

**Effects of chronic exposure to
ibuprofen and naproxen
on Florida flagfish (*Jordanella floridae*)
over one complete life-cycle**

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ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a class of drugs prescribed to relieve pain, fever and inflammation, and are among the most commonly consumed medications in Ontario. Approximately 70% of the ingested dose is excreted unchanged or as an active metabolite, much of which reaches the surface waters of lakes and rivers. NSAIDs function through the inhibition of cyclooxygenase (COX), an enzyme present in two isoforms in the body; the constitutively expressed COX-1 and the inducible COX-2. Traditional NSAIDs like ibuprofen inhibit both isoforms with little selectivity while newer variants such as naproxen preferentially inhibit COX-2. Both COX isoforms share a high similarity between humans and fish creating a potential for off target effects to exposed aquatic organisms. This research investigated the chronic effects of waterborne exposure to 0, 0.1, 1, 10 and 100 µg/L of a nonselective and selective NSAID (ibuprofen and naproxen, respectively) on Florida flagfish (*Jordanella floridae*) over one complete life-cycle. Chronic exposure concentrations were selected by performing a short term experiment which examined the hatchability of flagfish eggs using continuous semi-static exposure conditions. Growth, survivability and reproductive endpoints were assessed in the life-cycle study. A concentration-response relationship for both NSAIDs was detected during the first 28 days post-hatch, resulting in increased body length for F1 fish and their offspring with increasing concentrations. Exposure to 0.1 µg/L of both ibuprofen and naproxen resulted in a decrease in egg fertilization providing an experimental LOEC (lowest observable effect concentration) of 0.1 µg/L and NOEC (no observable effect concentration) of < 0.1 µg/L for both ibuprofen and naproxen based on the reproductive endpoint. This indicates that either NSAID has the potential to affect

the reproductive success of flagfish at concentrations at or below those commonly found in the environment.

Keywords: Ibuprofen, Naproxen, NSAID, Life-cycle, Flagfish, Growth, Reproduction

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LIST OF ABBREVIATIONS

AA – Arachidonic acid

COX – Cyclooxygenase

CTRL - Control

EC₅₀ – Median effective concentration for 50% of the test organisms

HSI – Hepatosomatic index

IBU - Ibuprofen

K – Condition factor

kd – kilodalton

K_{ow} – Octanol water coefficient

L –Litre

LC₅₀ – Lethal concentration at which 50% of the test organisms would be killed

LOEC – Lowest observable effect concentration

mg – Milligrams

mg/L – Milligrams per litre

mM – millimoles per litre

mm Hg – Millimetres of mercury

MS-222 – Tricane methane sulphonate

NOEC – No observable effect concentration

NPX – Naproxen

NSAID – Non steroidal anti-inflammatory drug

PG – Prostaglandins

PG D₂, F_{2α}, G₂, H₂, and I – Prostaglandins D₂, F_{2α}, G₂, H₂, and I

pK_a – Acid dissociation constant at logarithmic scale

PPCP – Pharmaceuticals and personal care products

TXA – Thromboxane

µg/L – Micrograms per litre

vtg – Vitellogenin, egg yolk protein

WWTP – Waste water treatment plant

1.0 LITERATURE REVIEW

1.1 Toxicity Testing

In 1962 the book *Silent Spring* by Rachel Carson took the quiet concern of research biologists regarding the effects of synthetic chemicals, particularly organochlorine pesticides, on the environment and catapulted it into public focus (Carson, 1962). This started a movement which provided the driving force for research into the new field of environmental toxicology and ecotoxicology (Rand *et al.*, 1995). However, this was certainly not the start of toxicity testing (Rand *et al.*, 1995). The long history of toxicity testing dates back to the 1800s where advances in pharmacology and toxicology were accompanied by basic toxicological research on rodents, fish and insects (Rand *et al.*, 1995). Other than these tests, it was not until the 1950's that modern methods of aquatic toxicity testing on fish were developed and applied (Rand *et al.*, 1995).

The establishment of the American Society for Testing and Materials (ASTM) and the American Public Health Association (APHA) in the 1950's saw the development of "standard methods" for testing chemicals on fish (Rand *et al.*, 1995). Calls for methodological standardization were necessary for regulatory purposes so that scientists could compare the results of chemical toxicity studies from different laboratories (Sprague, 1969).

There are many different types of toxicity test approaches which can be performed. Selection of one depends on the questions asked and the applicability of the

selected bioassays. Examples of common toxicity testing methodologies include acute, chronic, short-term sublethal, early life stage and bioaccumulation tests (Sprague, 1969; Rand *et al.*, 1995).

Toxicity testing on fish is one of the most effective methods for understanding the deleterious effects of environmental contaminants on aquatic ecosystems (van der Oost *et al.*, 2003). Fish are well suited as study species as they meet several requirements for effective test organisms. First, fish are ubiquitous in aquatic environments so that fish are likely present wherever contamination occurs, whether it be in lakes, rivers, streams or oceans (van der Oost *et al.*, 2003). Fish also play a major role in aquatic food webs where they generally occupy an intermediate or higher position. Fish are thus a conduit for energy transfer from lower trophic levels to higher ones (van der Oost *et al.*, 2003). Not only do a variety of aquatic predators feed on fish, but they also serve as a major food source for humans around the world (van der Oost *et al.*, 2003).

1.2 Chronic Exposures and Full Life Cycle Studies

The use of chronic exposure full life cycle studies has been validated in many laboratories (Holdway and Sprague, 1979; Holdway and Dixon, 1985; Parrott *et al.*, 2004; Parrott and Bennie, 2009). Full life cycle tests have been completed on a wide variety of species including the fathead minnow (*Pimephales promelas*), bluegill sunfish (*Salvelinus fontinalis*), American flagfish (*Jordanella floridae*), brook trout (*Salvelinus fontinalis*) and sheepshead minnow (*Cyprinodon variegates*) (Eaton, 1970; McKim and Benoit, 1971; Holdway and Sprague, 1979; Parrott and Bennie, 2009). Tests can begin with embryos or newly hatched fry less than 8 days post-hatch and continue until at least 28 days post-hatch of the subsequent generation (McKim, 1995). By covering all stages

of an organism's lifecycle as well as the critical early life stages of the next generation, a researcher using a full life cycle study has a full range of available endpoints including growth and development during critical and noncritical life stages, as well as reproduction (McKim, 1995). This is the most effective way to assess the long term effects of a contaminant and to determine the real concentration at which it has no effect (McKim, 1995).

A chronic exposure study by its nature has a much longer exposure period (generally >1/10th of the lifespan) than acute studies, and may lead to the discovery of unexpected effects not detectable when using shorter duration toxicity testing methodologies (Wayser *et al.*, 2011). Pharmaceutical contaminants present in the environment are rarely in concentrations high enough to cause acute effects (Lishman *et al.*, 2006). While they are broken down quickly, the rate of addition to the environment matches or exceeds the degradation rate resulting in pseudo-persistence (Han *et al.*, 2010; Wayser *et al.*, 2011). This constant low level contamination has the potential for population effects and for slowly altering ecosystem structure over successive generations, impacts that could not be assessed in acute studies (Walker *et al.*, 2006). Together this reinforces the need to study pharmaceuticals over the entire lifecycle of a species to mimic natural exposures and discover potential long term consequences (Wayser *et al.*, 2011).

In the methodological development and determination of endpoints for this thesis, the works of several authors were heavily cited (Holdway and Sprague, 1979; Holdway *et al.*, 1988; Patyna *et al.*, 1999; Parrott, 2005; Han *et al.*, 2010).

1.3 Fish Physiology

1.3.1 Uptake and Elimination Physiology

Pharmaceuticals and personal care products (PPCPs) pose a risk to fish as a result of their designed purpose. While PPCPs were developed for their biological activity in humans or animal (veterinary) groups, the functional and genetic conservation across vertebrates makes it likely that similar targets will be affected in fish (Corcoran *et al.*, 2010). Exposure of fish to contaminants can occur through a multitude of pathways; nonpolar pharmaceuticals will pass through biological membranes via diffusion to interact with their targets (Corcoran *et al.*, 2010). Waterborne and sediment bound contaminants can enter the organism across the dermal membrane or through the gills (Corcoran *et al.*, 2010). Contaminants can also enter the body orally through the consumption of contaminated food (Corcoran *et al.*, 2010). They can also pass from the maternal parent to the lipid reserves of the eggs (Corcoran *et al.*, 2010). During the early life stages, fish show a decreased ability to metabolize contaminants (Reid *et al.*, 1995) and as a result may experience increased effects of xenobiotics (Corcoran *et al.*, 2010). These younger fish also have lower energy reserves to offset the costs of contaminant metabolism (Holdway and Dixon, 1985).

Typically during exposure, contaminants will pass through the absorbing membrane where they are then incorporated into the blood (Kleinow *et al.*, 2008). From this point they will be transported throughout the body where they will have the opportunity to interact with the target sites on cells of organs or tissues (Kleinow *et al.*, 2008). Simultaneously the circulating contaminants will undergo biotransformations,

usually in the liver, to more hydrophilic compounds which facilitate their excretion from the body (van der Oost *et al.*, 2003). Biotransformations generally add reactive functional groups to increase ease of metabolism through oxidation, reduction and hydrolysis (van der Oost *et al.*, 2003). These actions are usually catalyzed by the mixed function oxidase system with the cytochrome P450 family of microsomal monooxygenase enzymes in the liver (van der Oost *et al.*, 2003).

1.3.2 Reproductive Physiology

Fish have a complex endocrine-based reproductive system which is affected by the interplay of hormones and external stimuli (Stacey and Goetz, 1982; Kime, 1999). Effects of external stimuli are species-specific though common examples of abiotic modifying factors which can have a direct impact on endocrine hormones include temperature, photoperiod and rainfall (Stacey and Goetz, 1982; Kime, 1999). These affect the primary internal control of reproduction in fish: the hypothalamic-pituitary-gonadal axis (Arcand-Hoy and Benson, 1998).

The hypothalamus controls and stimulates the synthesis and release of the hormone gonadotropin from the pituitary gland (Arcand-Hoy and Benson, 1998). This is regulated through the production of gonadotropin releasing hormone in the hypothalamus (Arcand-Hoy and Benson, 1998). Gonadotropin production is also regulated by prostaglandins (PG) (Stacey and Goetz, 1982). Gonadotropins are the principal hormones involved in fish reproduction and come in two forms, gonadotropin I and gonadotropin II (Arcand-Hoy and Benson, 1998). Gonadotropin I and II are analogous to luteinizing hormone and follicle stimulating hormone in mammals and fulfill similar roles in fish (Arcand-Hoy and Benson, 1998; Kime, 1999). Collectively this role is to

stimulate gonadal development and the production of steroid hormones (Kime, 1995; Arcand-Hoy and Benson, 1998; Kime, 1999).

The main target of the fish estrogen, estradiol, is the liver (Arcand-Hoy and Benson, 1998; Kime, 1999). In normally developing females, estradiol stimulates the liver to produce the yolk precursor protein vitellogenin (vtg) (Arcand-Hoy and Benson, 1998; Kime, 1999). Under the control of gonadotropin I, vitellogenin will circulate through the blood until it reaches the oocyte where it is incorporated (Arcand-Hoy and Benson, 1998; Kime, 1999). As the oocyte develops, gonadotropin I levels decline to be replaced by increasing levels of gonadotropin II (Arcand-Hoy and Benson, 1998). The gonadotropin binds to the granulosa cells of oocytes, stimulating the production and release of progestins which play a role in gonadal maturation as well as ovulation (Arcand-Hoy and Benson, 1998).

In male fish, gonadotropin I stimulates the production of the androgen 11-ketotestosterone in the Leydig cells (Kime, 1995; Arcand-Hoy and Benson, 1998; Kime, 1999). During the development of secondary sex characteristics and spermatogenesis, the blood concentration of gonadotropin I remains high to maintain the required level of androgen production (Arcand-Hoy and Benson, 1998). A sharp decline in gonadotropin I and an increase in gonadotropin II is accompanied by a drop in androgen levels and an increase in progestins (Arcand-Hoy and Benson, 1998). This is associated with the final maturation of sperm (Arcand-Hoy and Benson, 1998; Kime, 1999).

In both sexes, the development of mature gonads capable of producing viable gametes can take several months (Arcand-Hoy and Benson, 1998; Kime, 1999).

Throughout this time, the hypothalamic-pituitary-gonadal axis is under the control of a negative feedback mechanism (Figure 1) (Arcand-Hoy and Benson, 1998; Arukwe and Goksøyr, 2003).

Exposure to toxicants can have deleterious consequences on the reproductive health of exposed organisms (Kime, 1995). Visible signs of reproductive distress can come in the form of reduced fertility and fecundity and egg size for an exposed parental generation (Heckmann *et al.*, 2007). To study these effects, a suitable test species can be selected from the thousands of organisms exposed to contaminants, based on criteria such as size, ease of use, fecundity, susceptibility to toxins and availability of endpoints (Hill *et al.*, 2005). The cyprinodontidae family, in particular the American flagfish, has been found to be an excellent test species for use in a laboratory setting (Foster *et al.*, 1969; Woltering, 1984).

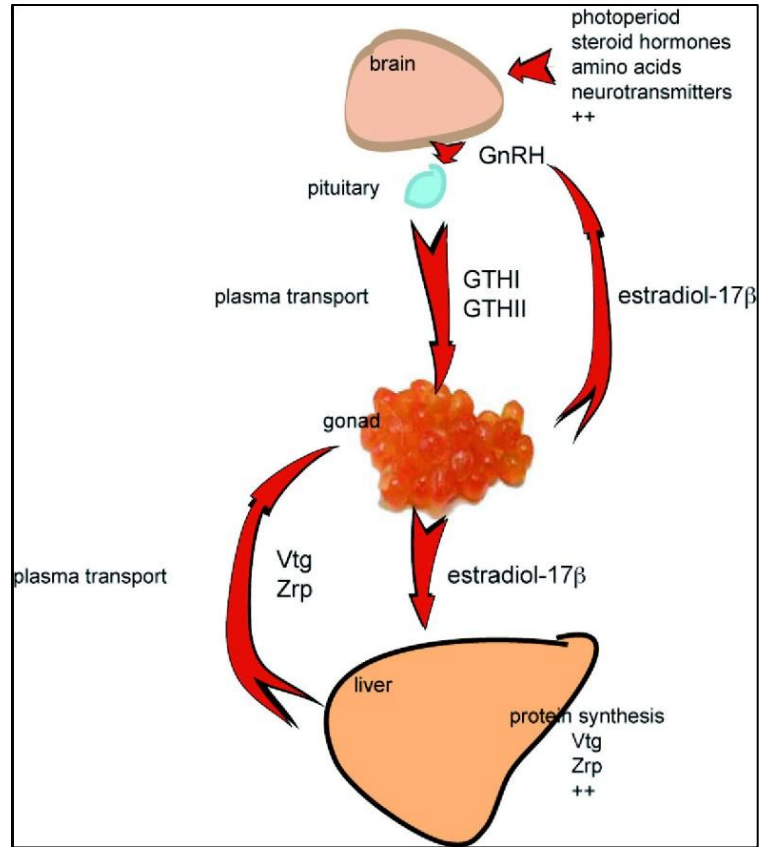


Figure 1: Representation of hypothalamus-pituitary-gonadal axis negative feedback in female teleost fish. Image from Arukwe and Goksøyr, 2003.

1.4 *Jordanella floridae* (American flagfish)

1.4.1 Classification, Habitat, Sexual Dimorphism

Jordanella floridae, commonly known as the American flagfish or Florida flagfish, is a fresh water species of the cyprinodontinae subfamily (Foster *et al.*, 1969). They are endemic to the shallow fresh, brackish and salt waters of central and southern Florida as well as coastal regions of the Yucatan to the Gulf of Mexico (Foster *et al.*, 1969; St Mary *et al.*, 2001). In the wild they live for approximately 1 year typically spawning between March and September when breeding temperatures range from 22 to 37°C (St Mary *et al.*, 2001).

Flagfish exhibit remarkable sexual dimorphisms which are easily distinguishable by the naked eye by approximately 70 days post hatch (Holdway and Sprague, 1979). Males will reach a maximum standard length of 50 mm and are characterized by up to nine red-orange horizontal stripes on alternate rows of their scales (St Mary *et al.*, 2001). When contrasted with the blue-green iridescence comprising the background of the fish, the body is reminiscent of the American flag which lends itself to the species' popular name (Crawford, 1975). A dark spot often surrounded by yellow pigmentation is found on the flank of the male, ventral to the origination of the dorsal fin (St Mary *et al.*, 2001). The females of the species lack the bright striping pattern of the males making the two sexes easily distinguishable. This is furthered by a distinct dark spot on the posterior edge of the dorsal fin on the female (Foster *et al.*, 1969).

1.4.2 Validity as a Test Species

Flagfish of the cyprinodontidae family have been validated as an excellent test species for use in laboratory studies (Foster *et al.*, 1969). The species fulfills many of the requirements for a successful model vertebrate for a reproductive study (Hill *et al.*, 2005). The maximum size of the adult flagfish is relatively small as previously noted (Foster *et al.*, 1969). This greatly increases the ease of handling larger experimental populations in a laboratory setting, increasing the statistical power of an experiment. The tendency to lay eggs in a single clutch permits a simple method of collection while the transparency of the eggs allows for observation of the developing embryo. Flagfish have a relatively short life-cycle of 90-120 days from egg to egg which, when combined with the ability to determine gender of individuals by approximately 70 days post-hatch, make this species an excellent candidate for reproductive studies (Foster *et al.*, 1969).

Flagfish have a long and rich history in the literature providing an excellent body of work to assess what normal growth and development looks like (Mertz and Barlow, 1964; Foster *et al.*, 1969; Spehar *et al.*, 1978; Holdway and Sprague, 1979; Holdway *et al.*, 1983; Holdway and Dixon, 1985; Holdway *et al.*, 1988; Smith *et al.*, 1991). Many of these authors have also contributed to the documentation of flagfish behavioural patterns, opening up another series of endpoints for study (Foster *et al.*, 1969; St Mary *et al.*, 2001; Bonnevier *et al.*, 2003; St Mary *et al.*, 2004; Klug *et al.*, 2008).

1.4.3 Behavioural Characterization

Jordanelle floridae have been identified as the only Cyprinodontae species to exhibit parental care (Bonnevier *et al.*, 2003). These behaviours are classified into 3

overarching types of behaviours: sexual behaviour, food seeking and comfort activities (Mertz and Barlow, 1964; Foster *et al.*, 1969).

Sexual behaviour occurs between a responsive, mature female and a dominant male who has taken up a guarding position over a suitable breeding substrate (Mertz and Barlow, 1964). The guarding male will initially defend his territory from intruding males and females by exhibiting chase behaviour (Mertz and Barlow, 1964). Chase is observed when a male leaves his territory to pursue other fish at speed, thereby driving them away (Mertz and Barlow, 1964; Foster *et al.*, 1969). Dominant males will also display their fins maximally while orienting their bodies so that one side is directed towards intruding fish (Foster *et al.*, 1969).

Presenting females will approach the guarded territory slowly with rapid, sporadic, low amplitude beats of the caudal fin accompanied by a blanching of their colouration (Mertz and Barlow, 1964). The male will initially persist with chase behaviour but the female's colouration and behaviour signals the male to desist in his aggression (Mertz and Barlow, 1964). The female will then orient her body 90° perpendicular to the male so that the caudal fin directly faces him; she then proceeds to back towards him (Mertz and Barlow, 1964). This position will be held by both fish as they rotate together in a circle for up to two minutes in a behaviour referred to as "T circling" (Mertz and Barlow, 1964). The female will break from the T position if the pair is not directly above the suitable spawning substrate (Mertz and Barlow, 1964). She will repeatedly initiate the T dance behaviour until the circling occurs overtop the substrate at which point they will progress to the next behavioural stage of spawning, the clasp (Mertz and Barlow, 1964; Foster *et al.*, 1969). Clasping is the spawning posture where

the male positions himself against the female in a sigmoidal curve at a 15° angle to the substrate (Foster *et al.*, 1969). During the clasp, the eggs are discharged and fertilized while both fish beat their caudal peduncles at a low amplitude and high frequency (Mertz and Barlow, 1964). Egg production by the female is influenced by gut pressure against the ovary created by digesting ample quantities of food (Foster *et al.*, 1969). Increased pressure aids in the expulsion of eggs (Foster *et al.*, 1969).

Flagfish food seeking behaviour consists of digging, nipping and ejecting (Foster *et al.*, 1969). Digging has been observed as a flagfish of either gender thrusts its snout into a substrate up to the preorbital region and then withdraws (Foster *et al.*, 1969). Grasping an object or substrate with an open mouth is classified as nipping (Foster *et al.*, 1969). If the grasped material from digging or nipping is then forcibly expelled from the mouth, this subsequent behaviour is ejecting (Foster *et al.*, 1969).

Yawning, chafing and gasping have all been described as the comfort behaviours of the flagfish. During a pause in forward swimming, the jaw may open maximally and be accompanied by erect fins and flared opercles; the yawn behaviour. Chafing is the sudden approach towards a solid object which is struck with the side of the body in a glancing fashion (Foster *et al.*, 1969). Gasping, the last comfort activity, has been observed when the flagfish approaches the surface of the water. The mouth will repeatedly open and close to gulp air. To maintain the position at the surface of the water, the gasping behaviour will often be accompanied by forward swimming (Foster *et al.*, 1969).

Fanning has been difficult to classify into a category, being observed during both sexual and food seeking behaviours (Foster *et al.*, 1969; St Mary *et al.*, 2001). During the fan, the male will orient his body at a 45° angle to the substrate (Foster *et al.*, 1969). The pectoral fins are beat to generate the fanning of the substrate along with a rear propulsive force (Foster *et al.*, 1969). This movement is concurrent with the beating of the caudal fin to prevent net movement of the fish (Foster *et al.*, 1969). As a sexual behaviour, fanning is thought to serve as a source of increased O₂ to developing offspring and cleaning of the breeding substrate (St Mary *et al.*, 2001). However, fanning has also been observed with few or no eggs, indicating that it may serve additional roles for flagfish (St Mary *et al.*, 2001). A secondary role for fanning seems to be associated with general food seeking in the absence of eggs or egg cannibalism (Foster *et al.*, 1969; St Mary *et al.*, 2001).

1.5 Overview of Pharmaceuticals and Personal Care Products (PPCPs)

A wide range of PPCPs are now on the market throughout the world (Jjemba, 2007). These drugs are specifically designed to affect the biochemical and physiological pathways of humans and animals allowing them to be used both in medical and veterinary applications (Jjemba, 2007). By design, many of these PPCPs have some form of persistence, to permit increased effect (Fent *et al.*, 2006). The majority of current work on PPCPs has focused on discovering their concentrations in the environment rather than understanding the possible non-target effects (Fent *et al.*, 2006). Many of the advances in this field of research have been driven forward by new analytical techniques and increased detection limits (Fent *et al.*, 2006). Examples include ultra performance liquid chromatography/mass spectroscopy and gas chromatography/

mass spectrometry, both of which require solid phase extraction to be performed on samples prior to the analysis of low concentrations (Metcalf *et al.*, 2003).

Huge quantities of PPCPs are being globally produced, prescribed and consumed. Research has shown that the contaminant profiles of lakes and rivers mirror the PPCP usage of local populations but only vary slightly from location to location (Ternes, 1998; Metcalfe *et al.*, 2003). NSAIDs, in particular, have consistently shown up in studies examining pharmaceutical contamination, and with prescription rates increasing by 15-20% yearly, the potential environmental impact from these contaminants is very large (Ternes, 1998; Metcalfe *et al.*, 2003; Lishman *et al.*, 2006; Jjemba, 2007).

