followed by 1% etodolac, but 1% etoricoxib showed a decreased GR compared to standard which could indicates a toxic potential toward fish at this concentration. No etoricoxib peak was detected on HPLC in feces samples which reflected, that, it was well absorbed by tilapias, extensively metabolized with no unchanged fraction excreted, or may undergo enter hepatic circulation, increasing further its toxic potential. No mortality was observed in adults mixed population. Mortality rates were 3.7% with 0.5% etodolac, 10% with 0.5% etoricoxib, 30% with 1% etodolac, 37%with 1%etoricoxib, and 50% with 2% etodolac treatments, respectively. It is clear evident that even administration of high concentrations of these drugs was well tolerated by fish.
Key Words: Etodolac, etoricoxib, COX2-inhibitors, growth rates, mortality.

Resumen. Los antiinflamatorios no esteroideos representan uno de los más comúnmente detectados compuestos en agua residual y agua superficial con escasa información relativa a los posibles riesgos ecotoxicológicos. Como en los mamíferos, Ciclooxigenasa COX es importante en la reproducción de los peces. Ya que los estudios en las células cancerosas humanas de mama demuestran que Ciclooxigenasa, inhibidores de la (COX) disminuyen la expresión del ácido ribonucleico ARN mensajero del aromatasa (ARNm) a nivel transcripcional, hemos probado los efectos de la suplementación de COX2-inhibidores (etodolaco y etoricoxib)en la dieta de la Tilapia sobre tasa de crecimiento, mortalidad durante el período crucial de diferenciación sexual. Determinación de etoricoxib en las heces de los peces utilizando fase-invertido deCromatografía líquida de alto rendimiento (RF-HPLC) con ELSD-PD Adetector, fue llevada a cabo. A la edad de 8 dias 30 Mezcla genética de *Oreochromis niloticus* larvas fueron sembradas por duplicado en 45 L acuarios en un sistema cerrado durante 6 meses. Los tratamientos incluian 5 diferentes dietas experimentales incluyendo 0,5% etodolac, 1% etodolaco, 2% etodolac, 0,5% etoricoxib, 1% etoricoxib concentraciones y una dieta estándar que actúa como control con dos repeticiones para cada grupo de 0,5% grupos de dietas. Peces se alimentaron con dietas experimentales durante 8 semanas y cambiaron a la dieta control después. Otro experimento se llevó a cabo utilizando 10 mezcla de adultos sembradas por triplicado en un sistema cerrado y, tratadas como en el caso anterior. Tasas de crecimiento (GR) fueron significativamente afectadas con la mayor GR obtenida con el

0,5% etodolaco. Sin embargo, ningún aumento o disminución del crecimiento en población mixta adulta fue observada. GR aumentó con las crecientes concentraciones con la mayor GR en los acuarios tratados con 2% etodolaco seguida de 1% etodolaco, pero 1% etoricoxib mostró una tasa de crecimiento disminuyó en comparación con el estándar que indica un potencial tóxico para los peces en esta concentración. Ningún pico de etoricoxib fue detectada por HPLC en muestras de heces fecales reflejando, que, se absorbe bien por el tilapia, extensamente metabolizado con ninguna fracción excretada inalterada, o puede someterse a entrar circulación hepática, aumentando aún más su potencial tóxico. No se observó mortalidad en población mixta de adultos. Las tasas de mortalidad fueron de 3,7% con 0,5% etodolaco, 10% con 0,5% etoricoxib, 30% con 1% etodolaco, 37% con 1% etoricoxib, y 50% con 2% etodolaco tratamientos, respectivamente. Es evidente que administración de altas concentraciones fue bien tolerado por los peces.
Palabras Clave: Etodolaco, etoricoxib, COX2-inhibidores, tasa de crecimiento, mortalidad.