The majority of PPCPs find their way into surface waters after they have been consumed by humans and enter the municipal sewer system (Jjemba, 2007). They are heavily utilized in households and hospitals where they are excreted through the urine or feces depending on the breakdown of the compound within the body (Jjemba, 2007; Glassmeyer *et al.*, 2008). Sewer systems transport the PPCPs to wastewater treatment plants (WWTPs) where they are subject to incomplete removal, eventually appearing in receiving surface waters via contaminated treated effluents (Glassmeyer *et al.*, 2008).

Veterinary applications also pose a significant risk of environmental contamination as animal excretions are often not subjected to treatment processes of any kind (Glassmeyer *et al.*, 2008). PPCP laden excretions from aquaculture will enter the surface water directly (Glassmeyer *et al.*, 2008). Agricultural PPCPs are excreted onto the soil by domestic animals where they leach into groundwater or contaminate surface runoff during rainfall, eventually entering surface water (Glassmeyer *et al.*, 2008).

Many of the most common pharmaceuticals are used both in human and veterinary applications (Boxall *et al.*, 2004). Antibiotics, hormones, NSAIDS and many other high volume drugs are contaminating surface waters (Ternes, 1998; Metcalfe *et al.*, 2003; Fent *et al.*, 2006; Jjemba, 2007; Glassmeyer *et al.*, 2008). More recent studies highlighting the endocrine disrupting impacts of PPCP on non-target organisms have increased the need for ecotoxicological investigations of these contaminants (Snyder *et al.*, 2003). Much of this toxicity testing has focused on the discovery of LC₅₀ and EC₅₀ values for PPCPs, producing useful reference information (Cunningham *et al.*, 2006). However, chronically exposed organisms like fish and other aquatic life never experience concentrations higher than ng/L or low µg/L, well below concentrations causing acute toxicity (Ternes, 1998; Metcalfe *et al.*, 2003; Cunningham *et al.*, 2006). There is a significant research gap in understanding the effects of PPCP contamination at environmentally relevant concentrations and exposure periods (Fent *et al.*, 2006). To address this gap, low concentration chronic exposure studies need to be conducted using model organisms representative of wild populations.

1.6 PPCP Removal - Waste Water Treatment Plant (WWTPs)

Wastewater treatment plants (WWTPs) function by subjecting the influent sewage water to primary and secondary treatment processes, followed by some sort of disinfection (Kim *et al.*, 2007). Primary treatment is designed to remove particulate matter from the wastewater (Kim *et al.*, 2007). Particulates are allowed to settle on the bottom while remaining suspended materials are prevented from flowing into the secondary treatment process through the use of screens. In secondary treatment, microbes are used to break down and remove organic matter (Kim *et al.*, 2007).

Disinfection is the final stage of the WWTP and is achieved through use of UV radiation or chlorine/bromine treatments (Kim *et al.*, 2007). It is during secondary treatment and disinfection that the PPCPs are potentially removed (Kim *et al.*, 2007).

Rates of PPCP removal during secondary treatment are subject to a variety of factors. Analysis of removal efficiency is primarily based on the difference between influent and effluent concentrations (Fent *et al.*, 2006). Factors that affect removal efficiency include the chemical properties of the pharmaceutical itself, the treatment processes employed, environmental temperature, light, the microbial community present in the activated sludge and the matrix effect (Farré *et al.*, 2007). PPCPs are generally removed from WWTP influents through two main elimination processes: adsorption and biodegradation (Fent *et al.*, 2006).

Adsorption in relation to sewage treatment is a process whereby the particulate matter in the water adheres to solids in the WWTPs secondary treatment, preventing them from entering the effluent. How much of the influent-dissolved PPCPs adsorb to the solids is mediated by the hydrophobic and electrostatic properties of the individual contaminants (Fent *et al.*, 2006). Adsorption has proved to be fairly effective with basic pharmaceuticals, but is not a viable option for those with greater complexity and relatively low pK_a (Fent *et al.*, 2006). Pharmaceuticals that fall into this category include the NSAIDs with pK_a values of 4.2 for naproxen and 4.9 for ibuprofen (Bjarnason *et al.*, 2007; Ziylan and Ince, 2007). Acidic pharmaceuticals such as these are in the dissolved state at the neutral pH of wastewater, preventing them from partitioning out of solution and onto the solids (Fent *et al.*, 2006). While higher rates of adsorption can be achieved

through decreasing pH in the treatment plants, adsorption remains a less effective method for the removal of dissolved PPCPs.

Biodegradation, however, is more effective in removing dissolved pharmaceuticals. It occurs in both the aerobic and anaerobic zones of activated sludge (Fent *et al.*, 2006). Hydraulic retention time is often the best predictor for the quantity of PPCPs removed through biodegradation as the microorganisms responsible for their breakdown are given more time to function (Fent *et al.*, 2006). One concern for biodegradation is that the potential exists for conjugated or non-conjugated polar metabolites to be cleaved by the microbes, releasing the active parent compound and actually increasing the concentration in the effluent (Fent *et al.*, 2006). Products of microbial digestion can also result in daughter compounds with increased toxicity (Fent *et al.*, 2006).

Disinfection processes also aid in the breakdown of PPCPs, but play a far lesser role when compared with secondary treatments (Kim *et al.*, 2007).

1.7 General Fate of PPCPs: Environmental Fate

Once in the environment, PPCPs have three potential fates: transport, sequestration and degradation (Jjemba, 2007). Transport is the movement of particulate and dissolved PPCPs throughout the water column. This has the benefit of diluting the drug which limits the risk of acute exposure and harm. However, the transport of PPCPs throughout the environment increases the number of organisms facing chronic exposure. Sequestration of PPCPs into matrices is an alternate fate. This occurs through bioconcentration, sorption and deposition of the compound (Glassmeyer *et al.*, 2008).

All can occur without prior degradation allowing the PPCP to be transported or degraded at a later time. Degradation alters the parent compound into one or more daughter compounds through biodegradation, hydrolysis or photolysis to name a few of the processes (Glassmeyer *et al.*, 2008). This can result in a less toxic form of the drug or, in the case of naproxen, a compound with significantly increased toxicity (Isidori *et al.*, 2005).

Prediction of which of the three fates will be favoured is based on the chemical properties of the compound (Jjemba, 2007). Molecular weight, solubility, K_{ow} , pK_a and the pharmacodynamics are all variables which affect predicted fates (Glassmeyer *et al.*, 2008). While the United States Food and Drug Administration (FDA) still utilizes this data to predict the fate of a contaminant and determine its ecotoxicological risk, predicted values are not significantly correlated with detected environmental concentrations (Jjemba, 2007). This further exemplifies the need for ecotoxicological studies of PPCP at environmentally relevant concentrations and exposures, as well as studies of PPCP environmental behaviour (Jjemba, 2007).

1.8 Nonsteroidal Anti-Inflammatory Drugs (NSAIDs)

NSAIDs are a chemically diverse group of pharmaceuticals with a similar mode of action: the inhibition of cyclooxygenase (COX). From the isolation of the first NSAID, salicylic acid, in 1829, the use of NSAIDs has expanded greatly and most are available without a prescription (Farré *et al.*, 2007). Therapeutically, NSAIDs are prescribed to reduce fever, cure headaches and alleviate the pain, redness and swelling of inflammation (Vane and Botting, 1998). NSAIDs are highly used for the treatment of these symptoms, particularly over opioids (Farré *et al.*, 2007). While they provide similar

symptomatic relief to opioids, NSAIDs do not produce sedation, respiratory depression nor do they have a risk of addiction (Farré *et al.*, 2007).

NSAIDs, like all pharmaceuticals, pose a risk for aquatic life as 30-90% of the administered dose is excreted unchanged or as an active metabolite through feces or urine (Metcalf *et al.*, 2003). These metabolites are particularly recalcitrant to degradation due to the chemical properties of NSAIDs and their salts (Farré *et al.*, 2007). High solubility of NSAIDs results in very low rates of removal during sedimentation and flocculation stages of WWTPs (Ziylan and Ince, 2007). Adsorption, another key mechanism relied on by WWTPs is almost completely ineffective on acidic pharmaceuticals with pK_a of 4.2-4.9 (Ziylan and Ince, 2007). As this is the pK_a range of NSAIDs, they easily pass through this stage of the WWTP (Ziylan and Ince, 2007). This leaves biotransformations in the activated sludge and disinfection as the primary methods of removal from WWTP influent (Ziylan and Ince, 2007).

Interestingly, the biological and chemical transformations responsible for the majority of NSAID removal are strongly affected by thermal conditions resulting in rates of removal that fluctuate with season (Ziylan and Ince, 2007). Lower temperatures in the winter drops the influent temperature, decreasing the efficiency and rates of reactions (Ziylan and Ince, 2007). This is coincidentally accompanied by the flu season which brings about a spike in the use of NSAIDs (Ziylan and Ince, 2007). The combination of flu season and low water temperatures results in significantly higher contaminant burdens during the winter, and documented in a number of Canadian sewage treatment plants (Metcalf *et al.*, 2003; Ziylan and Ince, 2007). This broadens the range of potential exposure concentrations for aquatic life (Metcalf *et al.*, 2003).

1.8.1 Mode of Action – Cyclooxygenase (COX)

The mode of action through which NSAIDs work within mammalian species is the inhibition of cyclooxygenase (COX) (Vane and Botting, 1998). Killifish species such as flagfish also possess COX, allowing NSAIDs to function through the same pathway in these organisms (Zou *et al.*, 1999; David and Pancharatna, 2009).

COX is a membrane bound haemo- and glycoprotein located primarily in the endoplasmic reticulum of prostanoid-forming cells responsible for the conversion of arachidonic acid (AA) into biologically active forms of PG (Vane and Botting, 1998). This process is achieved by first altering AA to become cyclic, and then by adding a 15-hydroxy group to form the unstable molecule prostaglandins G₂ (PGG₂). The conversion of PGG₂ into prostaglandins H₂ (PGH₂) is achieved by the reduction of the hydroperoxy group of PGG₂ resulting in the formation of the hydroxyl group on the PGH₂ (Vane and Botting, 1998; Simmons *et al.*, 2004; Blackwell *et al.*, 2010). Finally, the PGH₂ is utilized as a substrate by various isomerases and oxidoreductases to form the bioactive prostaglandins (PG) (Simmons *et al.*, 2004). These are prostaglandins I₂ (PGI), prostaglandins D₂ (PGD₂), prostaglandins F_{2α} (PGF_{2α}), prostaglandins E₂ (PGE₂) and thromboxane (TXA₂) (Simmons *et al.*, 2004).

PGs are lipids able to act in an autocrine and paracrine manner (Sales and Jabbour, 2003). Autocrine activity occurs when the released PGs act on the same cell such as in the GI tract. Paracrine activity occurs when nearby cells are affected by the release of PG such as in the reproductive system. The various biologically active forms are found in virtually all parts of the body (Al-Waili *et al.*, 2007). Interestingly, the synthesis of PG through COX mediated pathways, as well as the roles they play in the

bodies of fish are strikingly similar to their mammalian counterparts (David and Pancharatna, 2009).

COX exists in at least two isoforms, both of which have a molecular weight of 71 kd (Vane and Botting, 1998). They are distinguished as COX-1 and COX-2 based on slightly different activation sites as well as binding sites for arachidonic acid. One key difference is the size of the activation site. It is this size difference which various NSAIDs utilize to confer selectivity towards the inhibition of COX-1 or COX-2 (Vane and Botting, 1998).

COX-1, the constitutive form of COX, is expressed in many different tissue types (Al-Waili *et al.*, 2007). It plays various roles determined by the location in the body, all of which occur through COX mediated synthesis of PG (Flippin *et al.*, 2007). The COX-2 isoform is the induced form of COX and is expressed in fewer cell types (Al-Waili *et al.*, 2007). Specifically, this isoform serves to mediate the inflammation cascade as well as the resulting pain and fever response from stressors such as hypoxia and the presence of free radicals (Al-Waili *et al.*, 2007; Flippin *et al.*, 2007).

Both COX-1 and COX-2 have been sequenced for some fish species, and results have shown a high similarity with the mammalian DNA sequences (Zou *et al.*, 1999). Between the sequences present in rainbow trout (*Oncorhynchus mykiss*) and humans, the DNA sequences are 77% similar with COX-1 and 83-84% similar with COX-2 (Zou *et al.*, 1999). This similarity is nearly as high as that between humans and chickens, for which NSAIDs are widely used as a veterinary drug (Zou *et al.*, 1999; Boxall *et al.*,

2004). For humans and chickens, the sequence similarity is 77-78% for COX-1 and 89-90% for COX-2 (Zou *et al.*, 1999).

1.8.2 Ibuprofen and Naproxen

Several studies have examined the burden of PPCPs on the environment in an effort to discover the contaminant profiles of local Ontario surface waters. Similar to European studies, NSAIDs are ubiquitous in surface waters of North America and occur within the ng/L to low $\mu\text{g/L}$ range (Ternes, 1998; Metcalfe *et al.*, 2003; Lishman *et al.*, 2006). Of all detected pharmaceuticals, the NSAIDs ibuprofen and naproxen were consistently detected at higher concentrations in the effluents of more WWTPs than other drugs (Metcalfe *et al.*, 2003; Lishman *et al.*, 2006). Both NSAIDs are subject to high removal rates in WWTPs of up to 99%, yet ibuprofen is still detected at a median concentration of 4.0 $\mu\text{g/L}$ in Ontario surface waters and naproxen at 12.4 $\mu\text{g/L}$ by the same study (Metcalfe *et al.*, 2003).

Ibuprofen (2-(4-isobutylphenyl) propionic acid) is available without prescription and is the third most utilized drug in the world (Buser *et al.*, 1999). It is produced globally at several kilotons per year and is also one of the core medicines listed in the WHO's "Essential Drugs List" (Buser *et al.*, 1999; Han *et al.*, 2010). Ibuprofen has been placed on this list due to its wide use as an analgesic, antipyretic and anti-inflammatory (Han *et al.*, 2010). Ibuprofen is also prescribed to relieve the symptoms of arthritis and other rheumatic disorders (Han *et al.*, 2010). As with many pharmaceutical drugs, ibuprofen is specifically designed to be biologically active for use in human and veterinary medicine, hinting at the broadness of the COX target across species (Han *et al.*, 2010).

Ibuprofen is a nonselective NSAID with a COX-2/COX-1 inhibition ratio of 10/1 (Vane and Botting, 1998). Due to the vast number of cells and organs within the body that rely on COX-1 mediated PG production, the risk of non-target effects is high (Vane and Botting, 1998). Ibuprofen is a chiral molecule, though the pharmacological effects reside exclusively in the S enantiomer (Buser *et al.*, 1999). The drug is provided as a racemic mixture as the inactive r-enantiomer is converted to the active s-enantiomer *in vitro* (Buser *et al.*, 1999). It has a low vapour pressure of 1.86×10^{-4} mm Hg as well as a high solubility of 21 mg/L, indicating that once it is dissolved it will stay in that state (Ziylan and Ince, 2007). The log K_{ow} is 4.0 and the pK_a is 4.9 (Ziylan and Ince, 2007).

Ibuprofen can be excreted unchanged or in one of its many conjugates including hydroxyl-ibuprofen and carboxyl-ibuprofen (Ziylan and Ince, 2007). Interestingly, these biological metabolites are at least as toxic as the parent compound, both of which are suspected of endocrine disruption in humans and wildlife (Ziylan and Ince, 2007). When exposed to UV radiation, ibuprofen undergoes slow but significant photodegradation into one of several photoproducts (Castell *et al.*, 1987). This process is slow enough that it does not serve as a primary method of elimination in WWTPs or in the environment (Ziylan and Ince, 2007). Some of these photoproducts have been identified as having potential for hemotoxicity (Castell *et al.*, 1987). However, the rates of conversion from UV radiation in WWTPs and in surface waters are low enough that photoproduct concentrations necessary for toxicity are not reached (Castell *et al.*, 1987; Ziylan and Ince, 2007).

There is also the potential for degradation through oxidative, thermal and microbiological treatments resulting in a further production of byproducts (Caviglioli *et*

al., 2002). It is through these mechanisms that up to 92% of ibuprofen is degraded during the secondary treatment and disinfection stage of a WWTP (Ziylan and Ince, 2007). In the disinfection stage, the treatment must be via ozonation rather than chlorination for ibuprofen degradation to occur, as the latter is ineffective (Ziylan and Ince, 2007).

Naproxen (6-methoxy- α -methyl-2-naphthalene acetic acid), like ibuprofen, is another water soluble sodium salt belonging to the NSAID family and has been identified as one of the most pervasive pharmaceutical contaminants in Ontario surface waters (Lishman *et al.*, 2006). Unlike ibuprofen, naproxen selectively inhibits COX 2 over COX 1 at a higher ratio, the actual value of which is still controversial due to inconsistent results between laboratories. Reported ratios of COX-2/COX-1 inhibition have varied somewhat between laboratories with findings ranging from as low as about 3/1 to as high as 100/1 (Vane and Botting, 1998; Rao and Knaus, 2008; Solanki, 2010). However, the literature is consistent in that in the agreement that naproxen inhibits COX-2 significantly over COX-1 and is much more selective in the inhibition of COX than ibuprofen (Vane and Botting, 1998; Rao and Knaus, 2008; Solanki, 2010).

In terms of degradability, naproxen shares many similar attributes with ibuprofen (Ziylan and Ince, 2007). Its high solubility of 144 mg/L, low pK_a of 4.2 and low log K_{ow} of 3.3 renders it a challenging compound for removal in WWTPs and in the environment (Bjarnason *et al.*, 2007; Ziylan and Ince, 2007). Within the WWTP, naproxen is primarily degraded through various biodegradative processes which reduce the influent concentration by 36-99% by the time it becomes effluent (Ziylan and Ince, 2007). This occurs via various microorganisms during secondary treatment, and via chlorination and

ozonation during disinfection (Ziylan and Ince, 2007). However, the rate of consumption by the populace as well as heavy use in the veterinary community ensures that the effluent concentration still represents a significant contaminant loading in surface waters (Lishman *et al.*, 2006).

Naproxen is very sensitive to photodegradation and will generate several photoproducts which are 7-16 times more toxic than the parent compound (Isidori *et al.*, 2005). Despite a photo half life of 42 minutes, naproxen is not significantly degraded when UV radiation is used for disinfection in WWTPs (Packer *et al.*, 2003; Ziylan and Ince, 2007). Once in the surface waters, photodegradation is one of the primary fates of naproxen in the environment and serves as the main pathway of removal (Ziylan and Ince, 2007).

1.8.3 Prostaglandins Effects on Organs

The various forms of PG occur in different parts of the body with differing functionality. Pain and inflammation, two of the symptoms NSAIDs are indicated for, are a result of PGE₂ and PGI production, though PGE₂ has a stronger effect of the two (Simmons *et al.*, 2004). During the inflammatory responses, both PGE₂ and PGI are predominantly synthesized by the inducible COX-2 (Simmons *et al.*, 2004). The inflammation is then caused by the PG-induced vasodilatation, encouraging increased blood flow to the affected area (Simmons *et al.*, 2004). Pain response involves the same two PG isoforms, but does not result in the sensation of pain directly. Rather, it decreases the activation threshold of pain inducing mediators such as histamine (Simmons *et al.*, 2004).

Excess PG in the immune system can have an immunosuppressant effect (Simmons *et al.*, 2004). The PG responsible for this varies by species but it is generally accepted that PGE₂ is often involved (Simmons *et al.*, 2004). Other implicated PGs include PGI, PGD₂ and TXA₂, all of which when naturally present are produced by COX-1 in white blood cells (Simmons *et al.*, 2004).

PG synthesis is ubiquitous within the gastrointestinal tract. The PGs are synthesized by the epithelial and smooth muscles of the stomach (Simmons *et al.*, 2004). The EP1 receptor along the gastrointestinal tract is sensitive to PGE₂, responding with contractions of the smooth muscle (Simmons *et al.*, 2004). Along the gastrointestinal tract, PGE₂ and PGI have cytoprotective functionality, simultaneously reducing excretion of gastric acid and increasing the excretion of gastric mucus. This occurs through inducing vasoconstriction and vasodilatation for the acid and mucus excretory membranes respectively (Simmons *et al.*, 2004). Neither COX-1 nor COX-2 are exclusively responsible for the PG synthesis along the gastrointestinal tract, but a decrease in either enzyme increases the organism's sensitivity to colonic injury (Simmons *et al.*, 2004).

PGI, PGE₂ and PGF_{2α} are the primary cardiovascular prostanoids (Simmons *et al.*, 2004). PGI induces vascular relaxation while PGE₂ and PGF_{2α} have relaxing or constricting effects depending on the affected region of the vascular bed. The various roles PG can play in the cardiovascular system indicates that they play a key role in homeostasis, a finding supported in rainbow trout exposed to NSAIDs (Simmons *et al.*, 2004; Gravel and Vijayan, 2007; Gravel *et al.*, 2009). TXA₂ is also produced in the cardiovascular system and is the primary PG produced from platelet cells. This by-

product serves as a potent pro aggregator and vasoconstrictor (Simmons *et al.*, 2004). Normal cardiovascular epithelial cells express COX-1 to mediate the conversion of AA into the various PG though COX-2 production has been recognized as being able to be induced by stress (Simmons *et al.*, 2004).

The kidneys of vertebrates have varying concentrations of PG with the majority being PGE₂ and PGI₂ though there are some small quantities of TXA₂ (Simmons *et al.*, 2004). Increasing PG levels has a vasodilatation effect on the kidneys thereby increasing renal blood flow (Simmons *et al.*, 2004). However, while COX-1 is constitutively expressed in this section of the body, the primary pathway through which AA is metabolised is through cytochrome P450, an enzyme which also has a detoxifying role (Simmons *et al.*, 2004). Cytochrome P450 diverts the usual pathway of AA in the medullary cells of the loop of Henle in the nephron (Simmons *et al.*, 2004).

Reproduction is affected by several PGs including PGE₂, and PGF_{2α} (Simmons *et al.*, 2004). Of note are the results of COX-2 gene knockout mice studies which demonstrated infertility because the females did not ovulate (Simmons *et al.*, 2004). Generally this is due to PG's role in ovulation as evidenced by follicle rupture during ovulation being blocked by inhibition of PG synthesis (Stacey and Goetz, 1982). This finding was corroborated in 1976 in goldfish where intraperitoneal injections of indomethacin caused a complete termination of ovulation. Reproductive activity was restored with a subsequent injection of PGF_{2α} (Stacey, 1976). This finding was further supported in zebrafish where a decrease in PG production induced by a waterborne exposure to 100 µg/L of indomethacin stunted egg production (Lister and Van Der Kraak, 2008).

1.8.4 NSAID Toxicity on Survival, Growth and Reproductive Endpoints

The current state of knowledge for ibuprofen and naproxen toxicity is not well developed as testing has been limited and inconsistent. There is yet to be a strong consensus on the exact inhibition ratio of COX-2/COX-1 for either drug even in humans (Vane and Botting, 1998; Ziylan and Ince, 2007; Rao and Knaus, 2008; Solanki, 2010). Studies using fish have been performed to discover rudimentary toxicological information for these drugs finding LC_{50} and EC_{50} values in the mg/L range, well over environmentally relevant concentrations (Wayser *et al.*, 2011). Indeed several authors have highlighted the lack of environmental effects data for this class of drugs at environmentally relevant concentrations (Lawrence *et al.*, 2005).

Limited attempts to compare toxicity between pharmaceuticals in fish have been undertaken (Caminada *et al.*, 2006). In a study examining the effects of 24 hour incubations of fish hepatoma cells in solutions of various pharmaceuticals, EC_{50} data for ibuprofen and naproxen on the cell lines were determined (Caminada *et al.*, 2006). In this rare example of both drugs being simultaneously tested, it was established that the EC_{50} for ibuprofen (1.20 mM) was lower than that for naproxen (2.54 mM) indicating its higher toxicity (Caminada *et al.*, 2006). These findings were corroborated in studies on algae and daphnia which also found that ibuprofen had a higher toxicity than naproxen at a given concentration (Cleuvers, 2004). The EC_{50} on algae for ibuprofen and naproxen was 71.9 mg/L and 625 mg/L respectively while the EC_{50} on daphnia for ibuprofen and naproxen was 101.2 mg/L and 166.3 mg/L respectively (Cleuvers, 2004).

The effects of ibuprofen on the development of zebrafish eggs were examined in a recent study. While zebrafish typically can hatch as early as 3 days after fertilization,

significantly earlier than the 5-7 days for flagfish, this species is a comparable analog to flagfish for toxicological studies (Fogels and Sprague, 1977). Additionally, zebrafish show increased sensitivity to many reference toxicants including copper, pentachlorophenol and phenol when compared with the flagfish, a fact that must be considered when extrapolating information from zebrafish to flagfish (Fogels and Sprague, 1977).

Zebrafish in this study were assessed under progressive exposure concentrations of 1, 5, 10, 50 and 100 µg/L of ibuprofen (David and Pancharatna, 2009). Eggs (n = 10) were reared in a semistatic treatment in which solutions were renewed every 24 hours (David and Pancharatna, 2009). All ibuprofen exposures resulted in significantly increased cumulative mortality rates when compared with the controls (David and Pancharatna, 2009). This was assumed to be due to the need of eggs for PG to progress through the gastrulation and segmentation periods of embryo development (David and Pancharatna, 2009). Support for this assumption was observations of loss of organization in the development of the optical vesicles, brain, and somites as well as the extrusion of blastomeres into the chorionic space for fish exposed to 100 µg/L of ibuprofen (David and Pancharatna, 2009). Hatching was also significantly affected by the exposure concentration of the drug with increasing concentrations resulting in delays or failure to hatch altogether (David and Pancharatna, 2009). This effect was predominantly evident in embryonic exposures of 10, 50 and 100 µg/L (David and Pancharatna, 2009). An alarming finding for embryonic exposures of ≥ 10 µg/L was the detection of pericardial edema, decreased heart rate, and loss of pectoral fins (David and Pancharatna, 2009).

This critical life stage exposure indicates that the embryo stage of teleost species can be vulnerable to higher ibuprofen exposures (David and Pancharatna, 2009).

In a study on goldfish follicles, the NSAID indomethacin was shown to negatively impact the production of testosterone (Mercure and Van Der Kraak, 1996). Indomethacin is a nonselective COX inhibiting NSAID with an inhibition ratio between that of naproxen and ibuprofen (Mercure and Van Der Kraak, 1996; Vane and Botting, 1998). The fully grown ovarian follicles, maintained on a tissue growing plate, were assessed for PGE₂ and testosterone with the overall goal of discovering AA's effect on testosterone steroidogenesis (Mercure and Van Der Kraak, 1996). Ovaries were doused with 100 µM of AA, the effect of which was observed with and without the presence of 40 µM of indomethacin (Mercure and Van Der Kraak, 1996). AA alone was found to stimulate the production testosterone and PGE₂, a finding that was attenuated by the addition of indomethacin (Mercure and Van Der Kraak, 1996). Interestingly, when indomethacin was added without AA, testosterone production was stimulated in comparison to the control (Mercure and Van Der Kraak, 1996). These results serve to highlight the importance of AA and PGE₂, one of the major COX products, on steroidogenesis in fish (Mercure and Van Der Kraak, 1996).

A full lifecycle study of various PPCPs was conducted on fathead minnows with hatching success, larval survival, growth over time, length, weight, condition factor of adults, liver-somatic index, gonadosomatic index of adults, secondary sexual characteristics, hormone production by testes and ovaries, egg production, number of spawns, egg fertilization, egg hatching, and deformities in F1 offspring (Parrott and Bennie, 2009). Fish were grown until 7 days post-hatch after which they were transferred

into aquaria where they were exposed to a water control, an ethanol control or a pharmaceutical mixture of naproxen, gemfibrozil, diclofenac, ibuprofen, triclosan, salisilic acid and acetaminophen (Parrott and Bennie, 2009). The mixtures were present at nominal concentrations of 0.01, 0.03, 0.1, 0.3 or 1 µg/L (Parrott and Bennie, 2009). Measured concentrations compared with the nominal declined by 21-78% for all drugs except for acetaminophen which was undetectable in the treatment aquaria (Parrott and Bennie, 2009). Five measurements of growth as determined by weight were taken between 26 days post hatch and 93 days post hatch in addition to the other previously mentioned endpoints which were assessed (Parrott and Bennie, 2009). Among all of these analysis, only deformities in the fry of the 0.1 and 0.3 µg/L exposed fish were significantly different from the control (Parrott and Bennie, 2009). It was concluded from the findings that this spread of environmentally relevant concentration mixture did not significantly impact the growth, development and reproduction of the fathead minnow (Parrott and Bennie, 2009). However, while the concentrations used in this experiment were within the range of environmental relevance, they were in the lower range of what is detected (Ternes, 1998; Metcalfe *et al.*, 2003; Lishman *et al.*, 2006; Ziylan and Ince, 2007). Cleuvers (2004) indicated that NSAIDs have increased toxicity when in mixtures which may also help explain the observed effects in this study (Parrott and Bennie, 2009).

Reproductive endpoints as well as HSI and COX enzyme activity were specifically studied in Japanese medaka (*Oryzias latipes*) exposed to 1, 10 and 100 µg/L ibuprofen (Flippin *et al.*, 2007). Juvenile medaka approximately 2 months in age were exposed to ibuprofen for 5 weeks at which point breeding groups were established

(Flippin *et al.*, 2007). Breeding pairs were observed for 7 days for frequency of reproduction, clutch size, fertilization of eggs and average egg size. After sampling, HSI was assessed as well as COX activity (Flippin *et al.*, 2007).

An interesting trend was observed between clutch size and days with eggs. With increasing concentration of ibuprofen the number of days with eggs decreased but those days with eggs had increased clutch size (Flippin *et al.*, 2007). Analysis of HSI revealed it was significantly higher for females than for males though neither differed significantly from their sex specific controls (Flippin *et al.*, 2007). COX activity decreased in females following exposure to 10 and 100 µg/L. The overall effect on COX across both sexes was a decrease in the variability of detected levels following exposures (Flippin *et al.*, 2007). It was concluded from these findings that reproduction was significantly affected by ibuprofen exposure, though not in a way which would affect population growth in this species (Flippin *et al.*, 2007). These results do indicate however, that species which require a highly coordinated spawning time with their surroundings (e.g. cod or salmon) may experience a population scale effect as a result of ibuprofen exposure (Flippin *et al.*, 2007).

NSAIDs have also been implicated in the disruption of cortisol production in high acute exposures of 0.1 µg/mg (converted from 100mg/kg) in tilapia (*Oreochromis mossambicus*) as well as rainbow trout (*Oncorhynchus mykiss*) (van Anholt *et al.*, 2003; Gravel and Vijayan, 2007). Both studies were examples of oral exposures to NSAIDs as the salicylic acid was administered via laced food (van Anholt *et al.*, 2003; Gravel and Vijayan, 2007). Cortisol is a key hormonal response to stress in fish, the synthesis of which is mediated by PG in fish (Gravel *et al.*, 2009). This suggests that cortisol may be

a secondary target of NSAIDs in fish (Gravel *et al.*, 2009). Analysis determined that metabolic capacity of the liver was also reduced indicating that NSAIDs may disrupt homeostasis in rainbow trout (Gravel and Vijayan, 2007; Gravel *et al.*, 2009).

2.0 RESEARCH OBJECTIVES

2.1 Overall Objective

The overall objective of this project was to determine and compare the chronic toxicity of nonselective and selective nonsteroidal anti-inflammatory drugs (NSAIDs) on the growth, survivability and reproduction of Florida flagfish (*Jordanella floridae*).

2.2 Specific Objectives

This was accomplished by:

- 1) Determining the validity of sublethal concentrations of ibuprofen and naproxen for use in a chronic study. These chemicals are representative nonselective and selective NSAIDs respectively.
- 2) Assessing the toxicity of these NSAIDs over two successive generations of flagfish on the growth, survivability and reproduction as defined by survivability, morphological abnormalities, length, weight, condition factor, egg production fertilization, time to hatch and hatchability.

Null hypotheses tested in the full life cycle study were: Chronic exposure to the NSAIDs ibuprofen and naproxen will have no affect on growth, survivability or reproduction of flagfish.

3.0 MATERIALS AND METHODS

3.1 Laboratory Fish

Third generation from wild flagfish were used as the parental generation for all experimental fish. These fish were held in 70 L aquaria in the aquatic toxicology laboratory of the University of Ontario Institute of Technology. This stock population was maintained in 70 L glass aquaria with a photoperiod of 16 hours light, 8 hours dark; 0.5 hours of dusk and dawn were included in the light portion of the cycle.

3.2 Feed

Three feeds were used for initial breeding populations as well as throughout the chronic exposures. Tetramin Pro flake (Tetra, Oshawa Ontario) was fed to all adult and breeding populations once daily. The flake contained a minimum crude protein of 46.0 %, 12.0 % crude fat, 3.0 % crude fibre, 1.1 % phosphorus, 200 mg/kg ascorbic acid and maximum moisture of 8.0 % as listed on the packaging. All life stages from fry to breeding populations were fed fresh brine shrimp *nauplii* hatched from premium grade brine shrimp eggs (Brine Shrimp Direct, Oshawa Ontario) three times daily. Breeding populations were fed Bio-pure frozen brine shrimp (Hakari, Oshawa Ontario) three times daily and was also purchased at Big Al's in Oshawa Ontario. The frozen brine consists of 6.8 % crude protein, 1.5 % crude fat, and 1.2 % crude fibre, and has a maximum of 86.0 % moisture.

3.3 Temperature and pH

A waterproof thermometer (VWR) and used for daily temperature measurements. To ensure no cross contamination, 70 % denatured ethanol was applied to the thermometer for sterilization directly before a measurement was taken. Alteration of drug concentrations between tanks was minimized by measuring tanks in order of increasing concentration. pH of the general laboratory 25° C process water was assessed daily using the SevenEasy pH meter purchased from Mettler Toledo. This water was used both for the crystallization dish stage and 70 L aquaria stage of the experiment.

3.4 Parental Breeding Population

3.4.1 Breeding Tanks Setup

Two males and four females were selected from the general lab population for use in a breeding harem. Fish were selected so that one larger dominant male was present along with one smaller sub dominant male. Females were all selected according to size and health so that all females were similar in size and slightly smaller than the dominant male. Breeding harems were placed into 70 L aquaria. Aquaria contained an air stone and a breeding substrate. The breeding substrate was constructed out of a glass plate tightly wrapped with well washed green wool yarn. A rate of flow of 2.31 mL/sec was used to allow 5 daily turnovers of the aquarium water, achieving a molecular turnover of 99.9 % in the aquaria daily (Sprague, 1969). Breeding harems were fed 4 times daily to encourage egg production. First feed consisted of flake, fresh and frozen brine. Second feed occurring about 2.5 hours later was exclusively fresh brine. The third feed was fresh

and frozen brine while the final feed was once again just fresh brine. The offspring (F1) produced by the parental fish were used for both chronic and egg exposure experiments.

3.4.2 Egg Collection

Eggs were collected once every 24 hours. Breeding substrates were removed from the breeding tank while wearing shoulder length rubber gloves cleansed with ethanol between tanks. Extraction of the eggs was performed by gently rubbing the substrate by hand, expelling the eggs into a polycarbonate basin filled with 25° C laboratory water for counting. Those destined for experimental uses were transferred to sterile plastic Petri dishes containing rearing solution while all other eggs were discarded. Rearing solution is a mixture of 10 % NaCl, 0.30 % KCl, 0.40 % CaCl₂·2H₂O, 1.63 % MgSO₄·7H₂O, and 0.01 % methylene blue, dissolved into Milli-Q water. Increased salinity as well as presence of an antifungal solution in conjunction with the other ions allows an increased proportion of fertilized eggs to hatch (St Mary *et al.*, 2004).

After a four hour soaking period in rearing solution to allow the induction of methylene blue into unfertilized eggs as well as initial cleavages to occur, eggs were cleansed through gentle expulsion of rearing solution from a 1 mL bulb pipette. Larger debris was removed using tweezers under a light microscope. Eggs were then assessed for fertilization using a developmental map for Japanese medaka (*Oryzias latipes*) and deemed acceptable if they had reached stage 9, the late morula stage (Iwamastu, 2004). Only fertilized eggs were used for experimentation.

3.5 Glassware and Plastics Cleaning

All glassware for crystallization dish stage of the experiment was cleaned using a Miele Professional laboratory dish washer. The instrument was run on the intensive setting using LaboClean F detergent from NeoDisher. Glassware for this stage included glass crystallization dishes and beakers. Plastics were cleaned in the same manner as the glassware except the dishwasher was run on the plastics setting. Tanks for the aquaria stage of the experiment were cleaned using Liquinox as a cleaning agent and Vircon as a disinfectant.

3.6 Egg Exposure Study

Fertilized eggs were collected using the method described in Section 3.4.2 Egg Collection. Eggs were then transferred into 6 well Petri dishes containing 10 mL of rearing solution. Densities within each well were dependent on the number of eggs collected on a given day though no trials used less than 12 eggs per replicate. Once sorted, 10 mL of rearing solution and 0.1 mL of working stock were added to each well to reach the desired treatment concentrations. Nominal concentrations used were 0.1 $\mu\text{g/L}$, 1 $\mu\text{g/L}$, 5 $\mu\text{g/L}$, 10 $\mu\text{g/L}$, 50 $\mu\text{g/L}$ and 100 $\mu\text{g/L}$. All eggs used were between stage 9 (late morula) and stage 11 (late blastula) allowing for an estimated exposure starting age of 4-6 hours post fertilization (Iwamatsu, 2004).

Eggs were checked once daily. Wells were assessed for mortality, malformations, and successful hatch. Additionally, a total count of individuals within each well was preformed daily to ensure precision. Wells were subject to daily 95 % solution renewal. Once mostly drained, 10 mL of rearing solution and 0.1 mL of working stock was added

to recreate the desired treatment concentrations. The experiment was conducted twice, in duplicate for ibuprofen and naproxen.

3.7 Chronic Exposure Study

3.7.1 Egg Hatch and Larval Rearing

Eggs were collected using the parental breeding tanks described in Section 3.4 Parental Breeding Population and collected as described in Method 3.4.2 Egg Collection. Once fertilization was determined using the method described in Section 3.4.2 Egg Collection, eggs were transferred to plastic 50 mL Petri dishes filled with rearing solution with a density not exceeding 100. During gestation the petri dishes were subject to a 95 % daily solution change of the contained rearing solution. Once all eggs in a given Petri dish had hatched, the larva were transferred to a polycarbonate plastic basin filled with 25° C lab water. Eggs from a spread of 3 days were pooled in the polycarbonate basin to randomize individuals. This ensured that each treatment in the chronic exposure study used a random sample of individuals 8±1 days post hatch. Petri dishes and the polycarbonate basin were kept in a temperature control room set at 27° C allowing the semi-static liquid to maintain a temperature of 26 ± 0.5° C (St Mary *et al.*, 2004).

3.7.2 Transfer into Crystallization Dishes and First Series of Growth Measurements

Pooled 8±1 days post-hatch larval fish were used for the initiation of the chronic exposure. 30 individuals were transferred into each of the 300 mL crystallization dishes containing 200 mL of 25° C lab water. To this were added 2 mL of a concentrated stock created in the method described in section 3.8 Chemicals. Nominal exposure

concentrations of each drug used were 0.1, 1, 10 and 100 µg/L as well as a control of 0 µg/L. Both the ibuprofen and naproxen chronic exposure experiments were run in duplicate.

Fresh brine shrimp *nauplii* were fed to the fry 3 times daily. Excess food was siphoned and a 75% daily renewal (150 mL daily) was used to which 1.5 mL of working stock was added to reach a daily overall volume of 202 mL ± 5 %. Mortality and deformities were enumerated and recorded when they were observed. Water parameters were monitored as described in Section 3.3 Temperature and pH.

On day 14 and 28 all crystallization dishes were placed over 1 mm graph paper and photographed for an assessment of growth rate and overall size. Photographs were taken using a Fujifilm Finepix F80EXR camera. Lengths were assessed using Image J software. Analysis of each photograph was individually calibrated using the software to account for variations in the position of the camera. Only fish observed to be in a straight position were assessed to ensure accurate measurements were taken. All fish in the photographs were counted to provide a measurement of mortality.

3.7.3 Transfer into 70 L Aquaria, Feed Regime and Chronic Exposure

At day 29 fish were transferred into 70 L aquaria containing 40 L of the corresponding treatment. A flow rate of 2.31 mL/sec was established to allow 5 complete water changes daily, resulting in a 99.9% molecular turnover over a 24 hour period. Fish were fed brine shrimp *nauplii* 3 times daily. Feed was increased weekly to keep pace with the size of the fish. Excess food and debris were removed daily. Starting on day 57 and continuing for the rest of the chronic exposure, the feeding regime was altered to

include three daily feeds of frozen brine shrimp. On day 75 a breeding substrate was added to each aquarium and fish were monitored for guarding behaviour.

Chronic exposure was maintained through the use of a peristaltic pump which drew from stock bottles with solutions made up weekly. All other parameters were measured as described in Section 3.3 Temperature and pH.

3.7.4 Thinning and the 3rd, 4th and 5th Growth Assessment

On day 57, all replicates were thinned down to 16 fish with the actual number of fish euthanized being determined by the mortality over the first 2 months. Fish were assessed for wet weight, total length and condition factor. Weight was determined using an electronic scale with an associated error of ± 1 mg. Total length was determined using analog callipers with an associated error of ± 0.05 mm. Sex was determined when possible although the absence of secondary sex characteristics caused the majority of fish to appear as female. On day 85 an additional thinning was used to reduce all replicates to 2 males and 4 females to allow the formation of a breeding harem. All euthanized fish were assessed for wet weight, total length condition factor and sex. Egg production was recorded daily for each harem as well as the presence or absence of guarding behaviour at each feed. Breeding harems were sampled and assessed on day 113. At 30 days post hatch, all F2 fish were euthanized. Prior to sampling, all fish were euthanized using a solution consisting of MS-222 (180 mg) and sodium bicarbonate (360 mg) dissolved into 500 mL of water. Death was assessed through loss of equilibrium and a lack of response to external stimuli.

3.7.5 Egg Collection and Larval Rearing

Eggs were collected from breeding harems once daily in between the first and second feed. Once steady spawning had been achieved as defined by 4 days in a row with 30 or more eggs present on the breeding substrate (Beyger, 2009), a set of up to 45 eggs was collected and raised up to 30 days post hatch using the method outlined in Sections 3.4.2 Egg Collection and 3.7.1 Egg Hatch and Larval Rearing. Subsequent eggs collected over the next 19 days were collected in the method outlined in the next section.

3.7.6 Further Reproductive Assessment

Of the eggs collected daily from each breeding harem, 10 eggs were randomly selected and placed into a well of a 6 well Petri dish containing 10 mL of rearing solution. Daily observations were made using a 10x EZ410 microscope from Leica. 24 hours after collection, eggs which had not developed were considered unfertilized and removed. Remaining fertilized eggs were observed until hatch. Assessment for mortality, hatch and malformations were conducted each day. All mortalities were removed as they were observed and all malformations were photographed using the microscope's built in camera. During the gestation period all wells were subject to a 50 % daily renewal of rearing solution.

3.7.7 Condition Factor

Condition factor was used to assess the health of the fish. It was calculated using the following formula (Smolders *et al.*, 2002):

$$\text{condition factor} = (W \div L^3) \times 100$$

Where W is the total body weight of the fish in milligrams and L is the total length in mm.

3.8 Chemicals

Ibuprofen sodium ($C_{13}H_{17}O_2Na$) and naproxen sodium ($C_{14}H_{13}O_3Na$) were purchased from Sigma Aldrich Co (Figure 2). Salts were used to increase solubility. Stock solutions were prepared by weighing out the appropriate quantity of the drug determined by molecular weight of the pure chemical, then performing serial dilutions to reach the desired concentrations. Measurements were carried out using a PB50 3-S microbalance from Mettler Toledo and the drugs were dissolved into deionised water. Working solutions were prepared through serial dilutions of the stock. For both ibuprofen sodium and naproxen sodium, analytical standards were used ($\geq 98\%$).

For the egg exposure experiment working stocks for the 0.1, 1, 5, 10, 50 and 100 $\mu\text{g/L}$ treatments were 10 100, 5050, 1010, 505, 101 and 10.1 $\mu\text{g/L}$ respectively. During the crystallization dish stage of the chronic exposure experiment the same stock concentrations were used for the 0.1, 1, 10 and 100 $\mu\text{g/L}$ treatments. The working stock concentrations during the 70 L aquaria stage of the chronic exposure experiment for the 0.1, 1, 10 and 100 $\mu\text{g/L}$ treatments were 185.19, 18.5, 1.85 and 0.185 mg/L respectively.

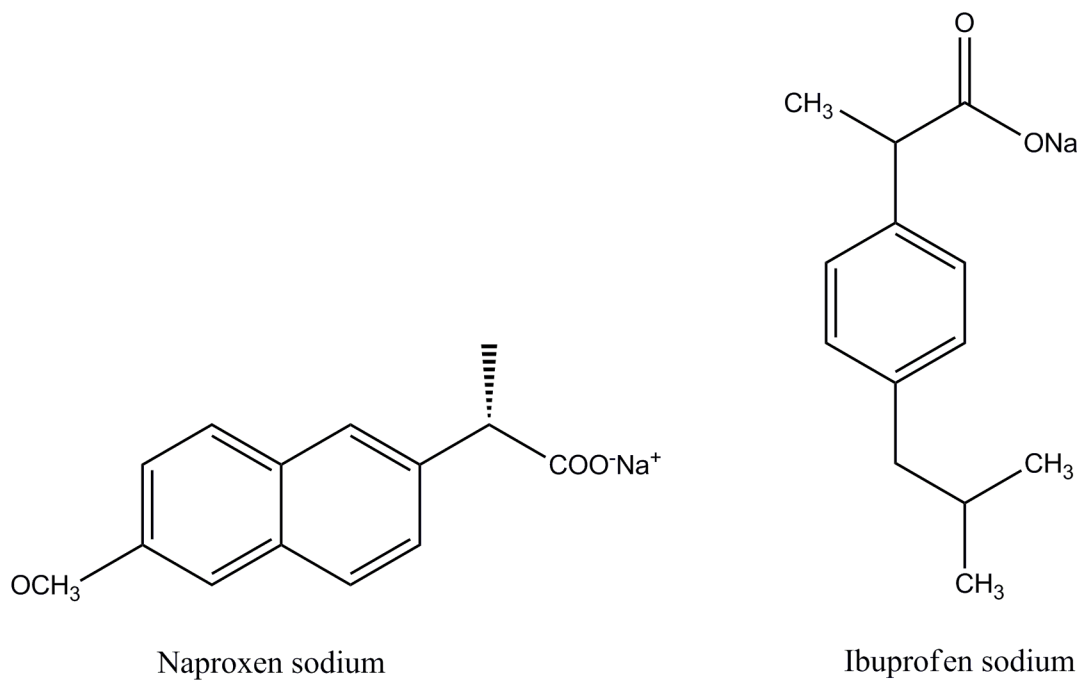


Figure 2: Ibuprofen sodium and naproxen sodium chemical structure.

3.9 Statistical Analysis

Data manipulation was performed using Microsoft Excel 2007. Graphs were created using Statistica 6 by Statsoft and Sigmaplot 11 by Systat Software Inc. All statistical analysis was performed using Statistica 6. Data was tested for normality using the Shapiro-Wilk's W test for normality. Data which failed this test was transformed using the equation $\log_{10}(x)$ or x^2 . Factorial analysis of variance (ANOVA) was used to analyze the differences between treatments, generations, sexes, drugs and sampling times. When significant differences were detected a Tukey's HSD post-hoc test gave a more detailed breakdown of where the differences existed. A type one error of $\alpha \leq 0.10$ was used to reduce the chance of type II error (false negative) and increase experimental power.