Introduction. Prostaglandins are biologically active derivatives of arachidonic acid and other polyunsaturated fatty acids that are released from membrane phospholipids by phospholipase A2. Prostaglandin G/H endoperoxide synthase, also known as cyclooxygenase is a key enzyme that catalyzes the conversion of arachidonic acid (ArA) to prostaglandins. When ArA is released from cell membranes by phospholipases mainly PLA2, it can be metabolized by prostaglandin G/H synthase. Prostaglandin G/H synthase is a membrane bound protein in the endoplasmatic reticulum of prostaglandin forming cells. The cyclooxygenase (COX) component catalyses the oxidation of the fatty acid arachidonic acid (ArA, 20:4n-6) to the intermediate prostaglandin G2 (PGG2), which is rapidly converted into PGs (PGE2, PGD2, PGF2) and thromboxanes (Simmons et al 2004). Prostaglandins play a physiological significance and role in intracellular signaling in mammals (Cha et al 2005). In teleosts, they have been reported to be produced in the ovaries (Goetz et al 1991). Non steroidal anti-inflammatory drugs (NSAIDs), specifically COX-2 inhibitors, are known to inhibit ovulation in mammals (Gaytan et al 2006). As in mammals, COX has been shown to play a role in reproduction in fish. In the Atlantic croaker *Micropogonias undulates* (Linnaeus, 1766) COX pathways may play a role in the maturation of ovarian follicles and ovulation through prostaglandin formation (Patino et al 2003). Results from the European sea bass *Dicentrarchus labrax* (Linnaeus, 1758) also indicate a similar role for prostaglandins in ovulation, with indomethacin inhibiting follicle maturation (Sorbera et al 2001). In the Japanese medaka *Oryzias latipes* (Temminck & Schlegel, 1846), exposure to low chronic levels of ibuprofen causes altered reproduction by decreasing the number of spawning events but increasing the number of eggs per spawning event (Flippin et al 2007). In an earlier study using non-selective COX-inhibitors ibuprofen and diclofenac we showed no significant differences in growth rate (GR) of 20 genetically females (XX) of *Oreochromis niloticus* (Linnaeus, 1758) fry following treatment with 1% diclofenac, 5% diclofenac, and 5% ibuprofen, but 7% in the control group, 36% in the 1% diclofenac group, 17% in the 0.5% ibuprofen group, and 22.2% in the 0.5% diclofenac group, respectively, never produced egg during the entire experimental period (Al-Qutob & Nashashibi 2009).

The main goal of our study is to predict the effect of etodolac, a non-selective COX-1 inhibitor with preferential COX-2 selectivity, and etoricoxib, a selective COX-2 inhibitor on growth during the crucial period of sexual differentiation. It is well known that there are differences in male vs female growth rates in *Oreochromis* species (Palada-de Vera & Eknath 1993; Toguyeni et al 1997). Monosex production or even incomplete female prevents reproduction and therefore feed energy could be diverted into growth instead of production of unwanted juvenile fish (Kwon et al 2001). Brueggemeier et al (1999) pointed to linear dependence between aromatase gene (CYP 19) expression and expression of COX-2 in mammary carcinoma. 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; Mocuţa et al 2010), we tested the hypothesis of possible cyclooxygenase inhibition during the crucial period that may modulate aromatase activity, thus altering sexual differentiation.

On the other hand, human and veterinary pharmaceuticals have been shown to occur in considerably high amounts in sewage treatment plant (STP) effluent and surface

water, with the non-steroidal anti inflammatory drugs representing one of the most commonly detected compounds (Metcalfe et al 2003). 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 samples using reversed-phase High Performance Liquid Chromatography (RF-HPLC) with Evaporative Light Scattering and Photo Diode array detector (HPLC-ELSD-PDA system).

Material and Method. The study was carried out at the Aquaculture Research Laboratory, in AL-Quds University, Jerusalem from 15th of November 2010 till 15th of May 2011. 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/min. All the aquaria were kept on 80 cm high platform to facilitate better observation and easy maintenance. Water temperature was maintained at 28 ± 1ºC. 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 produced through the indirect approach of producing mixed sex fish (Piferrer 2001).

Commercial feed was used as the control diet. Feed composition is shown in Table 1. Etodolac and etoricoxib were purchased from Sigma and Alderich products.

Table 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 *O. niloticus* larvae were stocked in duplicate, into aquariums each with a capacity of ≈ 45 L at aquatic and aquaculture lab, where the study was conducted. 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. All aquariums in the experiment were disconnected from the recirculation system in order to avoid cross- contamination. The test diets were prepared by mixing 600mg of etodolac with 120 g, 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 90mg of etoricoxib with 18 g, 9 g commercial feed, also to achieve 0.5% etoricoxib, 1% etoricoxib concentrations, 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. They were fed 10% of their weight during the 8th week of the experiment with etodolac and etoricoxib, after this period they were fed 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 8 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. Growth performance and diet nutrient utilization were analyzed in term of percent body weight gain (BWG), growth rate, feed conversion ratio (FCR), feed conversion efficiency (FCE), specific growth rate (SGR). 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.

Experiments done for fish growth rate

Experiment number (1): Mixed population of adults Nile tilapia treated with etodolac 0.5% and etoricoxib 0.5% at age more than 2 month: nine aquariums were chosen for the experiment from the recirculating system after being separated to prevent cross contamination. They were divided into three different groups and distributed randomly in all side of the system to achieve the same condition for each group. These groups included three for standard population without treatment, three with etodolac 0.5% treatment, and three for etoricoxib 0.5% treatment. Ten fish were stocked in each aquarium, the diet was given to fish in each aquarium once daily as 10% of the fish weight. Fish were weighted in each aquarium weekly and counted in each aquarium to determine survival rate during 8 weeks period. The 10% amount of diet given during the new week was calculated.
Experiment number (2): Mixed population of Nile tilapia treated with etodolac 0.5% and etoricoxib 0.5% at age of 8 days: Thirty fry fish were stocked in each aquarium, at age of 8 days, with duplicate aquariums for each group of etodolac 0.5%, etoricoxib 0.5%, and standard. The same procedure was done as in experiment one.
Experiment number (3): mixed population of Nile tilapia treated with etodolac 1%, etoricoxib 1% and etodolac 2% at age of 8 days: Thirty fish at age of 8 days post hatch were stocked in three aquariums in duplicate, one for each group of etodolac 1%, etoricoxib 1%, and standard. Only 20 fish were stocked in 2% etodolac duplicate aquarium, and they were all treated in the same way as the previous experiments (1 and 2).