4.0 RESULTS

4.1 Ibuprofen

4.1.1 Abiotic Parameters

Temperatures recorded during the crystallization dish stage were significantly lower than in the 70 L aquaria (Table 1). pH did not differ significantly during the experiment (Table 1).

Table 1: Average abiotic factors measured during chronic exposure. Values are written as means \pm standard error.

Generation	Experimental Stage	Temperature	pH
F1	Crystallization Dish	22.6 \pm 0.06	7.51 \pm 0.10
F1	70 L Aquaria	25.2 \pm 0.01	7.12 \pm 0.05
F2	Crystallization Dish	22.3 \pm 0.06	7.23 \pm 0.09

4.1.2 Egg Exposure Experiment

No significant differences in percent total hatch were detected in the ibuprofen egg exposure experiment (Figure 3).

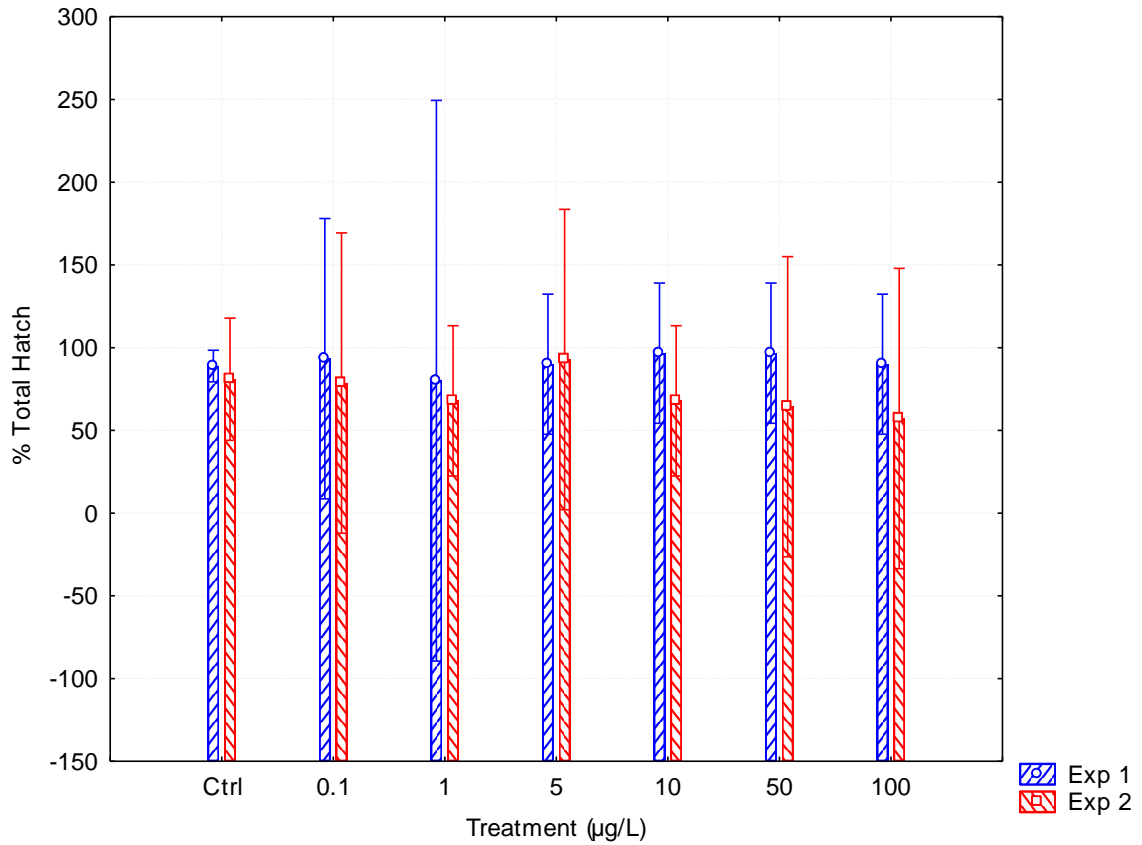


Figure 3: Impact of chronic ibuprofen exposure on the hatchability of eggs. Error bars denote standard error. Results for the experiments were statically different and so were analyzed separately. Sample size was 15 for experiment one and 14 for experiment two. No significant differences were detected between treatments ($p > 0.10$).

4.1.3 Growth

Replicates did not differ significantly at the first measurement period (14 ± 1 days post hatch) of the chronic ibuprofen experiment, allowing for pooling of the data. Flagfish chronically exposed to 1, 10 and 100 $\mu\text{g/L}$ of ibuprofen were significantly longer than control fish and those exposed to 0.1 $\mu\text{g/L}$ of ibuprofen (Figure 4).

At the second measurement period on day 28 ± 1 , the results of the replicates could not be pooled and so were analyzed separately. No treatments in either replicate A or B differed significantly from their respective controls (Figure 5).

During the first thinning at 54 ± 1 days, fish were assessed for length, weight and condition factor. Replicates were not significantly different from each other in any of these parameters and thus were pooled for further analysis. Condition factor could not be normalized and thus were analyzed using nonparametric statistics. No significant differences were detected for any of these analyses (Figure 6, 7 and 8).

On day 93 ± 1 , fish were assessed for length, weight and condition factor prior to the setup of breeding harems. Additionally, fish were now large enough to determine gender. Total length and wet weight were log transformed allowing for parametric analysis. Condition factor could not be normalized at this point and so was analyzed using nonparametric statistics. Data for replicates could not be pooled for both log total length (Figure 9) and log wet weight (Figure 10) and so were analyzed separately. Data for sexes could also not be pooled for log total length and log wet weight as males were significantly longer than females in both replicates A and B. During the further analysis between replicates, sexes and treatments for log total length, the only significant

difference detected was between females exposed to 1 µg/L in replicate B and males exposed to 10 µg/L in replicate A (Figure 9). No significant differences were detected for log wet weight (Figure 10). Results for sexes and replicates were not significantly different with regards to condition factor and so all data was pooled. While there were no differences from the control, fish exposed to 100 µg/L had a significantly higher condition factor than fish exposed to 1 µg/L (Figure 11).

On day 121 ± 1 the breeding harems were sampled and assessed for total length, wet weight and condition factor. The results from all three analyses did not significantly differ between replicates and so the data was pooled. Data for total length and wet weight were transformed to allow for parametric analysis. Data for sexes with regard to log total length and log wet weight could not be pooled as males were significantly longer and heavier than females (Figures 12 and 13). Analysis of log total length revealed no significant differences between treatments within each sex (Figure 12). Females exposed to 1, 10 and 100 µg/L were not significantly different from the smaller males exposed to 10 µg/L (Figure 12). Females exposed to 10 µg/L were also not significantly different from males exposed to 100 µg/L (Figure 12). Analysis of wet weight also revealed that there were no significant differences between treatments within each sex (Figure 13). Females exposed to 1, 10 and 100 µg/L were not significantly different from males in the control and those exposed to 1 and 10 µg/L (Figure 13). Females exposed to 10 µg/L were also not significantly different from males exposed to 100 µg/L (Figure 13). Although the results of males and females could not be pooled for the analysis of condition factor nor was the data able to be normalized, nonparametric statistics did not reveal any significant differences between groups (Figure 14).

Analysis of the second generation fish was unable to be conducted on fish exposed to 0.1 $\mu\text{g/L}$ as insufficient eggs were laid to permit statistical analysis. At the first measurement of total length for these second generation fish on day 14 ± 1 , the results of the two replicates were not significantly different and so were pooled. Fish exposed to 100 $\mu\text{g/L}$ were significantly longer than control fish but not significantly longer than fish exposed to 1 or 10 $\mu\text{g/L}$ (Figure 15).

On day 28 ± 1 , the second generation fish were analyzed once again and assessed for total length. The replicates were not significantly different allowing their results to be pooled. Fish exposed to 10 and 100 $\mu\text{g/L}$ were significantly longer than fish exposed to 1 $\mu\text{g/L}$ and fish in the control treatment (Figure 16).

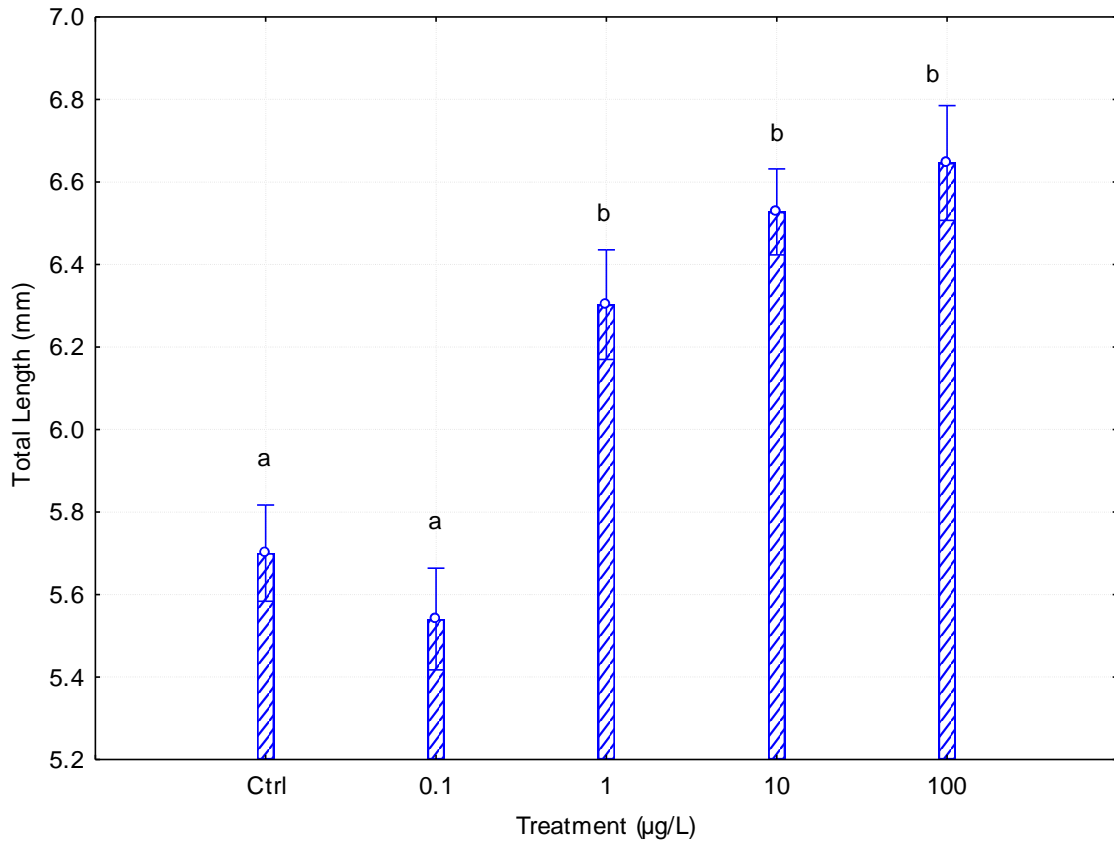


Figure 4: Total length of 14 ± 1 day-old flagfish of a chronic ibuprofen exposure experiment. Error bars denote standard error. The results of two replicates were pooled. Sample sizes for control (Ctrl), 0.1, 1, 10 and 100 $\mu\text{g/L}$ were 37, 40, 37, 38 and 34, respectively. Bars without a letter in common are significantly different from each other ($p \leq 0.10$).

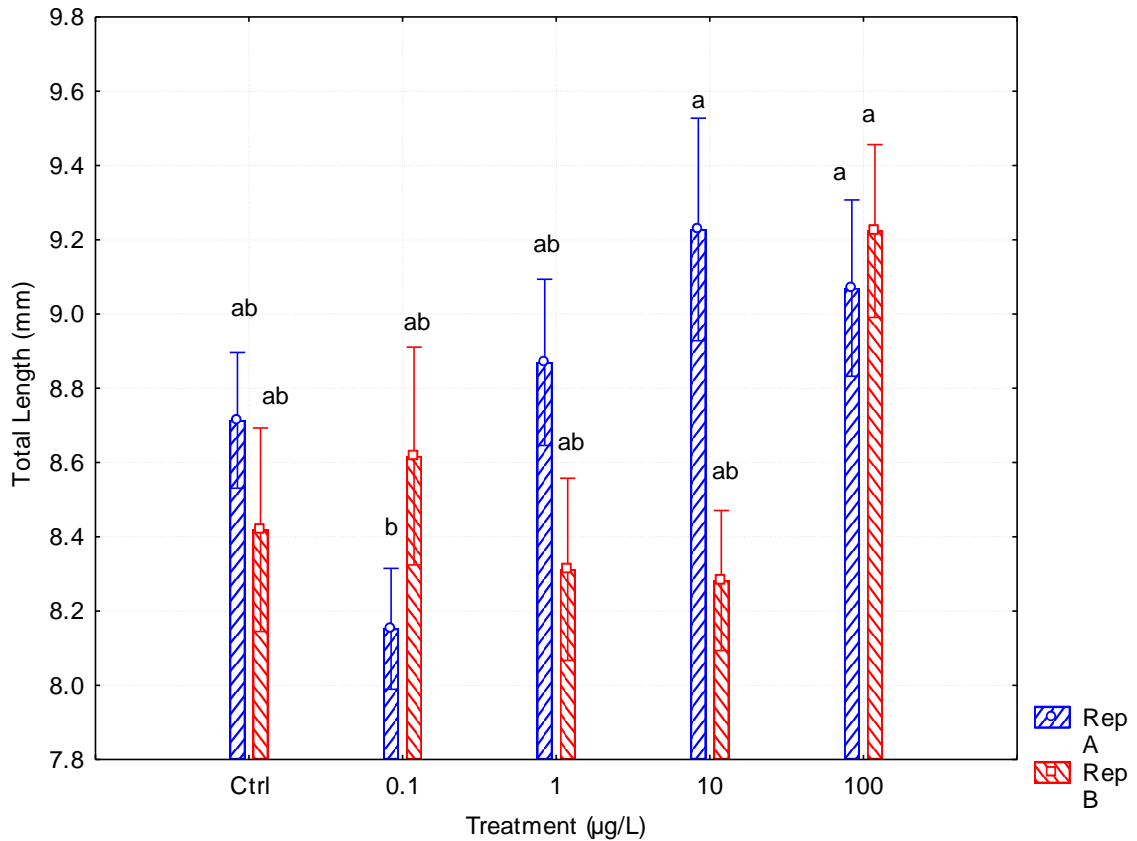


Figure 5: Total length of 28 ± 1 day-old flagfish of a chronic ibuprofen exposure experiment. Error bars denote standard error. The results of two replicates could not be pooled. Sample sizes for replicate A Ctrl, 0.1, 1, 10 and 100 $\mu\text{g/L}$ were 24, 25, 24, 16 and 22, respectively. Sample sizes for replicate B control (Ctrl), 0.1, 1, 10 and 100 $\mu\text{g/L}$ were 18, 19, 27, 24 and 16, respectively. Bars without a letter in common are significantly different from each other ($p \leq 0.10$).

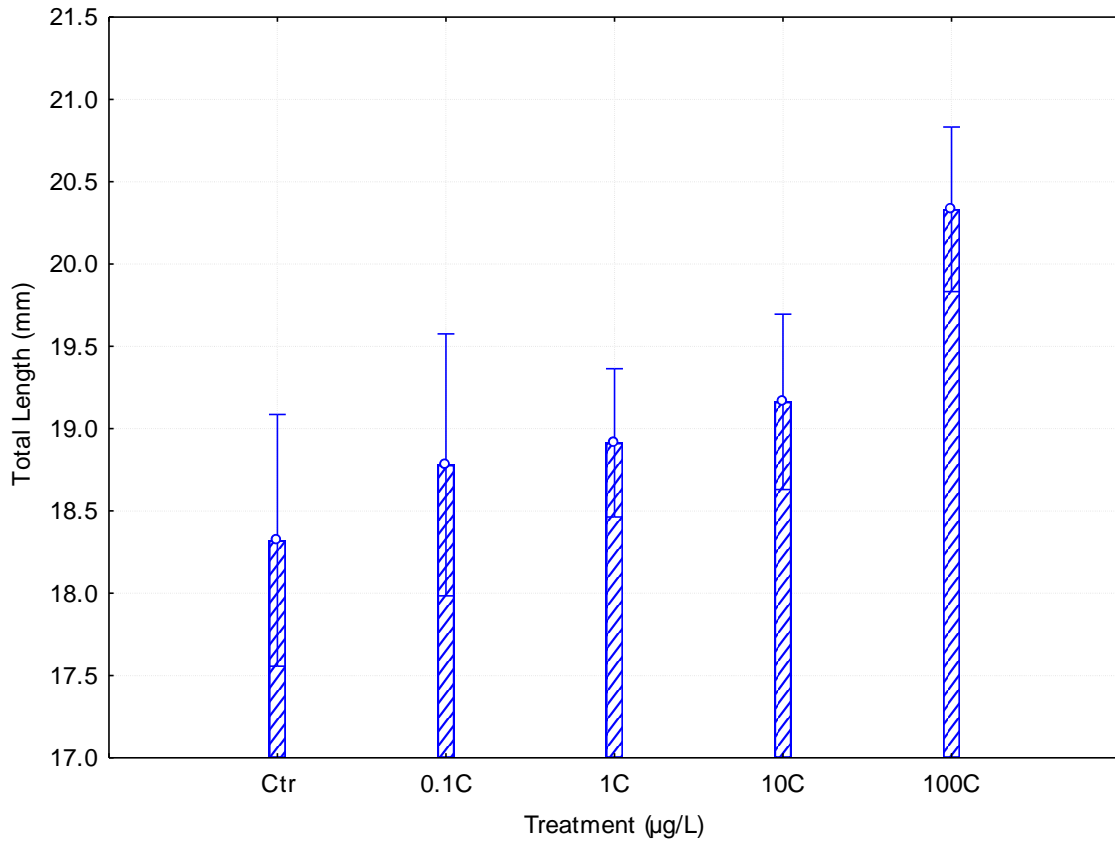


Figure 6: Total length of 54 ± 1 day-old flagfish of a chronic ibuprofen exposure experiment. Error bars denote standard error. The results of two replicates were pooled. Sample sizes for control (Ctrl), 0.1, 1, 10 and 100 µg/L were 21, 16, 22, 16 and 14, respectively. No significant differences were detected ($p > 0.10$).

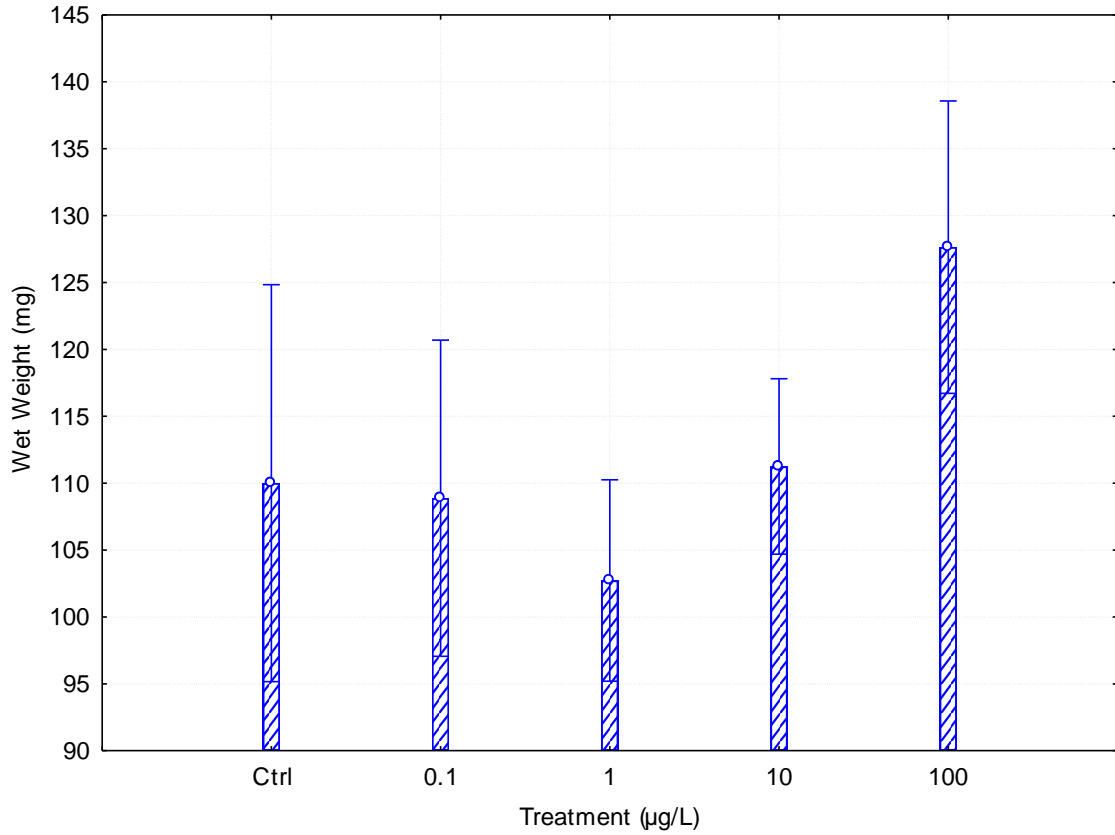


Figure 7: Wet weight of 54 ± 1 day-old flagfish of a chronic ibuprofen exposure experiment. Error bars denote standard error. The results of two replicates were pooled. Sample sizes for control (Ctrl), 0.1, 1, 10 and 100 µg/L were 21, 16, 22, 16 and 14, respectively. No significant differences were detected ($p > 0.10$).

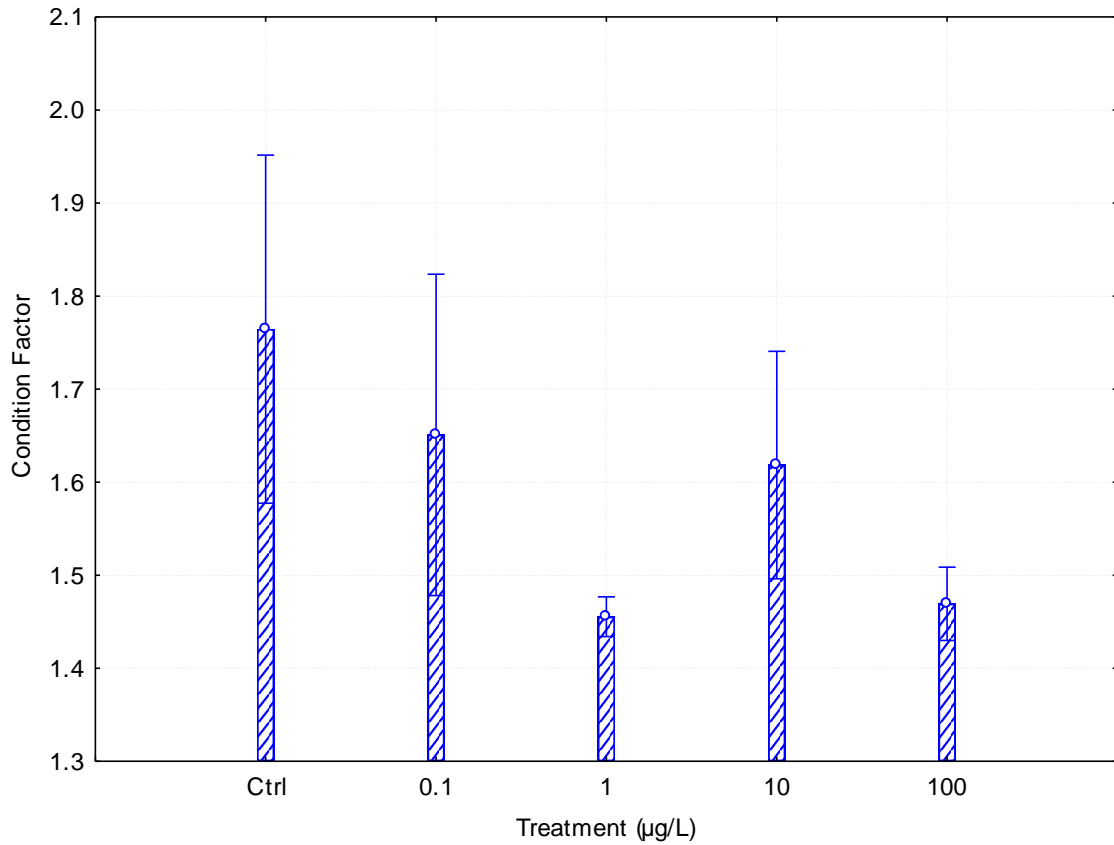


Figure 8: Condition factor of 54 ± 1 day-old flagfish of a chronic ibuprofen exposure experiment. Error bars denote standard error. Data could not be normalized. The results of two replicates were pooled. Sample sizes for control (Ctrl), 0.1, 1, 10 and 100 µg/L were 21, 16, 22, 16 and 14, respectively. No significant differences were detected ($p > 0.10$).