Determination of etoricoxib in fish feces by HPLC. Four months age Nile tilapia were separated in one aquarium from the recirculating system and given diet with 1% etoricoxib. Feces samples each with triplicate were collected after half an hour, 1 hour, 2 hours, 3 hours, 4 hours, and 24 hours, respectively. Etoricoxib determination was carried out using reversed-phase HPLC with 2424 Evaporative Light Scattering and 996 Photo Diode Array detector (HPLC–ELSD-PDA, Water Alliance HPLC system) in the analysis center lab in Al Quds University. A simple reverse phase HPLC method was 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. Linearity 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 was obtained by plotting the peak area versus concentration.

Results and Discussion

Growth performance parameters. The growth performance parameters (BWG, FCR, FCE, SGR, and GR) of *O. niloticus* fed with etodolac (non-selective COX-1 inhibitor with preferential COX-2 selectivity) and etoricoxib (selective COX-2 inhibitor), respectively, were significantly affected by COX-2 inhibitors administration with Nile tilapia diet, with the highest growth obtained with the 0.5% etodolac, followed by etoricoxib 0.5%, followed by untreated sample (Table 2, Figure 1). No significant differences were found in the average weights and growth rates ($p>0.05$), in mixed population adult Nile tilapia. In

humans, NSAIDs are reported to cause a gradual weight gain attributed to the effect of medication itself or due to its effect on other factors (Boullata & Armenti 2004). Our discussion will be based on three points of view, the aromatase theory, the interfering with thyroid axis and the modulated gastrointestinal blood flow (GBF).

Table 2
Body weight gain and other important growth parameters in mixed population Nile tilapia treated with etodolac 0.5% and etoricoxib 0.5% at age of 8 days

	BWG (g)	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

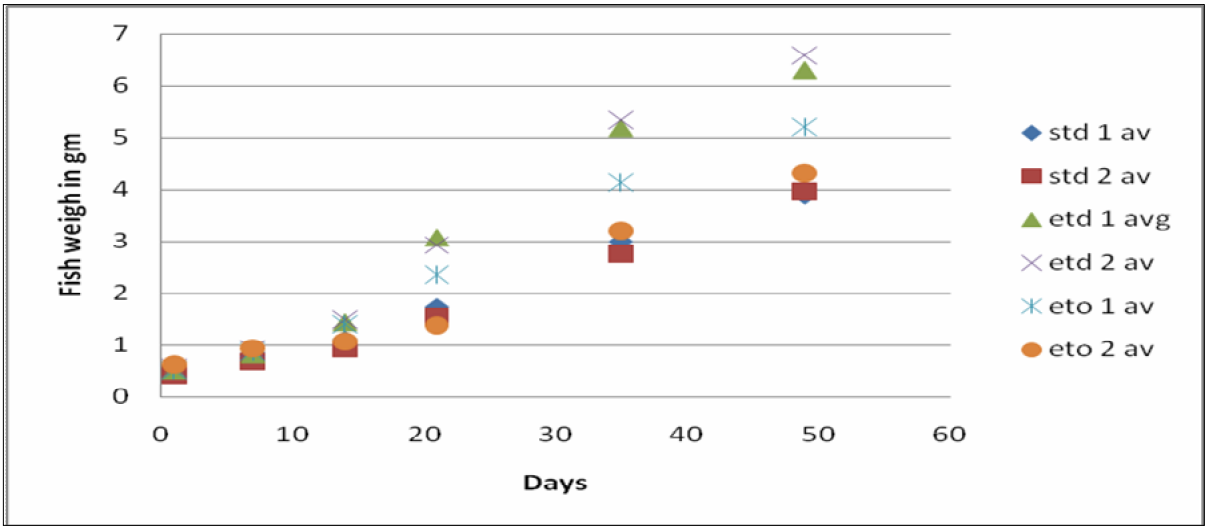

Figure 1. Growth rate of mixed population of Nile tilapia treated with 0.5% etodolac and 0.5% etoricoxib mixed with food at age of 8 days (std1 - standard 1%, std2 - standard 2%, etd1 - etodolac 1%, etd2 - etodolac 2%, eto1 - etoricoxib 1%, eto2 - etoricoxib2%).

Our observation of significant differences in growth rates of mixed fry Nile tilapia compared to adult mixed population following exposure to 0.5% etodolac and 0.5% 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 alteration in reproduction following exposure to non selective Cox inhibitors (Al-Qutob & Nashashibi 2009).