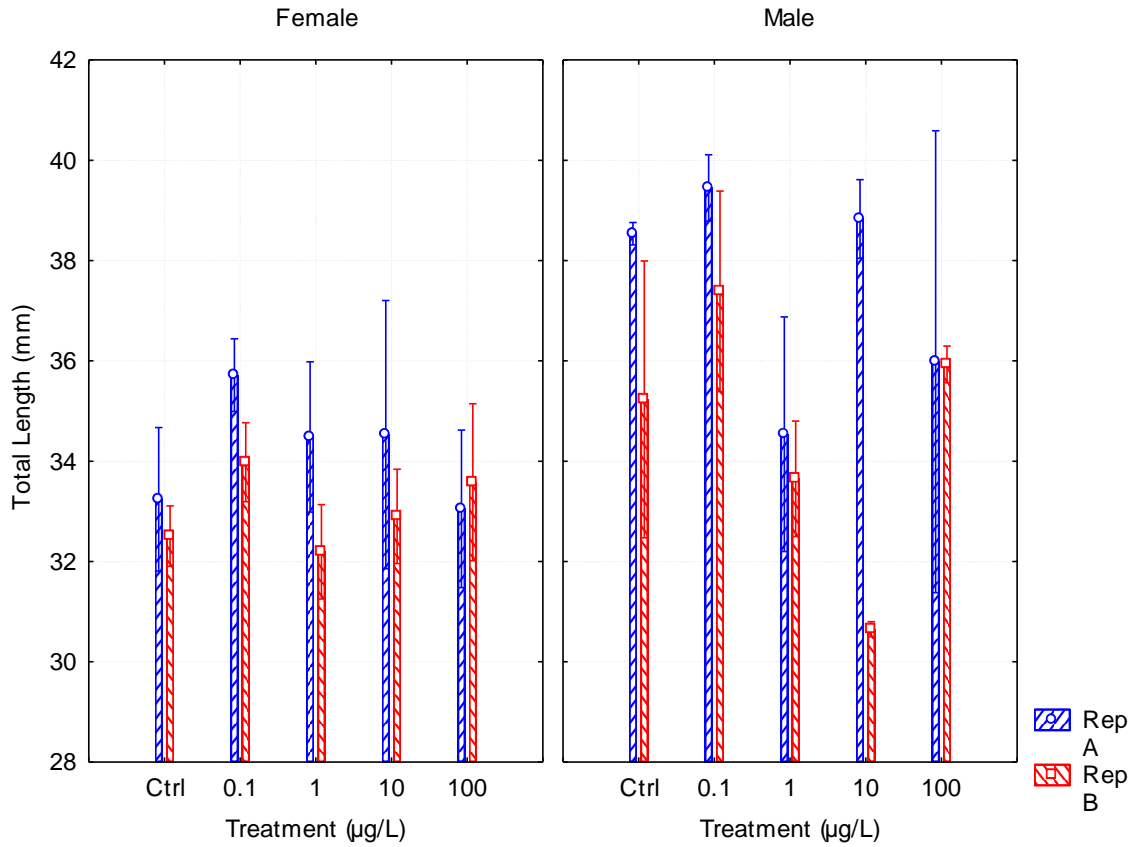


Figure 9: Total length (logged for analysis) of 93 ± 1 day-old flagfish of a chronic ibuprofen exposure experiment. Error bars denote standard error. The results of two replicates and sexes could not be pooled. Sample sizes are given in table 2. No significant differences were detected ($p > 0.10$).

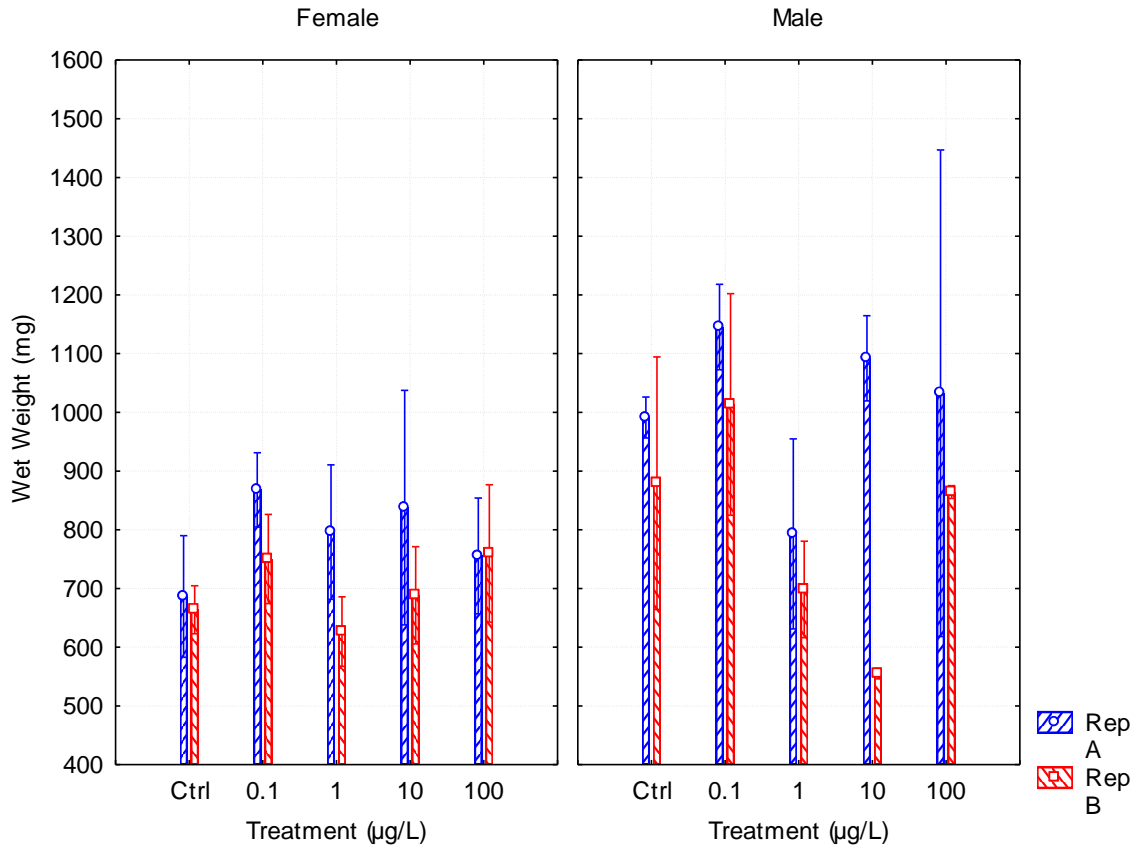


Figure 10: Wet weight (logged for analysis) of 93 ± 1 day-old flagfish of a chronic ibuprofen exposure experiment. Error bars denote standard error. The results of two replicates and sexes could not be pooled. Sample sizes are given in table 2. No significant differences were detected ($p > 0.10$).

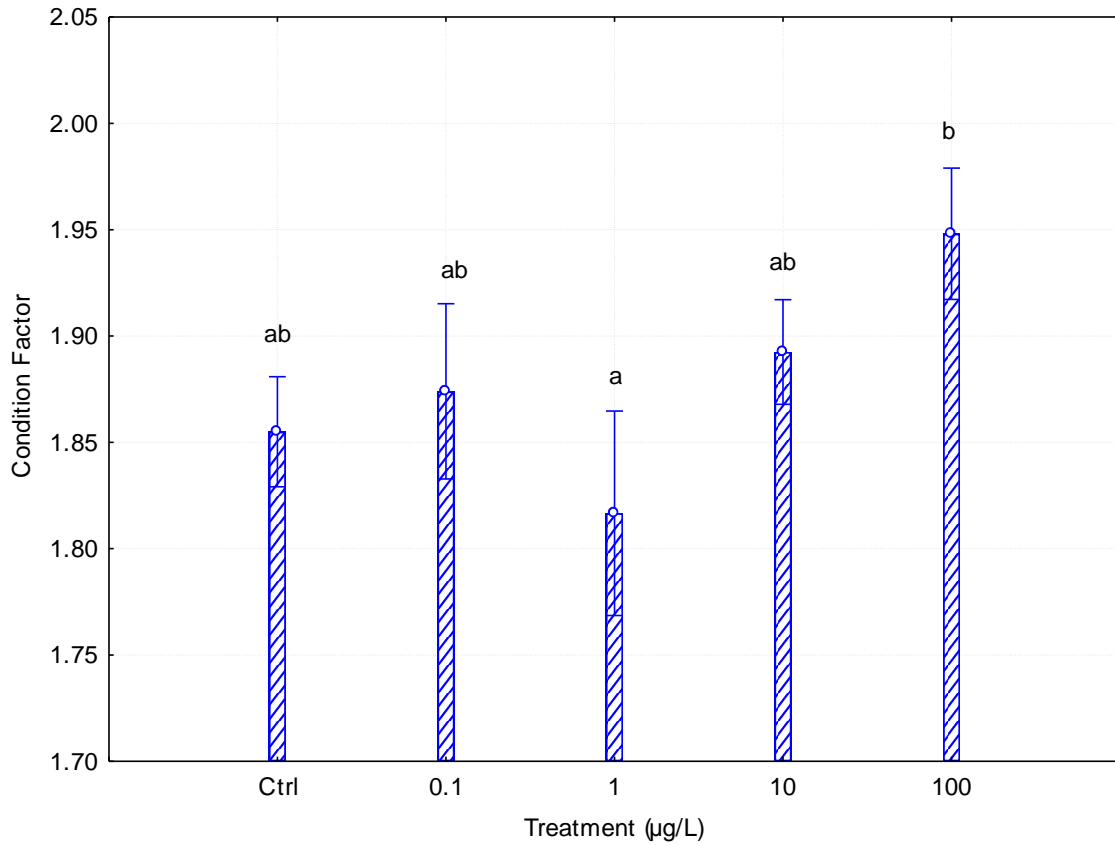


Figure 11: Condition factor of 93 ± 1 day-old flagfish of a chronic ibuprofen exposure experiment. Error bars denote standard error. Data could not be normalized. The results of two replicates and sexes were pooled. Sample sizes are given in table 2. Bars without a letter in common are significantly different from each other ($p \leq 0.10$).

Table 2: Sample size used for total length, wet weight and condition factor at day 93 \pm 1 of the chronic ibuprofen experiment.

Treatment ($\mu\text{g/L}$)	Replicate A		Replicate B	
	Female	Male	Female	Male
Control	5	4	7	3
0.1	3	3	5	4
1	6	4	7	3
10	3	5	7	2
100	4	3	5	5

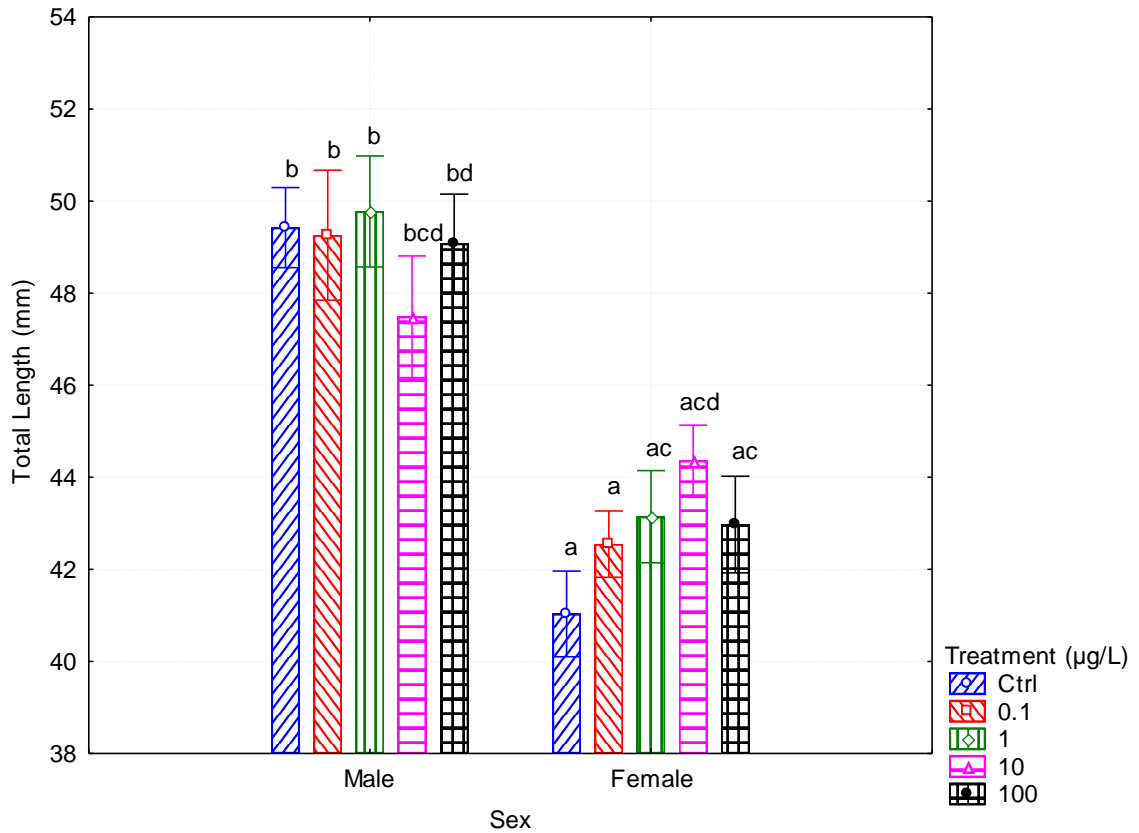


Figure 12: Total length (logged for analysis) of 121 ± 1 day-old flagfish of a chronic ibuprofen exposure experiment. Error bars denote standard error. The results of two replicates were pooled. The results of the two sexes could not be pooled. Sample sizes are given in table 3. Bars without a letter in common are significantly different from each other ($p \leq 0.10$).

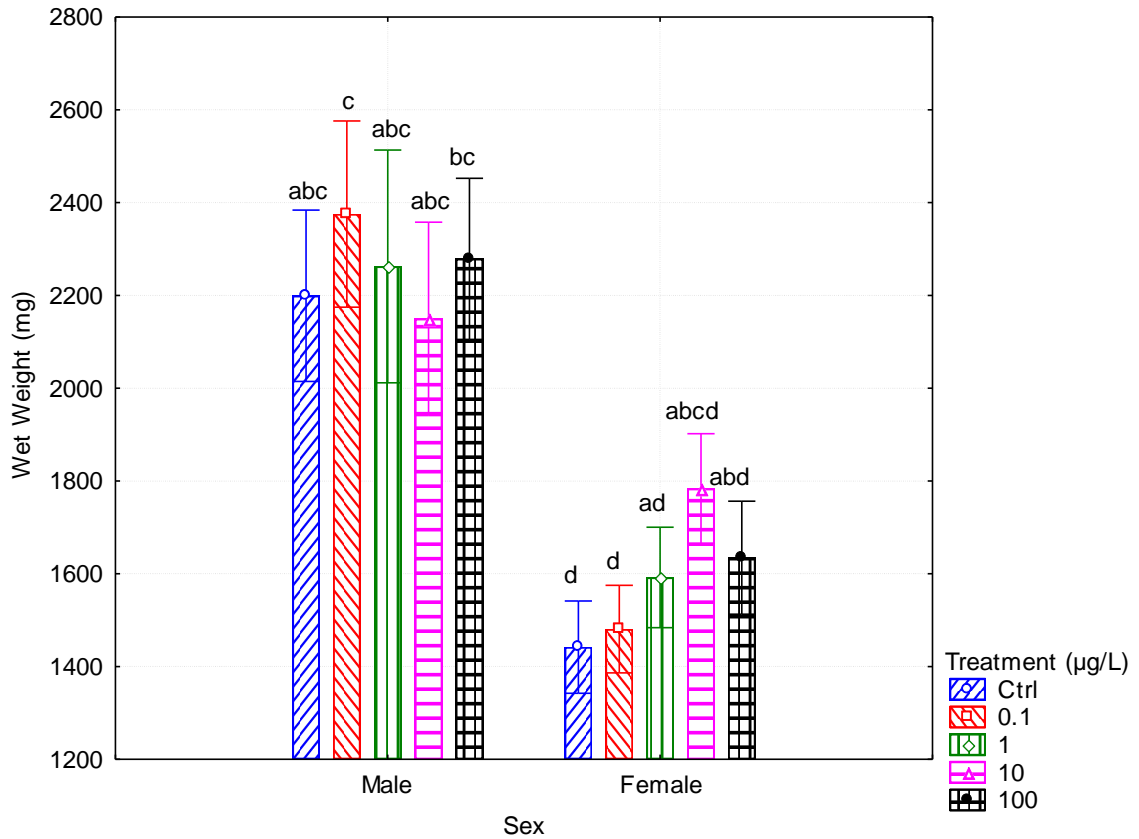


Figure 13: Wet weight (logged for analysis) of 121 ± 1 day-old flagfish of a chronic ibuprofen exposure experiment. Error bars denote standard error. The results of two replicates were pooled. The results of the two sexes could not be pooled. Sample sizes are given in table 3. Bars without a letter in common are significantly different from each other ($p \leq 0.10$).

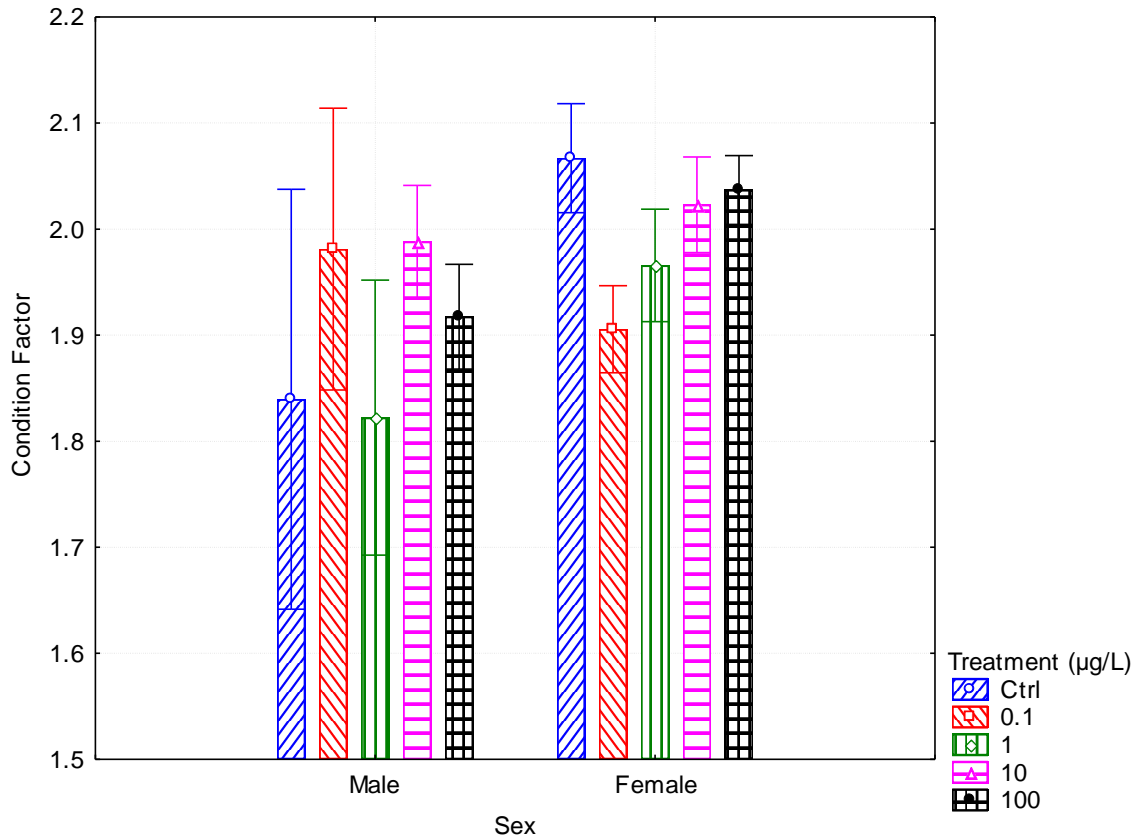


Figure 14: Condition factor of 121 ± 1 day-old flagfish of a chronic ibuprofen exposure experiment. Error bars denote standard error. Data could not be normalized. The results of two replicates were pooled. The results of the two sexes could not be pooled. Sample sizes are given in table 3. No significant differences were detected ($p > 0.10$).

Table 3: Sample size used for total length, wet weight and condition factor at day 121 \pm 1 of the chronic ibuprofen experiment.

Treatment ($\mu\text{g/L}$)	Female	Male
Control	8	4
0.1	7	5
1	8	4
10	8	4
100	8	4

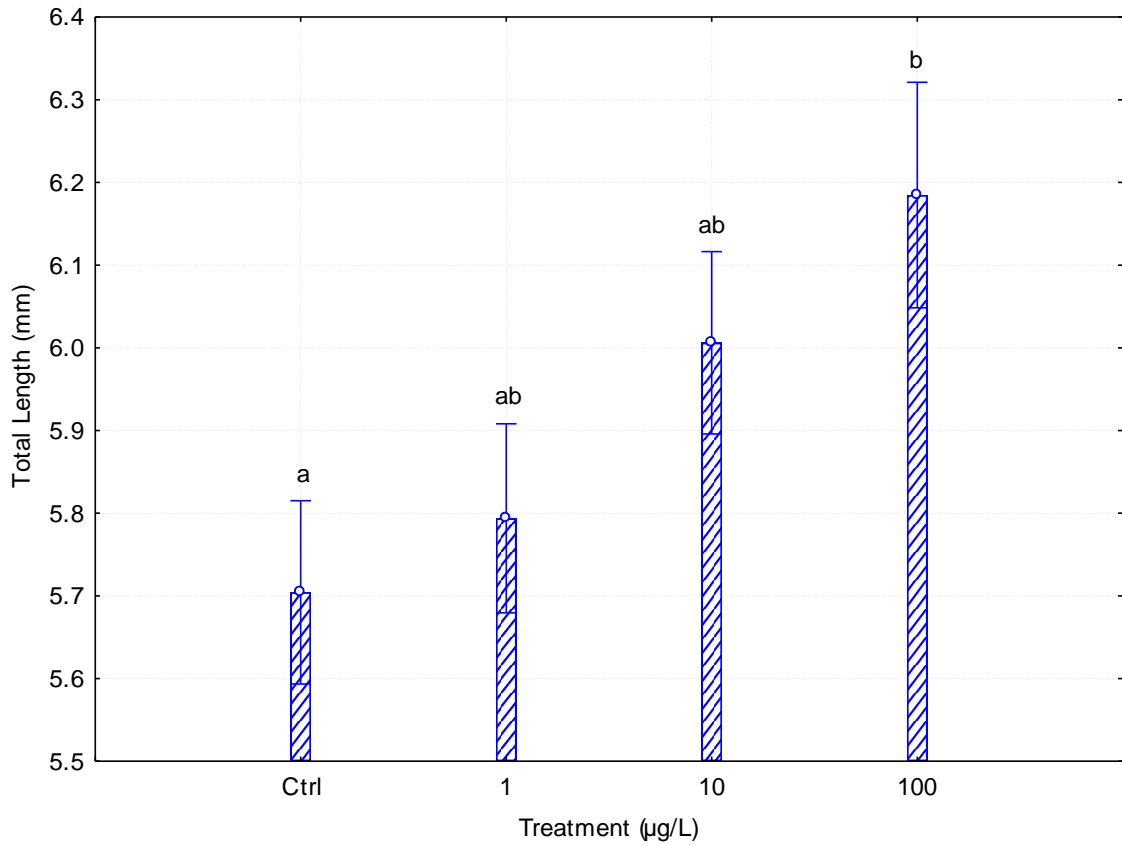


Figure 15: Total length of 14 ± 1 day-old flagfish of a chronic ibuprofen exposure experiment from parents also chronically exposed to the same ibuprofen concentration. Error bars denote standard error. The results of two replicates were pooled. Sample sizes for control (Ctrl), 1, 10 and 100 $\mu\text{g/L}$ were 42, 36, 40 and 25 respectively. Bars without a letter in common are significantly different from each other ($p \leq 0.10$).

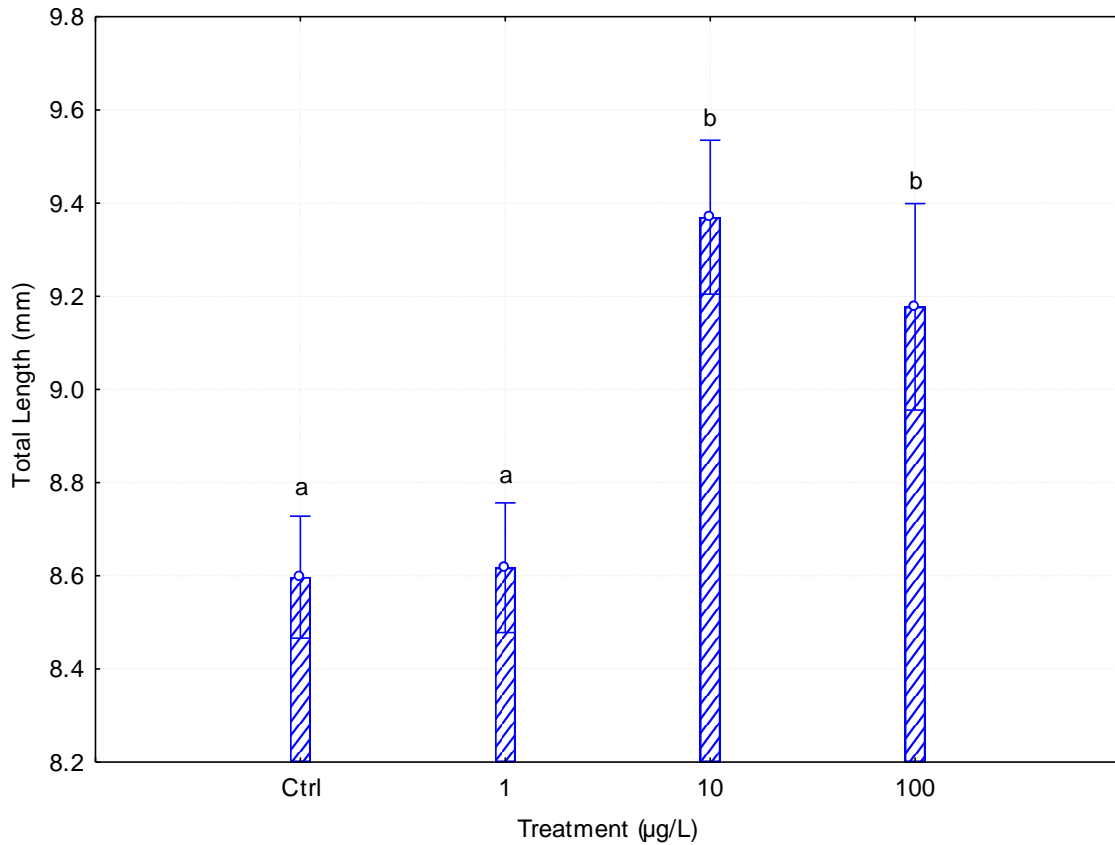


Figure 16: Total length of 28 ± 1 day-old flagfish of a chronic ibuprofen exposure experiment from parents also chronically exposed to the same ibuprofen concentration. Error bars denote standard error. The results of two replicates were pooled. Sample sizes for control (Ctrl), 1, 10 and 100 µg/L were 34, 32, 35 and 29 respectively. Bars without a letter in common are significantly different from each other ($p \leq 0.10$).

4.1.4 Reproduction

Egg production replicates were significantly different requiring replicates A and B to be analyzed separately. Time to first laying was generally unaffected by chronic exposure to ibuprofen (Table 4). The assessment of days on which eggs were laid revealed that in both replicates A and B fish exposed to 0.1 $\mu\text{g/L}$ of ibuprofen laid eggs on fewer days than any other treatment. Daily egg production revealed few differences between treatments, none being significantly different from at least one of the replicate control treatments (Table 4). However, within replicate A, fish exposed to 1 and 100 $\mu\text{g/L}$ had significantly higher daily egg production than fish in the control (Table 4). Within replicate B, no treatment was significantly different from the control (Table 4).

The results of fertilization success or time to hatch did not differ significantly between replicates and so the results of the data were pooled. Fish exposed to 0.1 $\mu\text{g/L}$ had a significantly lower fertilization success rate than the controls and all other treatments (Figure 17). Of the eggs which were fertilized, there was no significant difference in the proportion which hatched (Figure 18). 90% of all fertilized eggs hatched by 144 hours post fertilization (Figure 18).

Table 4: Impact of chronic exposure to ibuprofen on reproductive capacity as defined by egg production. Daily egg production is given as a value \pm standard error. Values without a letter in common are significantly different from each other ($p \leq 0.10$).

	Treatment ($\mu\text{g/L}$)	Time to First Egg Laying (d)	Number of Days Where Eggs Were Laid (d)	Daily Egg Production (eggs/d)	Total Number of Eggs Laid (eggs)
Replicate A	Control (0)	99	13	$8.00 \pm 2.13^{\text{ab}}$	168
	0.1	97	4	$11.2 \pm 3.44^{\text{ab}}$	235
	1	96	18	$47.7 \pm 11.5^{\text{d}}$	1002
	10	96	19	$34.8 \pm 8.11^{\text{bc}}$	730
	100	96	19	$40.0 \pm 7.83^{\text{cd}}$	840
Replicate B	Control (0)	96	17	$24.8 \pm 5.96^{\text{abcd}}$	520
	0.1	99	6	$0.710 \pm 0.430^{\text{a}}$	15
	1	96	17	$38.4 \pm 8.86^{\text{cd}}$	807
	10	96	13	$20.5 \pm 4.59^{\text{abc}}$	431
	100	96	14	$9.43 \pm 3.32^{\text{ab}}$	198

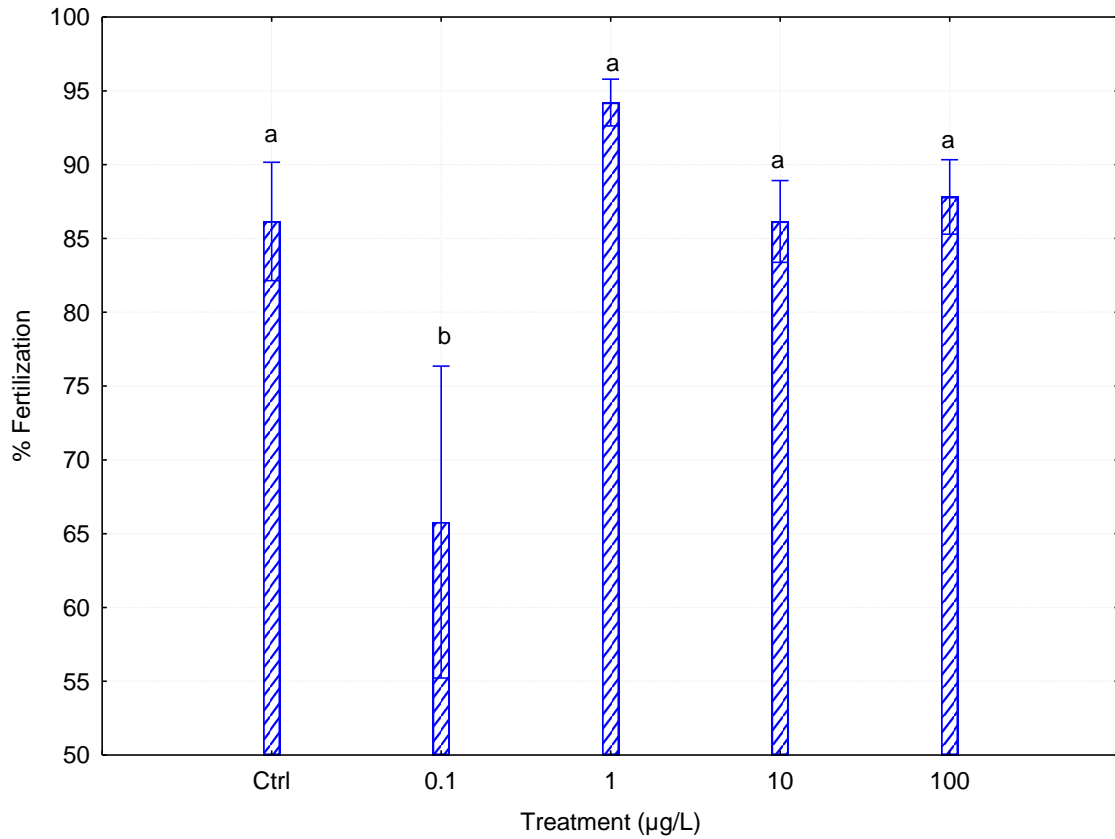


Figure 17: Impact of chronic ibuprofen exposure on percent egg fertilization. Error bars denote standard error. Sample sizes for control (Ctrl), 0.1, 1, 10 and 100 µg/L were 13, 8, 23, 22 and 17, respectively. Bars without a letter in common are significantly different from each other ($p \leq 0.10$).

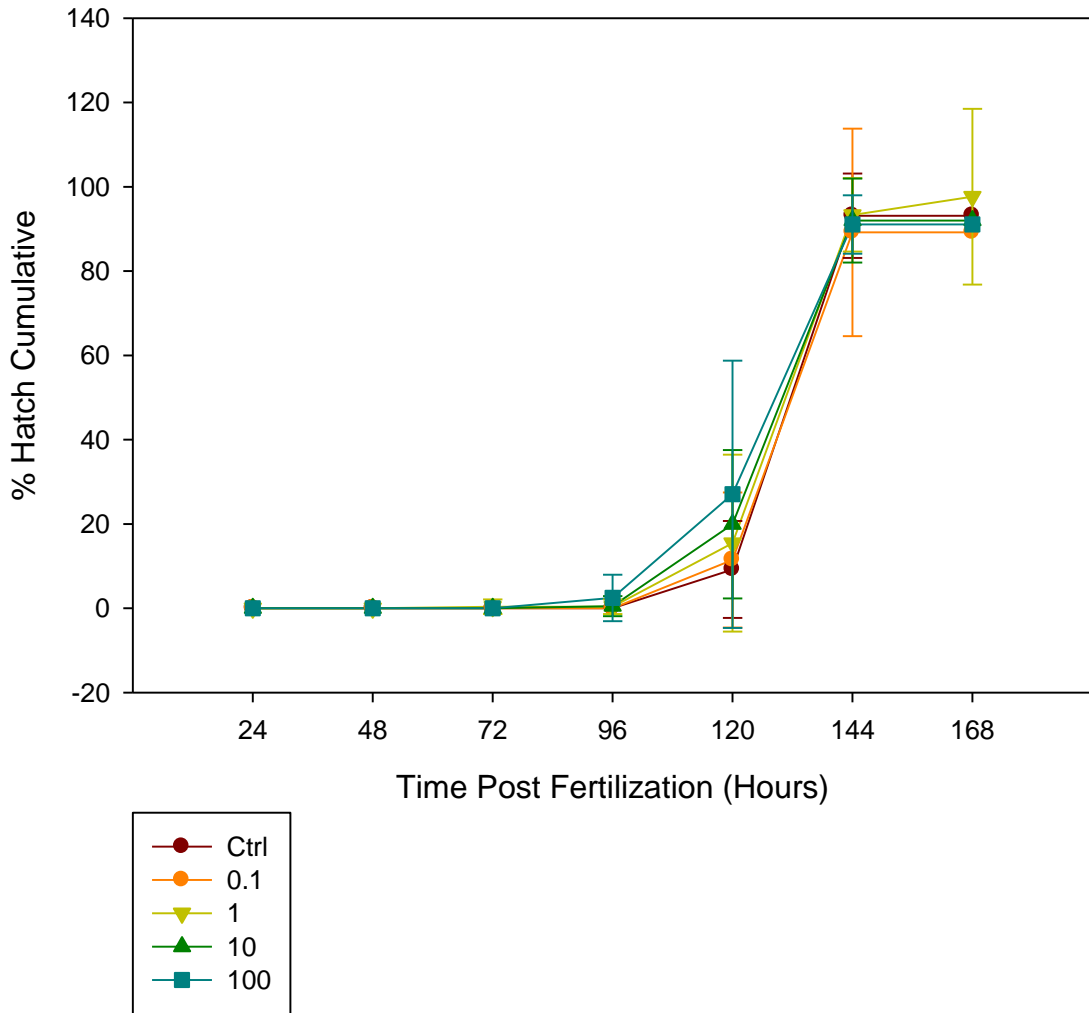


Figure 18: The effect of parental ibuprofen exposure on time to cumulative hatch of young. Error bars denote standard error. The results of two replicates were pooled. Sample sizes for control (Ctrl), 0.1, 1, 10 and 100 $\mu\text{g/L}$ were 13, 8, 23, 22 and 17, respectively. No significant differences were detected ($p > 0.10$).

4.1.5 Mortality

No significant mortalities were found between treatments or time points throughout the experiment (Figure 19).

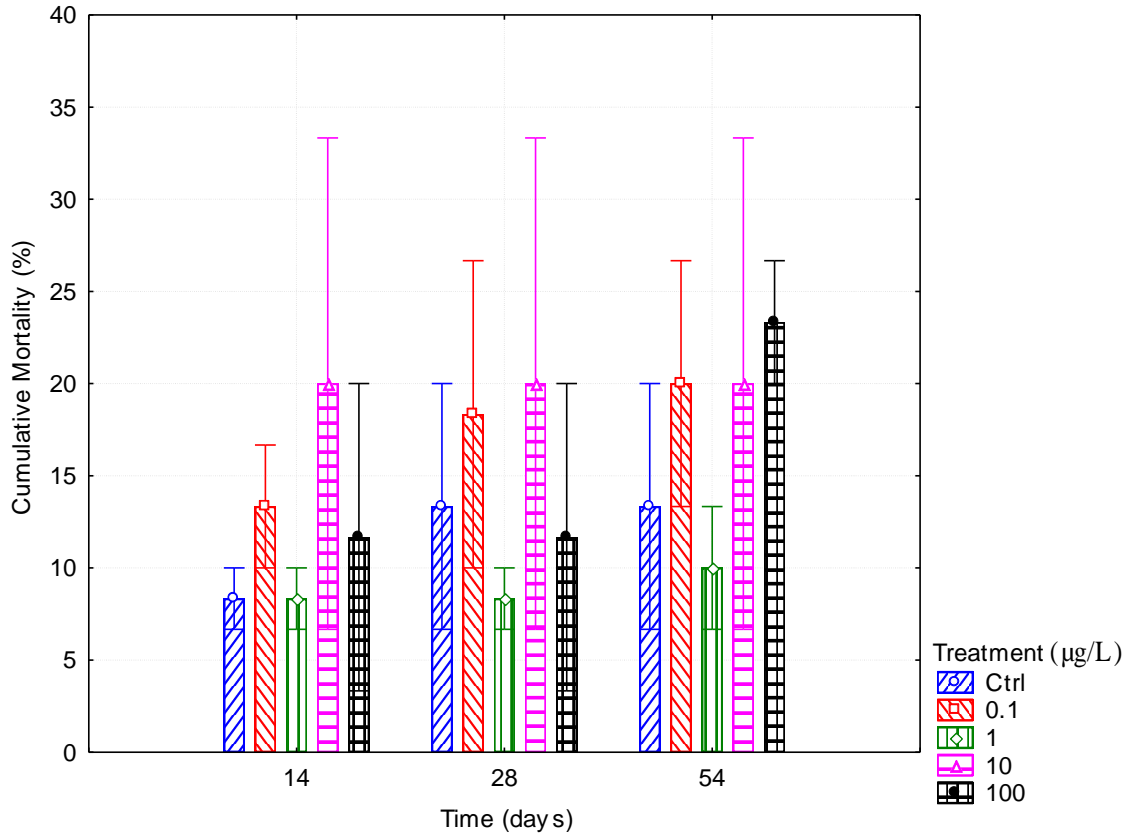


Figure 19: Cumulative mortality for 14, 28 and 54 ± 1 day-old flagfish of a chronic ibuprofen exposure experiment. Error bars denote standard error. The results of two replicates were pooled. Sample size for all treatments was 60. No significant differences were detected ($p > 0.10$).

4.2 Naproxen

4.2.1 Abiotic Parameters

Temperatures recorded during the crystallization dish stage were significantly lower than in the 70 L aquaria (Table 5). pH did not differ significantly during the experiment (Table 5).

Table 5: Average abiotic factors measured during chronic exposure. Values are written as means \pm standard error.

Generation	Experimental Stage	Temperature	pH
F1	Crystallization Dish	22.4 \pm 0.04	7.45 \pm 0.09
F1	70 L Aquaria	25.1 \pm 0.01	7.11 \pm 0.05
F2	Crystallization Dish	22.4 \pm 0.06	7.14 \pm 0.08

4.2.2 Egg Exposure Experiment

No significant differences in percent total hatch were detected in the naproxen egg exposure experiment (Figure 20).

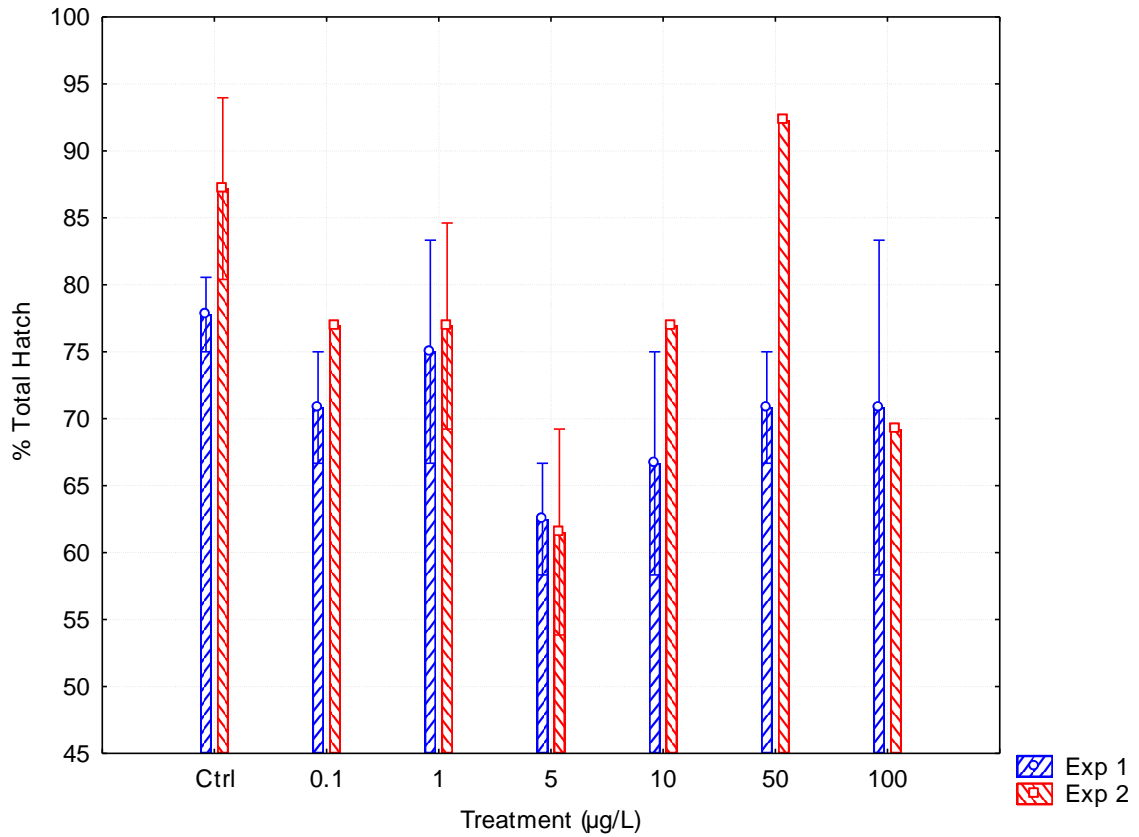


Figure 20: Impact of chronic naproxen exposure on percent egg hatchability. Error bars denote standard error. Results for the experiments were statically different and so were analyzed separately. Sample size was 15 for experiment one and 14 for experiment two. No significant differences were detected between treatments ($p > 0.10$).

4.2.3 Growth

At the first measurement period (14 ± 1 days post hatch) of the chronic naproxen experiment, replicates did not differ significantly allowing for pooling of the data. At 14 ± 1 days, flagfish chronically exposed to $100 \mu\text{g/L}$ of naproxen were significantly longer than controls as well as 0.1 , 1 and $10 \mu\text{g/L}$ exposed fish (Figure 21). Fish exposed to 1 and $10 \mu\text{g/L}$ were both significantly longer than fish exposed to $0.1 \mu\text{g/L}$ but not different from controls (Figure 21).

At the second measurement period on day 28 ± 1 , replicates were not significantly different allowing for the pooling of the results. Only fish exposed to $100 \mu\text{g/L}$ were significantly longer than controls; all other treatments did not differ significantly (Figure 21). Within the exposure treatments, fish exposed to $1 \mu\text{g/L}$ were significantly longer than those exposed to $0.1 \mu\text{g/L}$ (Figure 22).

At the third measurement period on day 54 ± 1 , sampled fish were assessed for total length, wet weight and condition factor. Wet weight was log transformed to normalize the data. Replicates were not significantly different for any of the measurements allowing for the pooling of the data. There were no differences between treatments in either total length or log wet weight (Figures 23 and 24). Condition factor of fish exposed to 0.1 and $1 \mu\text{g/L}$ did not differ significantly from the control (Figure 25), while the condition factor of fish exposed to $10 \mu\text{g/L}$ was significantly larger than controls but not different from condition factor of fish exposed to 0.1 and $1 \mu\text{g/L}$ (Figure 25). Condition factor of fish exposed $100 \mu\text{g/L}$ was significantly larger than all other treatments except for fish exposed to $10 \mu\text{g/L}$.

Prior to the setup of the breeding harems on day 93 ± 1 , fish were assessed for total length, wet weight and condition factor. For all analyses, replicates were significantly different and thus were analyzed separately. Sexes also differed significantly for the analysis of total length and wet weight. No significant differences were detected between treatments for the analysis of total length (Figure 26). The analysis of wet weight and condition factor also revealed no significant differences to the control between treatments or genders within replicates (Figure 27 and 28).