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 follicle stimulating hormone (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 release in female tilapia is under the control of the hypothalamic decapeptide Gonadotropin releasing hormone (GnRH) and the feedback inhibition loop at the gonadal-pituitary-axis (Aizen et al 2007; Yaron et al 2003).

The somatic growth of teleosts is controlled by the growth axis consisted of hypothalamus–pituitary–liver, i.e. the GH/IGF-I axis. Growth hormone (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 & 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), and 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 Luteinizing hormone (LH) and FSH release are inhibited by GH immune neutralization, suggesting that endogenous GH may act in a para-crine manner regulating gonadotroph functions. Similarly, 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, gonadotropin 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 GtH I (FSH-like) gonadotrophs are adjacent but slightly peripheral to them, whereas the GtH II (LH-like) gonadotrophs outlay these cells. 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 aromatized before eliciting these effects (Melamed et al 1999).

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). Thyroid status itself is one of the most potent regulators of iodothyronine deiodinase expression. The study of Mol 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 D1 activity, as well as D1 mRNA expression, is up regulated in hypothyroid tilapia (Van der Geyten 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). A study done on thirty eight dogs that were treated for orthopedic disorders were given etodolac (10 to 13.3 mg/kg, orally, once daily) for 14 days, serum T4 concentration decreased significantly after etodolac administration with increased canine thyroid stimulating hormone (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). 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 acetyl salicylic acid (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. It is also worth to mention that the thyroid axis (TRH, TSH, and T3) stimulates both synthesis and release of GH in tilapia, thus interfering with this axis could indirectly alter GH secretion (Melamed et al 1999).

Results showed no increase or decrease of growth in mixed population adult Nile tilapia treated with 0.5% etodolac and 0.5% etoricoxib. 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 (Brown et al 2004). Thyroid hormones assist in control of osmoregulation, metabolism, somatic growth, development, and posthatching metamorphosis (Janz & Weber 2000). Metamorphosis in flounders *Paralichthys olivaceus* (Temminck & Schlegel, 1846) and *P. dentatus* (Linnaeus, 1766), and flatfish *Hippoglossus hippoglossus* (Linnaeus, 1758) is associated with a dramatic spike in thyroxin (T4) concentrations (Tagawa et al 1990; Schreiber & Specker 1999; Saele et al 2006). These results support our hypothesis that COX-inhibitors could modulate aromatase enzyme during the crucial period of sexual differentiation as we discussed previously. 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 increase in gastrointestinal blood flow (GBF) after the ingestion of a normal sized meal ranges from around 70% in the sea bass (Axelsson et al 2002) to over 150% in the sea bass and the rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) according to Dupont-Prinet et al (2009) and Grans et al (2009). The postprandial increase in GBF 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. 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.

After the meal has been digested i.e. enzymatically broken down into smaller components by carbohydrases, lipases and proteases, it is likely that these hydrolyzed products that induce the subsequent GI hyperemia. The 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 2010). NSAIDs may enhance the proteolytic activity of the gut by increasing proteases activity, thus accelerating the hyperemia event (Rust 2002).

It is well known that prostaglandins have diverse effects in fish circulation as 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 (Stensløkken et al 2002). Thus, blocking prostaglandins synthesis abolishes their effect 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 & Perry 1992).

Mortality vs toxicity. The different concentrations affected the growth rate in a concentration dependent manner (Figure 2). 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. The 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. Gills alterations may interfere with normal respiratory functions and renal changes might lead to impaired osmoregulation (Evans et al 2005). Since etoricoxib 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 pecies (Woodward & Strange 1987).