Harems were sampled on day 121 ± 1 and assessed for total length, wet weight and condition factor. Replicates for all three analyses did not differ significantly allowing the results to be pooled. Total length and wet weight of males and females were significantly different and thus analyzed separately (Figures 29 and 30). There were no significant differences in total length between treatments within sexes (Figure 29). Female controls and those exposed to $100 \mu\text{g/L}$ also did not differ significantly from any of the males (Figure 29). Females exposed to 1 and $10 \mu\text{g/L}$ were not significantly different from males exposed to 0.1 and $100 \mu\text{g/L}$ (Figure 29). There were no significant differences in wet weight between treatments within sexes (Figure 30). Males exposed to 0.1 and $100 \mu\text{g/L}$ were not significantly different from any of the females (Figure 30). The female controls and females exposed to $100 \mu\text{g/L}$ were not significantly different from any of the male treatments (Figure 30). There were no differences between treatments except for males exposed to $1 \mu\text{g/L}$ being the largest group from any treatment and females exposed to $0.1 \mu\text{g/L}$ being the smallest (Figure 30). There were no significant differences in condition factor between treatments detected (Figure 31).

Analysis of second generation fish was unable to be conducted on fish exposed to 100 µg/L as insufficient eggs were laid to permit statistical analysis. At the first measurement on day 14 ± 1 of total length for these second generation fish, replicates were not significantly different and so were pooled. Data were square root transformed to achieve normality. Fish exposed to 0.1 µg/L were significantly smaller than all other treatments (Figure 32).

On day 28 ± 1 , the second generation fish were again assessed for total length. The replicates were not significantly different allowing their results to be pooled. No treatment differed significantly from the control although fish exposed to 10 µg/L were significantly longer than those exposed to 1 µg/L of naproxen (Figure 33).

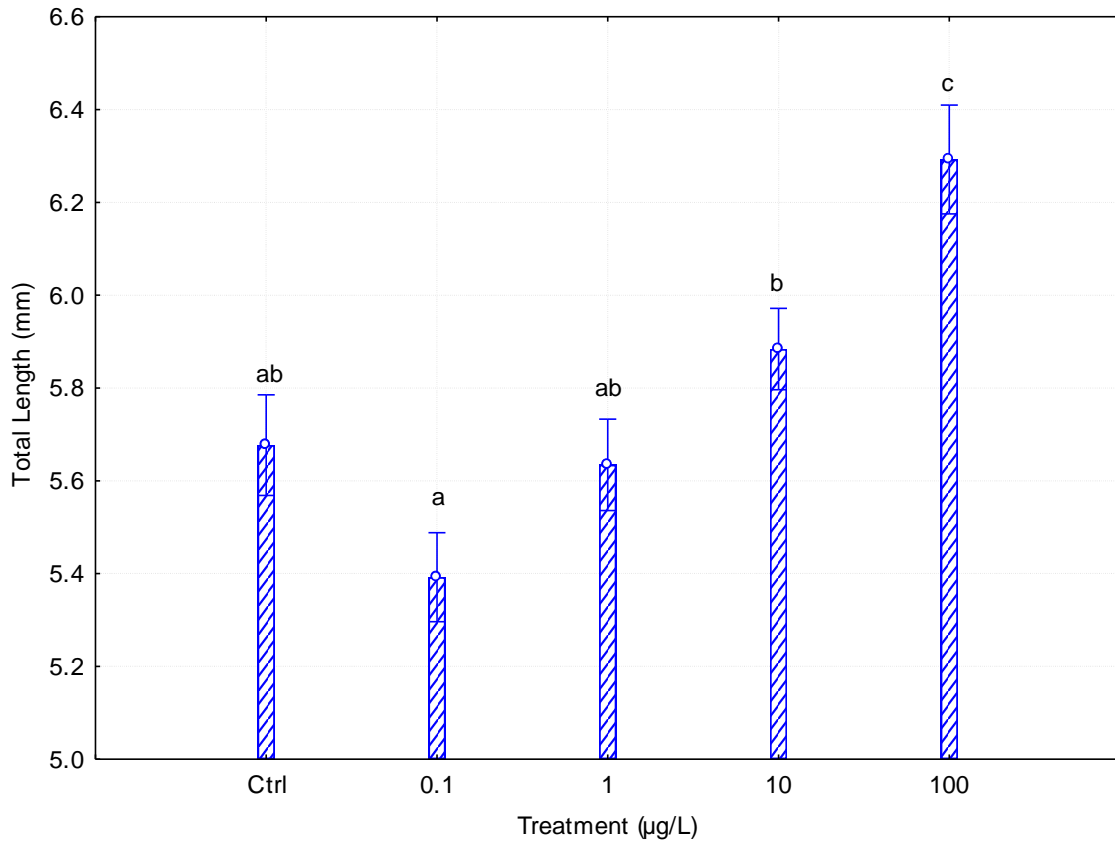


Figure 21: Total length of 14 ± 1 day-old flagfish of a chronic naproxen exposure experiment. Error bars denote standard error. The results of two replicates were pooled. Sample sizes for control (Ctrl), 0.1, 1, 10 and 100 $\mu\text{g/L}$ were 51, 55, 42, 48 and 49, respectively. Bars without a letter in common are significantly different from each other ($p \leq 0.10$).

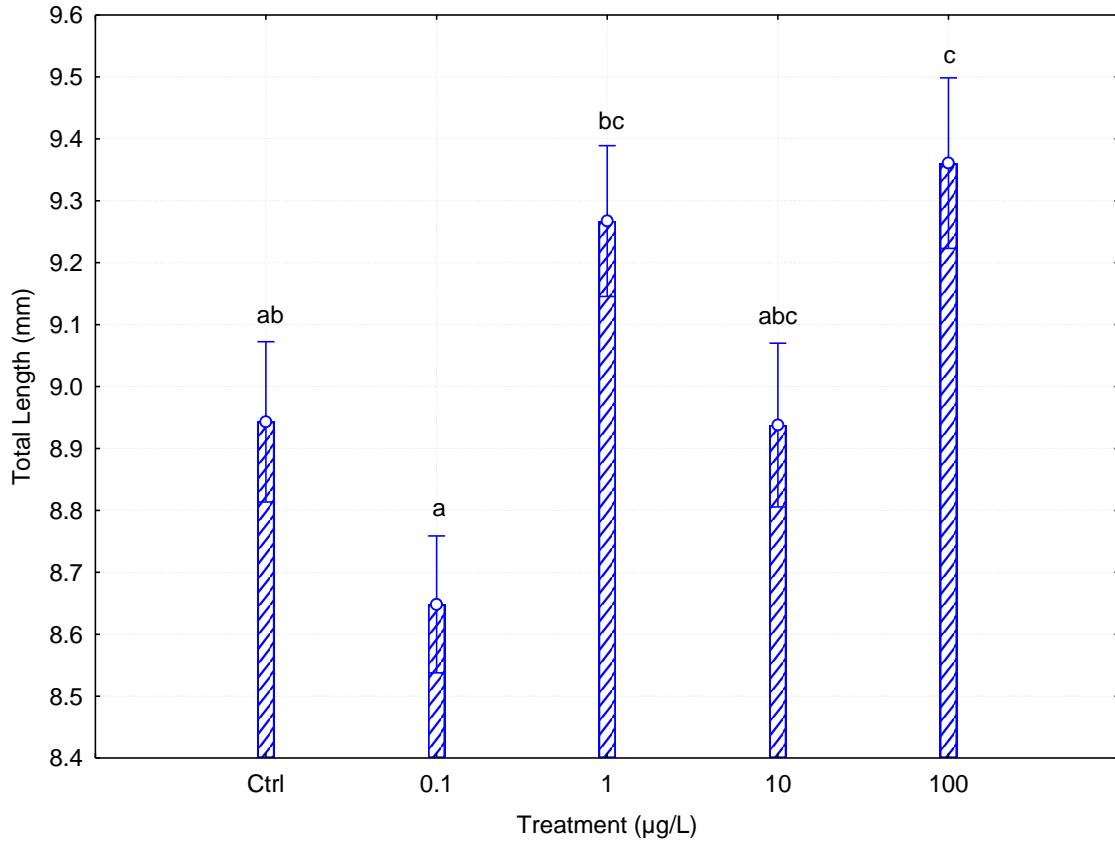


Figure 22: Total length of 28 ± 1 day-old flagfish of a chronic naproxen exposure experiment. Error bars denote standard error. The results of two replicates were pooled. Sample sizes for control (Ctrl), 0.1, 1, 10 and 100 µg/L were 41, 45, 33, 24 and 34, respectively. Bars without a letter in common are significantly different from each other ($p \leq 0.10$).

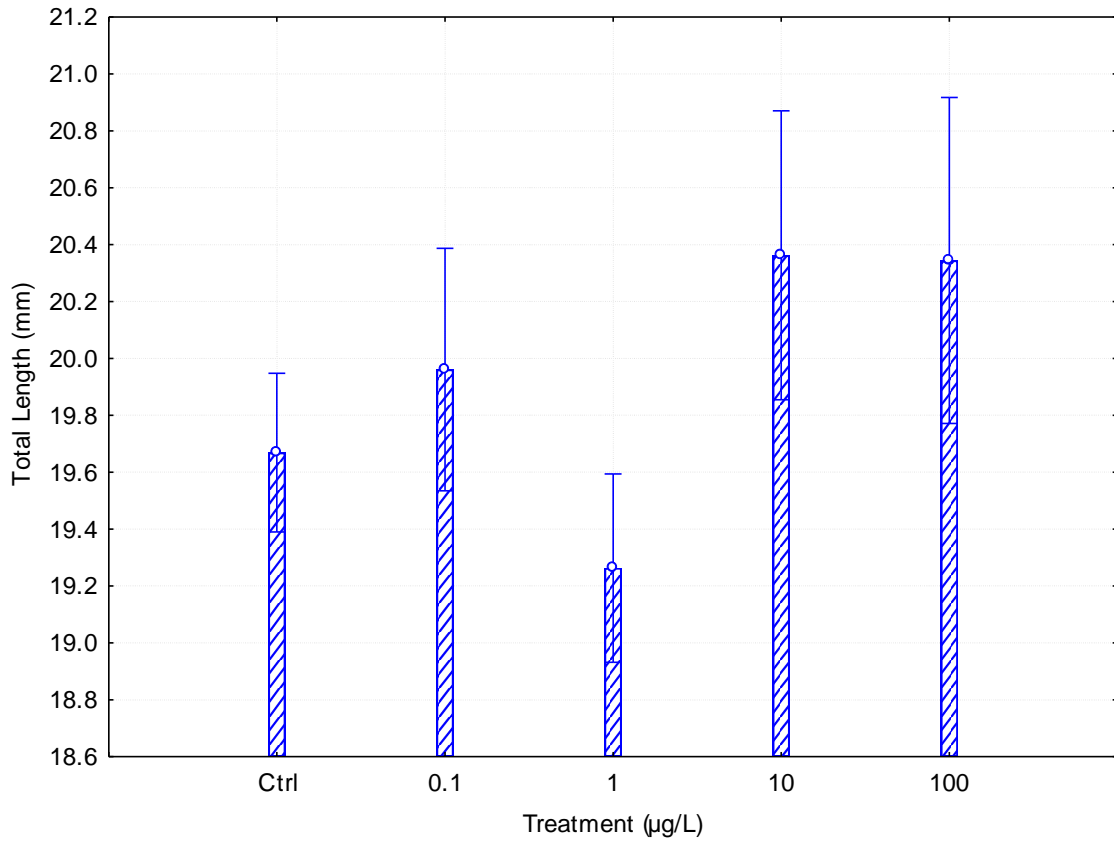


Figure 23: Total length of 54 ± 1 day-old flagfish of a chronic naproxen exposure experiment. Error bars denote standard error. The results of two replicates were pooled. Sample sizes for control (Ctrl), 0.1, 1, 10 and 100 µg/L were 24, 23, 23, 12 and 9, respectively. No significant differences were detected ($p > 0.10$).

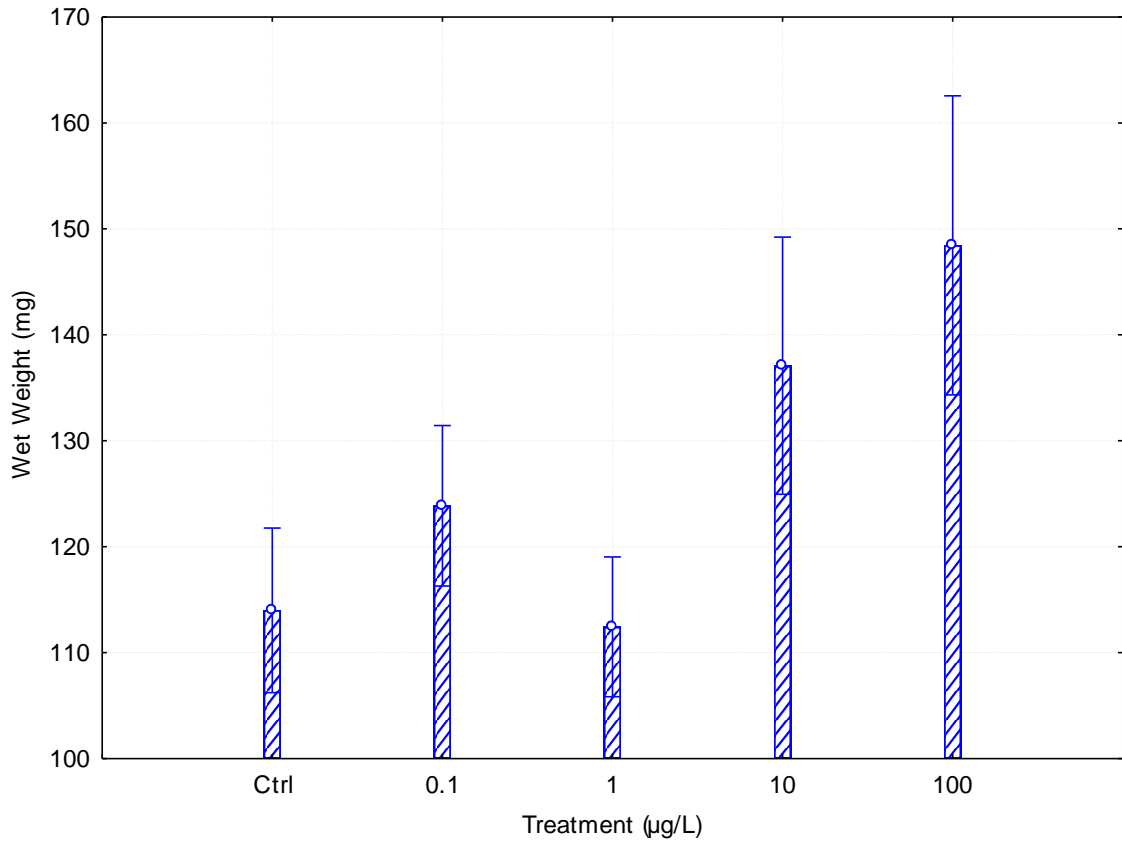


Figure 24: Wet weight (logged for analysis) of 54 ± 1 day-old flagfish of a chronic naproxen exposure experiment. Error bars denote standard error. The results of two replicates were pooled. Sample sizes for control (Ctrl), 0.1, 1, 10 and 100 µg/L were 24, 23, 23, 12 and 9, respectively. No significant differences were detected ($p > 0.10$).

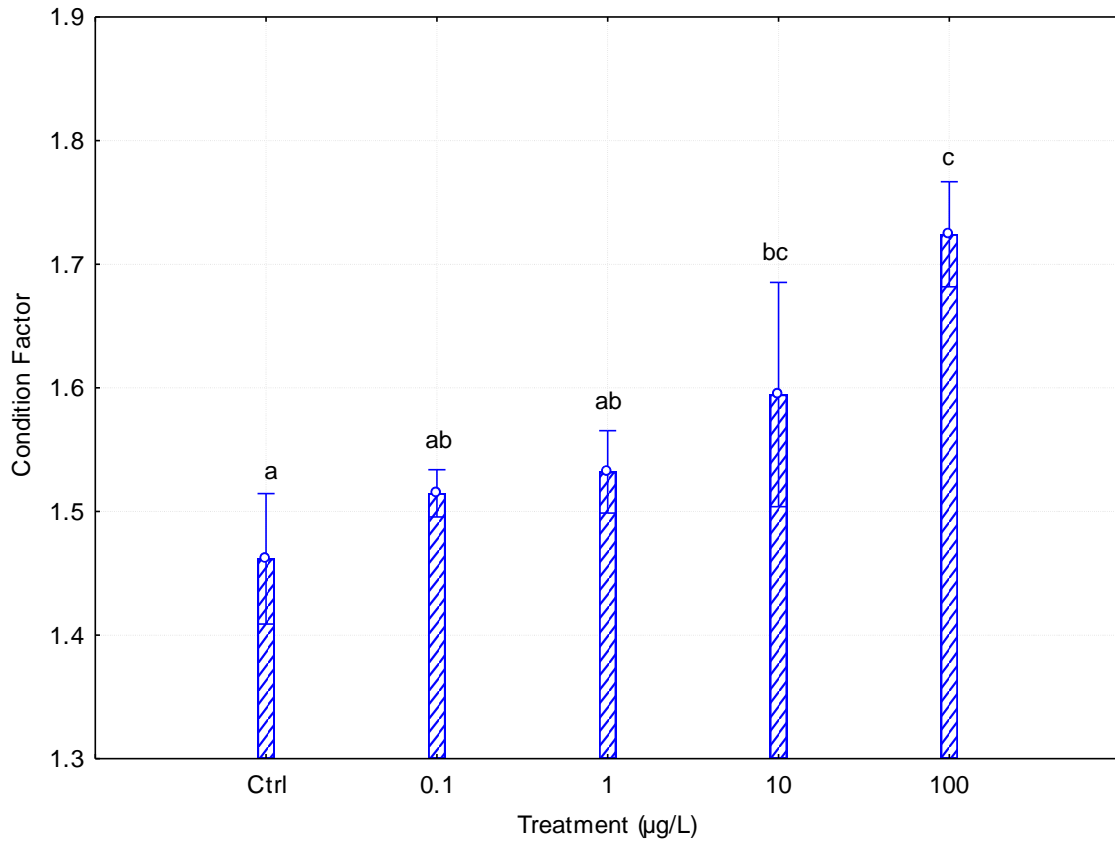


Figure 25: Condition factor of 54 ± 1 day-old flagfish of a chronic naproxen exposure experiment. Error bars denote standard error. The results of two replicates were pooled. Sample sizes for control (Ctrl), 0.1, 1, 10 and 100 µg/L were 24, 23, 23, 12 and 9, respectively. Bars without a letter in common are significantly different from each other ($p \leq 0.10$).

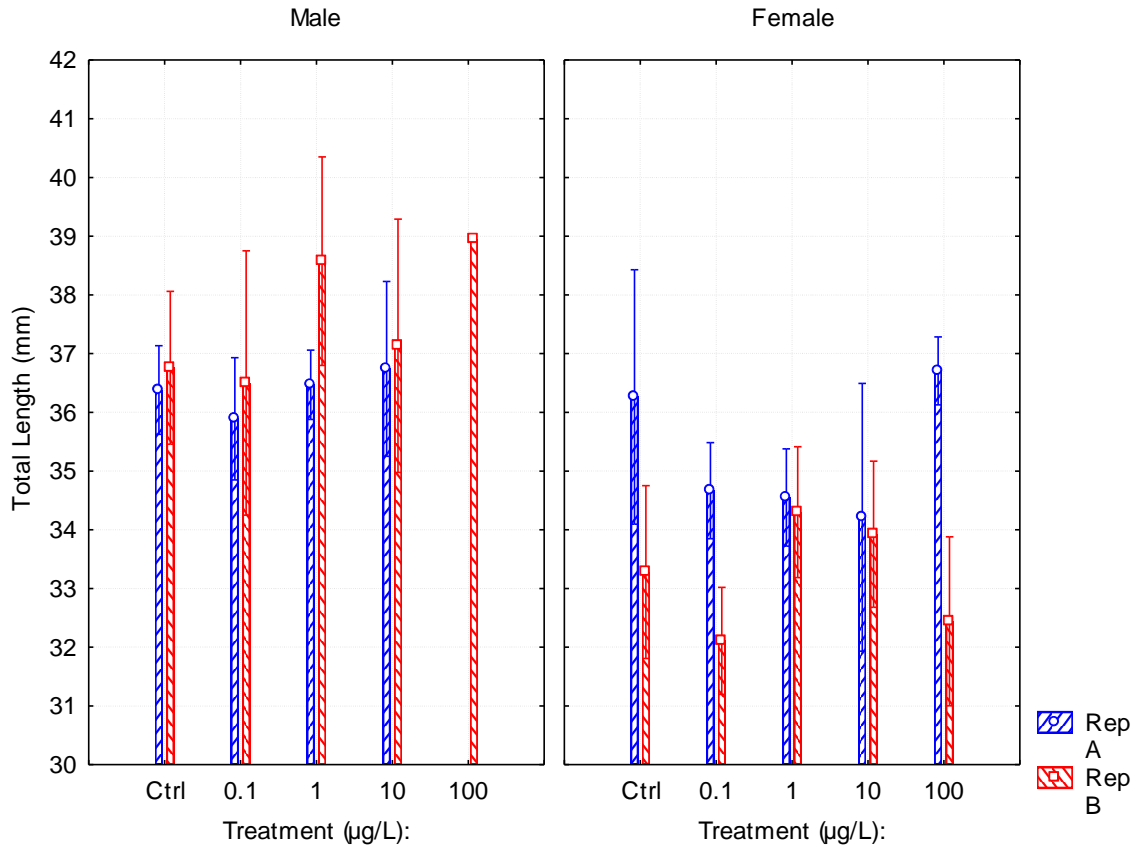


Figure 26: Total length of 93 ± 1 day-old flagfish of a chronic naproxen exposure experiment. Error bars denote standard error. The results of two replicates and sexes could not be pooled. Sample sizes are given in Table 6: No significant differences were detected ($p > 0.10$).

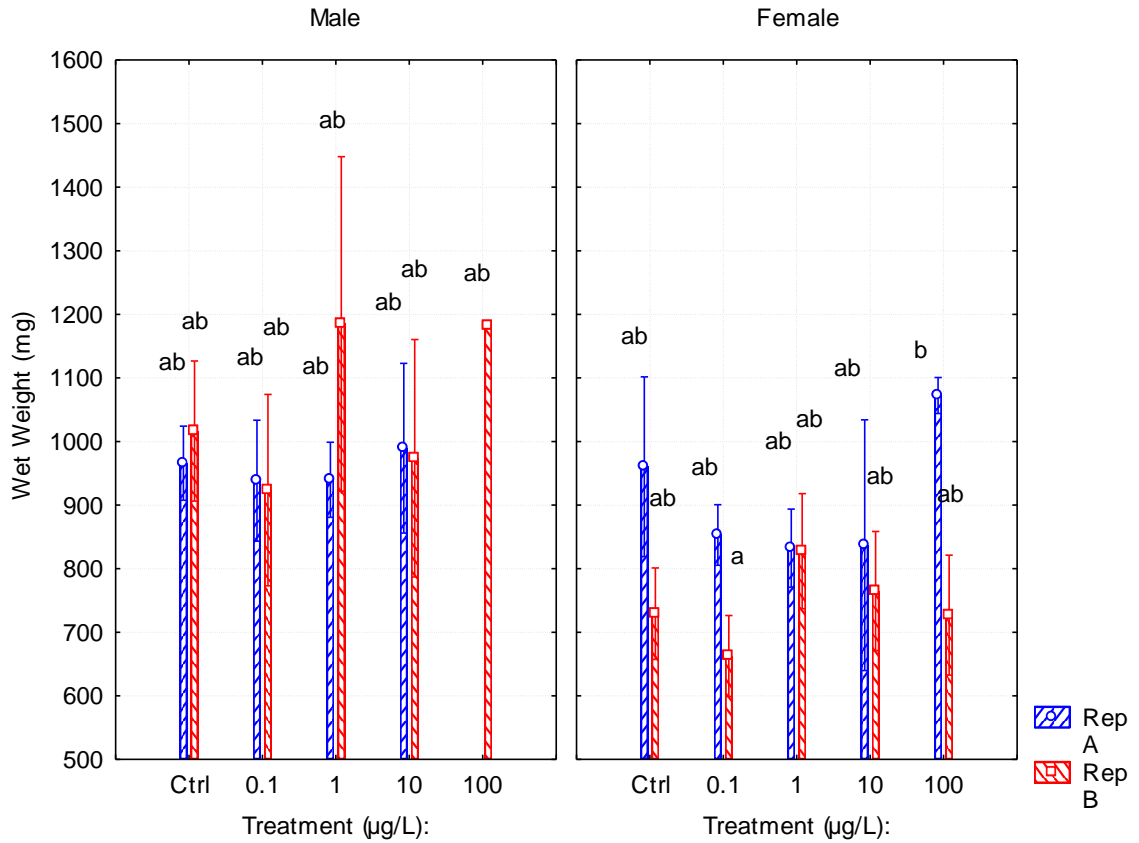


Figure 27: Wet weight of 93 ± 1 day-old flagfish of a chronic naproxen exposure experiment. Error bars denote standard error. The results of two replicates and sexes could not be pooled. Sample sizes are given in Table 6: Bars without a letter in common are significantly different from each other ($p \leq 0.10$).