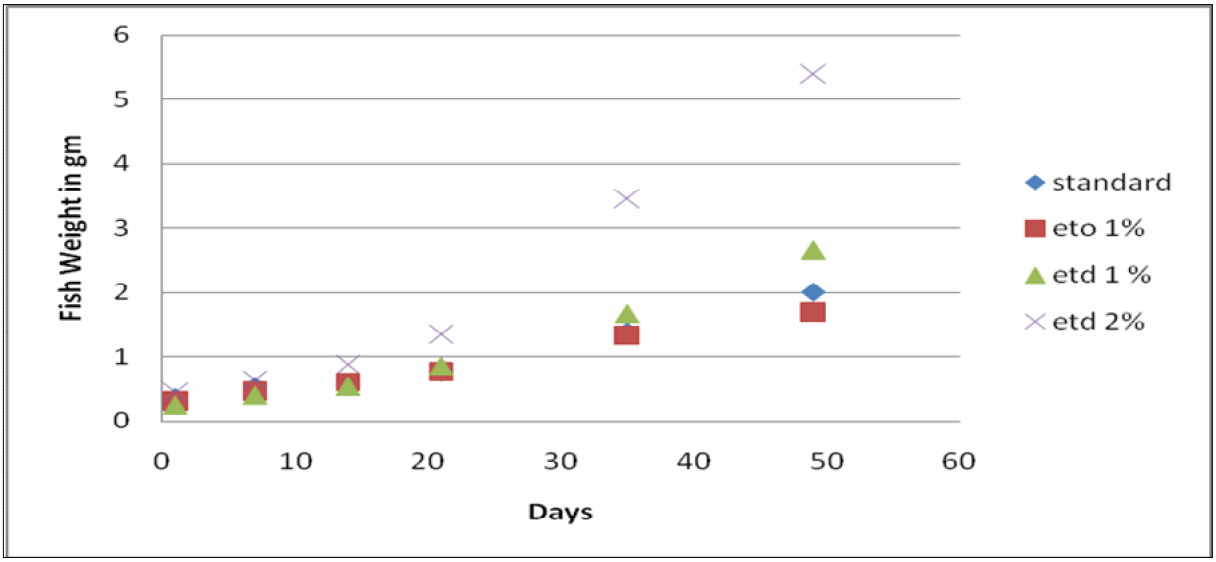

Figure 2. Growth rate of mixed population Nile tilapia treated with 1% etodolac, 1% etoricoxib, 2% etodolac mixed with food at age of 8 days (eto1% - etoricoxib 1%, etd1% - etodolac 1%, etd2% - etodolac 2%).

In reference to Figure 3, 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%, and 83.3%, respectively) during the experimental feeding. Higher mortalities were shown as concentrations increase with 1% etoricoxib, 1% etodolac and 2% etodolac treatments (Figure 4). 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 (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 & Nashashibi 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 crustacean *Daphnia magna* Straus, 1820, and the mollusk *Planorbis carinatis* Müller, 1774 which had been reported to be in mgL^{-1} range (Pounds et al 2008). Other study carried on adult Japanese medaka *O. latipes* showed 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).

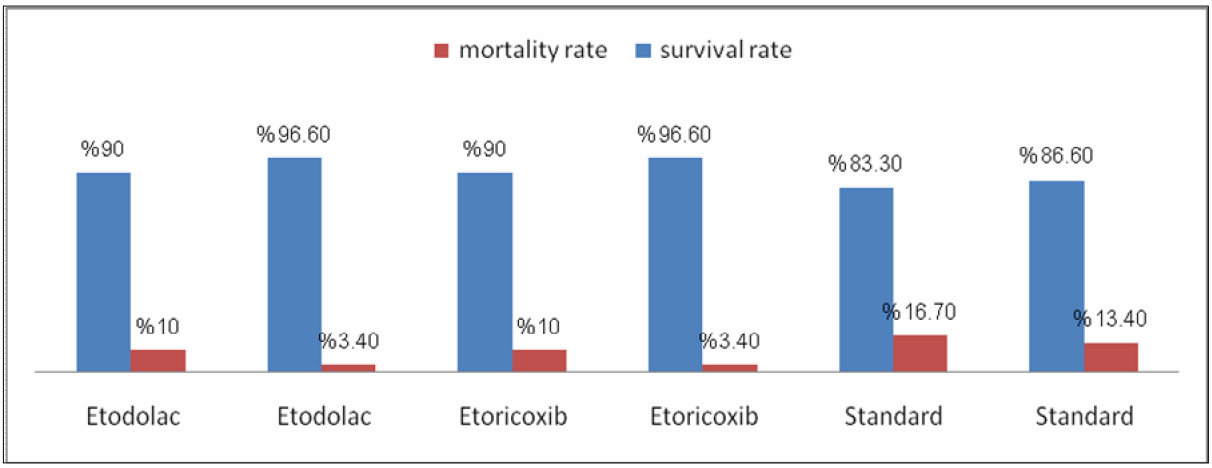

Figure 3. Survival and mortality rates during treatment with 0.5% etodolac and 0.5% etoricoxib.

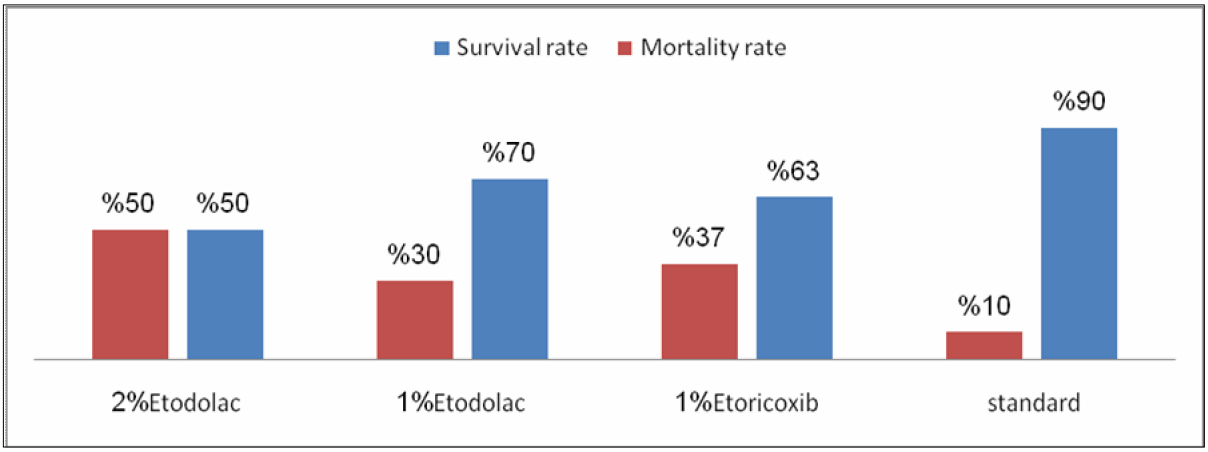

Figure 4. Survival and mortality rates during treatment with different concentrations of etoricoxib 1%, etodolac 1%, and etodolac 2%.

Determination of etoricoxib in fish feces. In reference to Figure 5 the retention time of etoricoxib 100 ppm injected sample was 4.18 min. However, the feces sample injected to HPLC system, showed no peak for etoricoxib (Figure 6). 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%) via 6-methyl hydroxylation (major) and 1-N-oxidation.

These metabolites are either excreted directly, or are metabolized further to secondary metabolites that are also excreted largely by urine (70%) and by feces (20%) with less than 2% recovered unchanged as etoricoxib. The 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) (Rodrigues et al 2003).

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 (Chauret et al 2001). Moreover, the involvement of multiple P450s, 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 (Kassahun et al 2001).

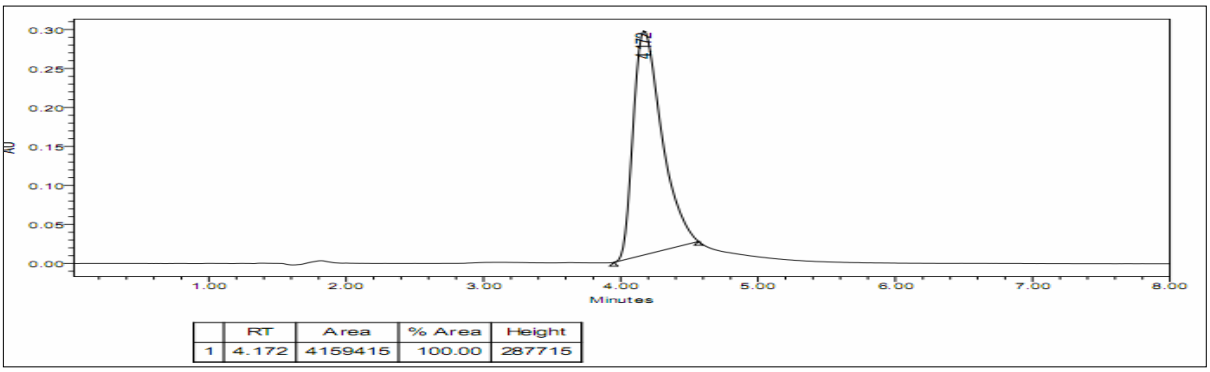

Figure 5. HPLC result from etoricoxib 100 ppm injected sample.

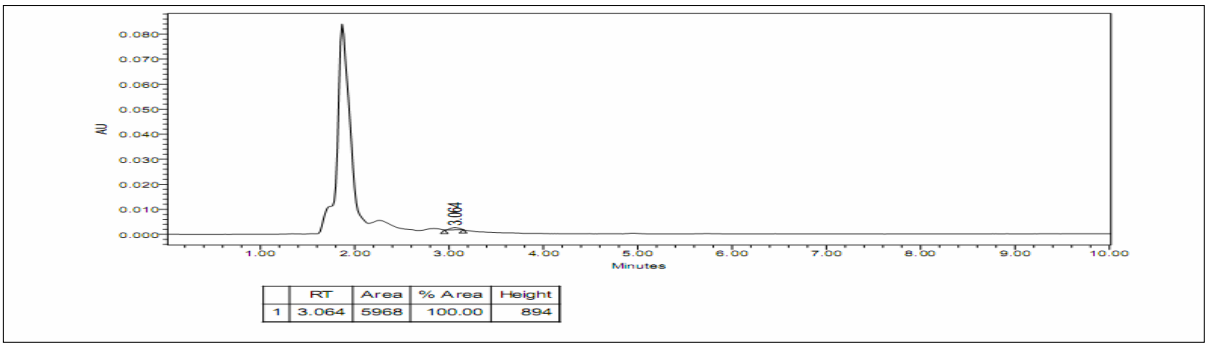

Figure 6. HPLC result from tilapia feces injected sample showing no etoricoxib peaks.

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, could indicate that etoricoxib was either extensively metabolized by liver or some of the drug have undergone enterohepatic circulation (Figure 6). 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).

Conclusions. The highest growth rate was with 2% etodolac, followed by 1% etodolac, but optimum dose with lower mortality (3.4% and 10%) was with 0.5% etodolac, and 0.5% etoricoxib which show SGR (%) of 4.93 and 4.64, respectively. The 1% etoricoxib showed a decreased growth rate compared to standard which could indicates a toxic potential of etoricoxib toward fish at this concentration. The 0.5% etodolac and 0.5% etoricoxib could be the optimum dose for improving growth rate with lower mortality. 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. 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. It is clear evident from mortality rates that even administration of high concentrations of these drugs was well tolerated by fish. Since there is a possibility to undergo enterohepatic circulation fish living downstream the wastewater treatment plants 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.

References

Aizen J., Kasuto H., Golan M., Zakay H., 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.

Al-Qutob M., 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 **2**(4):381-390.

Axelsson M., Altimiras J., 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.

Boullata J. I., Armenti V. T., 2004 Handbook of drug–nutrient interactions. Bendich A. (ed), pp. 393-394, Humana Press.

Brown B. S., Adams A. B., Cyr G. D., Eales G. J., 2004 Review: contaminant effects on the teleost fish thyroid. Environ Toxicol Chem **23**:1680-1701.

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

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

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.

Cha Y. I., Kim S. H., Solnica-Krezel L., Dubois R. N., 2005 Cyclooxygenase-1 signaling is required for vascular tube formation during development. Dev Biol **282**:274-283.

Chauret N., Yergey J. A., Brideau C., Friesen R. W., Mancini J., Riendeau D., Scheigetz J., Silva J., Styhler A., Trimble L., Nicoll-Griffith D., 2001 Invitro metabolism considerations including activity testing of metabolites, in the discovery and selection of the COX-2 inhibitor etoricoxib (MK-0663). Bioorg Med Chem Lett **11**:1059–1062.

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**(5):2563-2570.

Dupont-Prinet A., Claireaux G., 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 and Integrative Physiology **154**:233-240.

Eales J. G., Brown S. B., Cyr D. G., Adams B. A., Finnson K. R., 1999 Deiodination as an index of chemical disruption of thyroid hormone homeostasis and thyroidal status in fish. In: Environmental Toxicology and Risk Assessment: Standardization of Biomarkers for Endocrine Disruption and Environmental Assessment, Eight Volume, ASTM STP 1364, Henshel D. S., Black M. C., Harrass M. C. (eds), West Conshohocken, PA, American Society for Testing and Materials, pp. 136-164.

Evans D. H., Piermarini P. M., 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.

Flippin J. L., Huggett D., 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.

Gaytan M., Morales C., Bellido C., Sanchez-Criado J. E., Gayton F., 2006 Non-steroidalanti-inflammatory drugs (NSAIDs) and ovulation: lessons from morphology. Histol Histopathol **21**:541–556.

Grans A., Albertson F., Axelsson M., 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.
Goetz F. W., Hsu S.-Y., Selover A., 1991 Stimulation of prostaglandin synthesis in fish follicles by a phorbol ester and calcium ionophore. J Exp Zool **259**:355-364.
Grosser T., Yusuff S., Cheskis E., Pack M. A., FitzGerald G. A., 2002 Developmental expression of functional cyclooxygenases in zebra fish. Proc Natl Acad Sci USA **99**:8418-8423.
Il'yasova D., Colbert L., Harris B. T., Newman B. A., Bauer C. D., Satterfield S., Kritchevsky B. S., 2005 Circulating levels of inflammatory markers and cancer risk in the health aging and body composition cohort. Cancer Epidemiol Biomarkers Prev **14**:2413–2418.
Jackson A. J., Capper B. S., Matty A. J., 1982 Evaluation of some plant proteins in complete diets for the tilapia, *Sarotherodon mossambicus*. Aquaculture **27**:97-109.
Janz D. M., Weber L. P., 2000 Microscopic functional anatomy: endocrine system in the laboratory fish. Ostrander G. K. (ed), Chapter 25, pp. 415-435, Academic Press, London. ISBN 0-12-529650-9.
Kassahun K., McIntosh I., Shou M., Walsh D. J., Rodeheffer C., Slaughter D. E., Geer L. A., Halpin R. A, Agrawal N., Rodrigues A. D., 2001 Role of human liver cytochrome P4503A in the metabolism of etoricoxib, a novel cyclooxygenase-2 selective inhibitor. Drug Metab Dispos **29**: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.
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.
Lands W. E. M., 1991 Biosynthesis of prostaglandins. Ann Rev Nutr **11**:41–60.
Melamed P., Gur G., Rosenfeld H., Elizur A., Yaron 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.
Metcalfe C. D., Koenig B. G., Bennie D. T., Servos M., Ternes T. A., Hirsch R., 2003 Occurrence of neutral and acidic drugs in the effluents of Canadian sewage treatment plants. Environ Toxicol Chem **22**:2872-2880.
Mocuţa D., Pop T., Szasz F, Lazăr E., 2010 Precancerous cervical lesions and immunomarkers for their prognosis. Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii **20**(3):87-93
Mol K. A., Van der Geyten S., Kühn E. R., Darras V. M., 1999 Effects of experimental hypo-and hyperthyroidism on iodothyronine deiodinases in Nile tilapia, *Oreochromis niloticus*. Fish Physiology and Biochemistry **20**(3):201–207.
Ness T. A., Torres S. M., Kramek E. A., Blauvelt M. M., 2003 Effect of dosing and sampling time on serum thyroxine, free thyroxine, and thyrotropin concentrations in dogs following multidose etodolac administration. Veterinary Therapeutics Research in Applied Veterinary Medicine **4**(4):340-349.
Palada-de Vera M. S., Eknath A. E., 1993 Predictability of individual growth rates in tilapia. Aquaculture **111**:147-158.
Patiño R., Yoshizaki G., Bolamba D., 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.
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.

Randall D. J., Perry S. F., 1992 Catecholamines. In: Fish physiology. Hoar W. S., Randall D. J., Farrell A. P. (eds), Vol. xii B, pp. 255-300, San Diego, CA: Academic.
Rodrigues A. D., Halpin R., Geer L., Cui D., Woolf E. J., Matthews C., Gottes Diener K., Larson P. J., Lasseter K. C., Agrawal N. G. B., 2003 Absorption, metabolism and excretion of etoricoxib, a potent and selective cyclooxygenase-inhibitor. Healthy Male Volunteers DMD **31**:224–232.
Rust M. B., 2002 Nutritional physiology. In: Fish nutrition, 3rd edition. Halver J. E., Hardy R. W. (eds), pp. 367-452, Academic Press, Amsterdam.
Saele O., Silva N., Pittman K., 2006 Post-embryonic remodeling of neurocranial elements: a comparative study of normal versus abnormal eye migration in a flate fish, the Atlantic halibut. Journal of Anatomy **209**:31-41.
Schreiber A. M., Specker J. L., 1999 Metamorphosis in the summer flounder, *Paralichthys dentatus*: thyroidal status influences salinity tolerance. J Exp Zool **284**:414-424.
Seth H., Gräns A., Axelsson M., 2010 Cholecystokinin CCK as a potential regulator of cardiac function and postprandial gut blood flow in rainbow trout *Oncorhynchus mykiss*. Am J Physiol Regul Integr Comp Physiol **298**:1240-1248.
Simmons D. L., Botting R. M., Hla T., 2004 Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. Pharmacol Rev **56**:387–437.
Sorbera L. A., Asturiano J. F., Carrillo M., 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 prostaglandin F2a and prostaglandin E2 in Atlantic cod *Gadus morhua*. J Comp Physiol B **172**:363–369.
Tagawa M., Miwa S., Inui Y., de Jesus E. G., Hirano T., 1990 Changes in thyroid hormone concentrations during early development and metamorphosis of the flounder, *Paralichthys olivaceus.* Zoological Science **7**:93-96.
Toguyeni A., Fauconneau B., Boujard T., Fostier A., Kühn E. R., Mol K. A., Baroiller J. F., 1997 Feeding behaviour and feed utilisation in tilapia, *Oreochromis niloticus*: effect of sex-ratio andrelationship with the endocrine status. Physiol Behav **62**:273-279.
Van Anholt R. D., Spanings T., Koven W., Wendelaar Bonga S. E., 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):R1098-1106.
Van der Geyten S.,Toguyeni A., Baroiller J. F., Fauconneau B., Fostier A., Sanders J. P., Visser T. J., Kühn E. R., Darras V. M., 2001 Hypothyroidism induces type I iodothyronine deiodinase expression in tilapia liver. General and Comparative Endocrinology **124**:333-342.
Woodward C. C., Strange R. J., 1987 Physiological stress responses in wild and hatchery-reared rainbow trout. Trans Am Fish Soc **116**:574-579.
Yaron Z., Gur G., Melamed P., Rosenfeld H., Elizur A., Levavi-Sivan B., 2003 Regulation of fish gonadotropins. Int Rev Cytol **225**:131–185.
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.

Received: 29 September 2011. Accepted: 23 November 2011. Published online: 19 December 2011.
Authors:
Mutaz Al-Qutob, Faculty of Science and Technology, Department of Earth and Environmental studies, Al-Quds University, P. O. Box 19164, Jerusalem – Israel. E-mail: qutob@planet.edu.
Iman Al-Hirsh, Aquatic and Aquaculture Laboratory, Al-Quds University, P. O. Box 19164, Jerusalem – Israel. E-mail: amona_299@yahoo.com.
Tharwat Nashashibi, Aquatic and Aquaculture Laboratory, Al-Quds University, P. O. Box 19164, Jerusalem – Israel. E-mail: tharwatnash33@hotmail.com.
How to cite this article:
Al-Qutob M. A., Al-Hirsch I., Nashashibi T. S., 2011 The effects of COX2-inhibitors (etoricoxib and etodolac) on growth rate and mortality in Nile tilapia (*Oreochromis niloticus*). AACL Bioflux **4**(5):691-703.