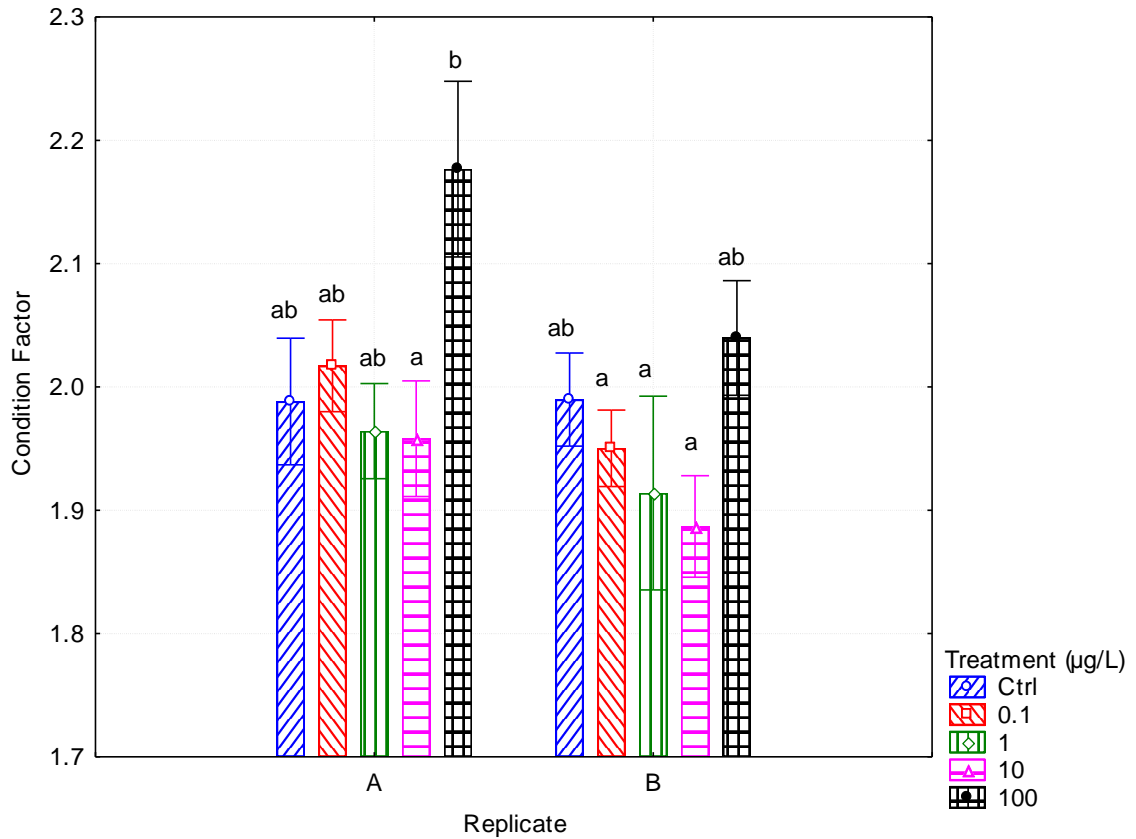


Figure 28: Condition factor of 93 ± 1 day-old flagfish of a chronic naproxen exposure experiment. Error bars denote standard error. Data could not be normalized. The results of two replicates could not be pooled. Results of the sexes were pooled. Sample sizes are given in Table 6. Bars without a letter in common are significantly different from each other ($p \leq 0.10$).

Table 6: Sample size used for total length, wet weight and condition factor at day 93 \pm 1 of the chronic naproxen experiment.

Treatment ($\mu\text{g/L}$)	Replicate A		Replicate B	
	Female	Male	Female	Male
Control	4	5	5	5
0.1	3	5	6	2
1	4	5	6	2
10	4	5	6	3
100	8	0	6	1

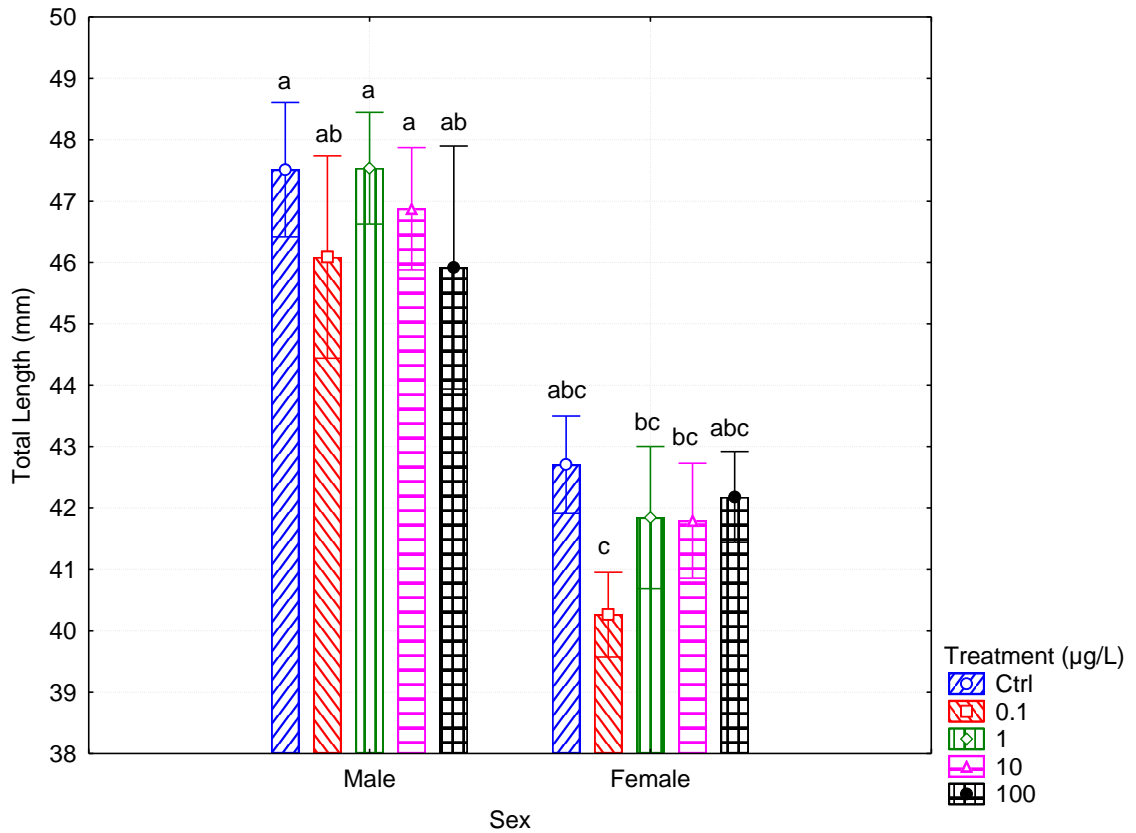


Figure 29: Total length (logged for analysis) of 121 ± 1 day-old flagfish of a chronic naproxen exposure experiment. Error bars denote standard error. The results of two replicates were pooled. The results of the two sexes could not be pooled. Sample sizes are given in Table 7. Bars without a letter in common are significantly different from each other ($p \leq 0.10$).

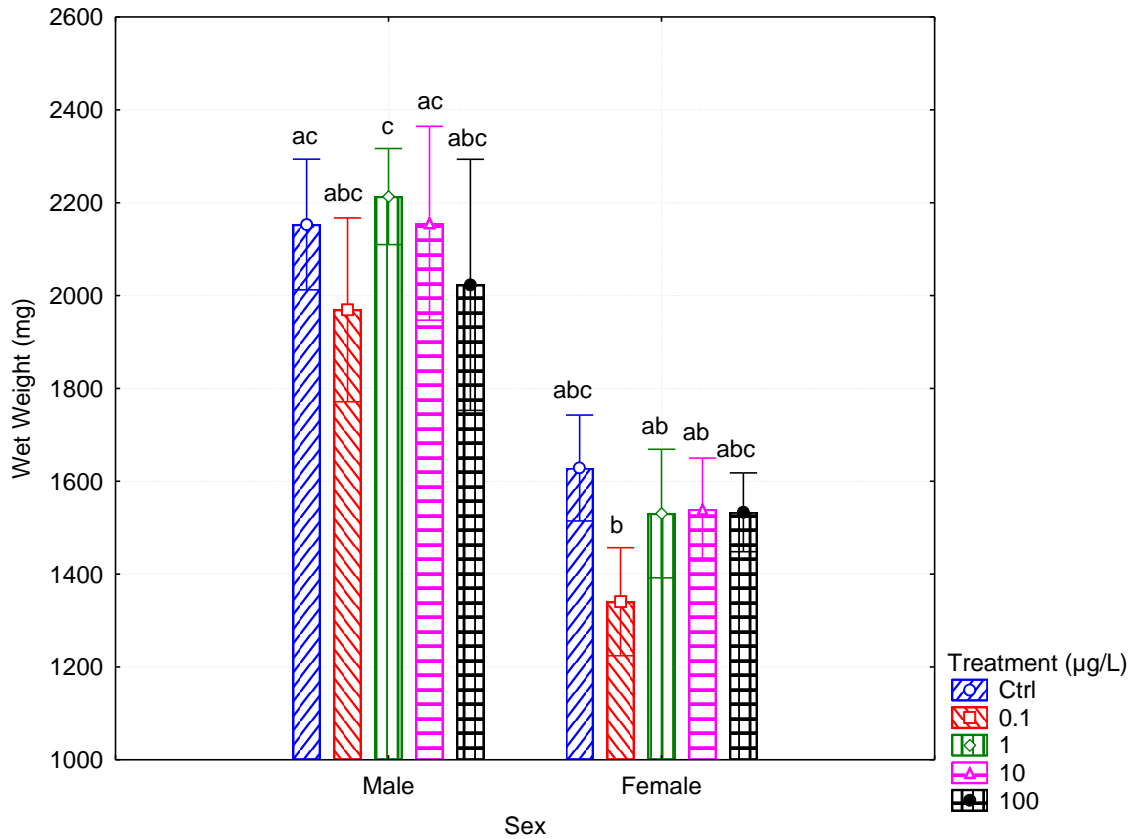


Figure 30: Log wet weight of 121 ± 1 day-old flagfish of a chronic naproxen exposure experiment. Error bars denote standard error. The results of two replicates were pooled. The results of the two sexes could not be pooled. Sample sizes are given in Table 7. Bars without a letter in common are significantly different from each other ($p \leq 0.10$).

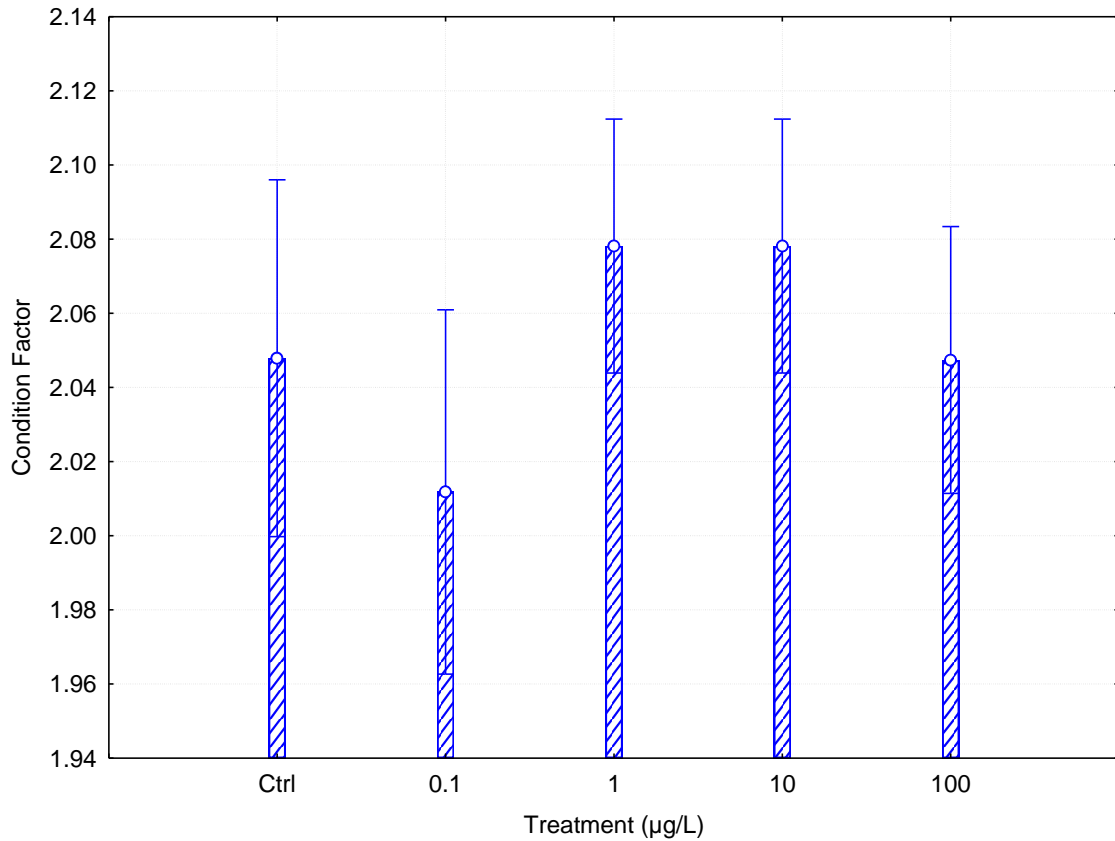


Figure 31: Condition factor of 121 ± 1 day-old flagfish of a chronic naproxen exposure experiment. Error bars denote standard error. The results of two replicates and sexes were pooled. Sample sizes are given in Table 7. Bars without a letter in common are significantly different from each other ($p \leq 0.10$).

Table 7: Sample size used for total length, wet weight and condition factor at day 121 \pm 1 of the chronic naproxen experiment.

Treatment ($\mu\text{g/L}$)	Female	Male
Control	8	4
0.1	7	4
1	8	4
10	8	4
100	5	3

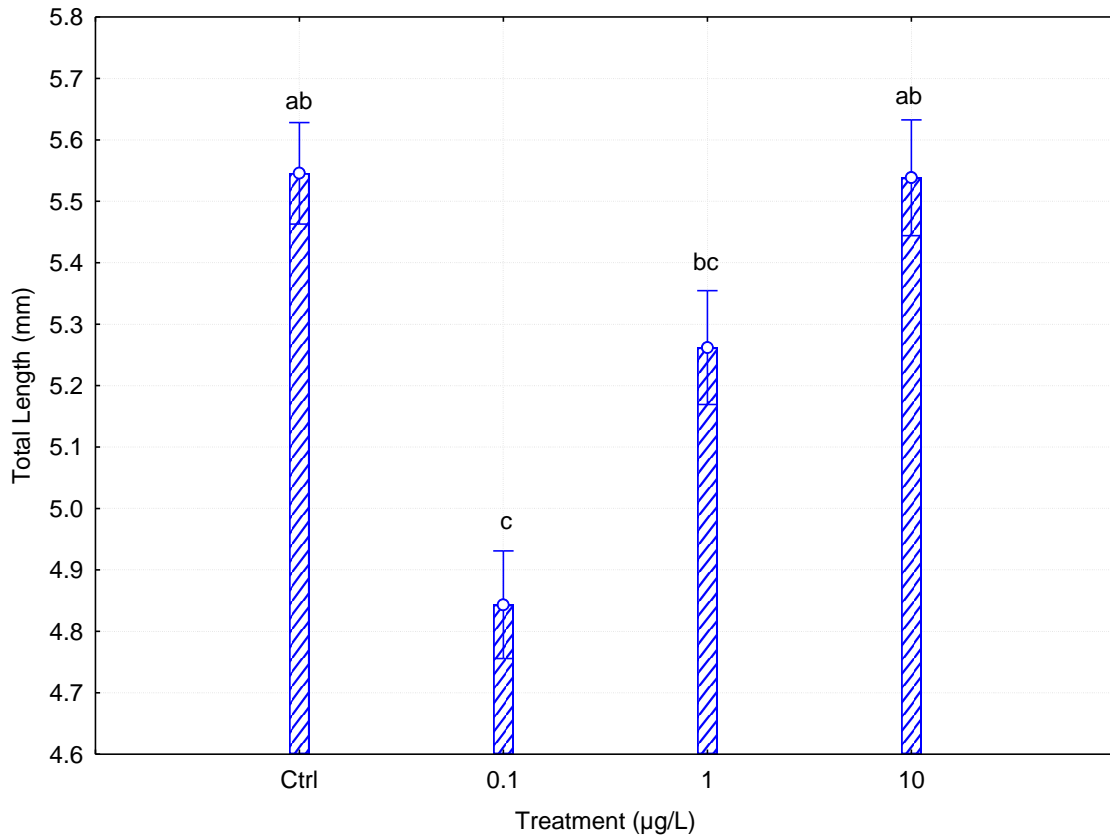


Figure 32: Total length (squared for statistical analysis) of 14 ± 1 day-old flagfish of a chronic naproxen exposure experiment from parents also chronically exposed to the same naproxen concentration. The results of two replicates were pooled. Error bars denote standard error. Sample sizes for control (Ctrl), 0.1, 1 and 10 $\mu\text{g/L}$ were 41, 36, 36 and 35, respectively. Bars without a letter in common are significantly different from each other ($p \leq 0.10$).

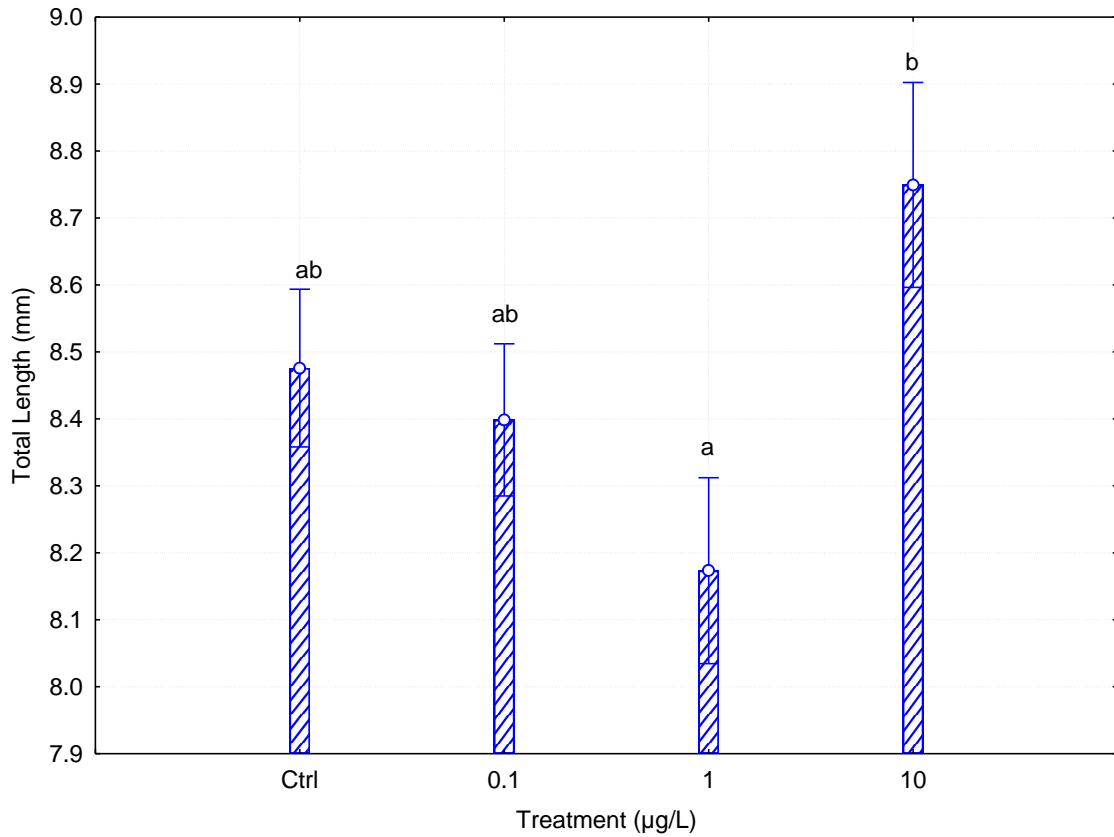


Figure 33: Total length of 28 ± 1 day-old flagfish of a chronic naproxen exposure experiment from parents also chronically exposed to the same naproxen concentration. Error bars denote standard error. The results of two replicates were pooled. Sample sizes for control (Ctrl), 0.1, 1 and 10 µg/L were 34, 33, 28 and 30, respectively. Bars without a letter in common are significantly different from each other ($p \leq 0.10$).

4.2.4 Reproduction

Replicate daily egg production was significantly different and thus had to be analyzed separately. In replicate A, significantly fewer eggs were produced relative to the control at all concentrations of naproxen except for 10 µg/L (Table 8). Daily egg production for all treatments was extremely variable though the egg production at least one replicate from each treatment overlapped with that of a control (Table 8). Fish exposed to 100 µg/L consistently produced very few eggs over a small number of days in both treatments (Table 8). Time to first egg laying was not significantly affected by chronic naproxen exposure although fish exposed to 100 µg/L in replicate B took the longest to begin laying eggs relative to any other treatment (Table 8).

The results of fertilization success or time to hatch did not differ significantly between replicates and so the data were pooled. Fish exposed to 0.1 µg/L had a significantly lower fertilization success than controls and fish exposed to 1, 10 and 100 µg/L of naproxen (Figure 34). Of the eggs which were fertilized, there was no significant difference in hatchability or time to hatching with about 90% of all fertilized eggs hatching by 144 hours post fertilization (Figure 35).

Table 8: Impact of chronic exposure to naproxen on reproductive capacity as defined by egg production. Daily egg production is given as a value \pm standard error. Values without a letter in common are significantly different from each other ($p \leq 0.10$).

	Treatment ($\mu\text{g/L}$)	Time to First Egg Laying (d)	Number of Days Where Eggs Were Laid (d)	Daily Egg Production (eggs/d)	Total Number of Eggs Laid (eggs)
Replicate A	Control (0)	100	15	$25.7 \pm 7.29^{\text{bcd}}$	539
	0.1	not achieved	0	0^{a}	0
	1	100	6	$0.71 \pm 0.32^{\text{a}}$	15
	10	97	17	$49.0 \pm 8.17^{\text{de}}$	1028
	100	98	2	$0.10 \pm 0.07^{\text{a}}$	2
Replicate B	Control (0)	96	18	$49.5 \pm 10.99^{\text{e}}$	1040
	0.1	97	18	$40.7 \pm 7.15^{\text{cde}}$	854
	1	98	16	$11.7 \pm 4.02^{\text{ab}}$	245
	10	99	16	$21.3 \pm 4.30^{\text{abc}}$	448
	100	106	6	$2.48 \pm 1.19^{\text{ab}}$	52

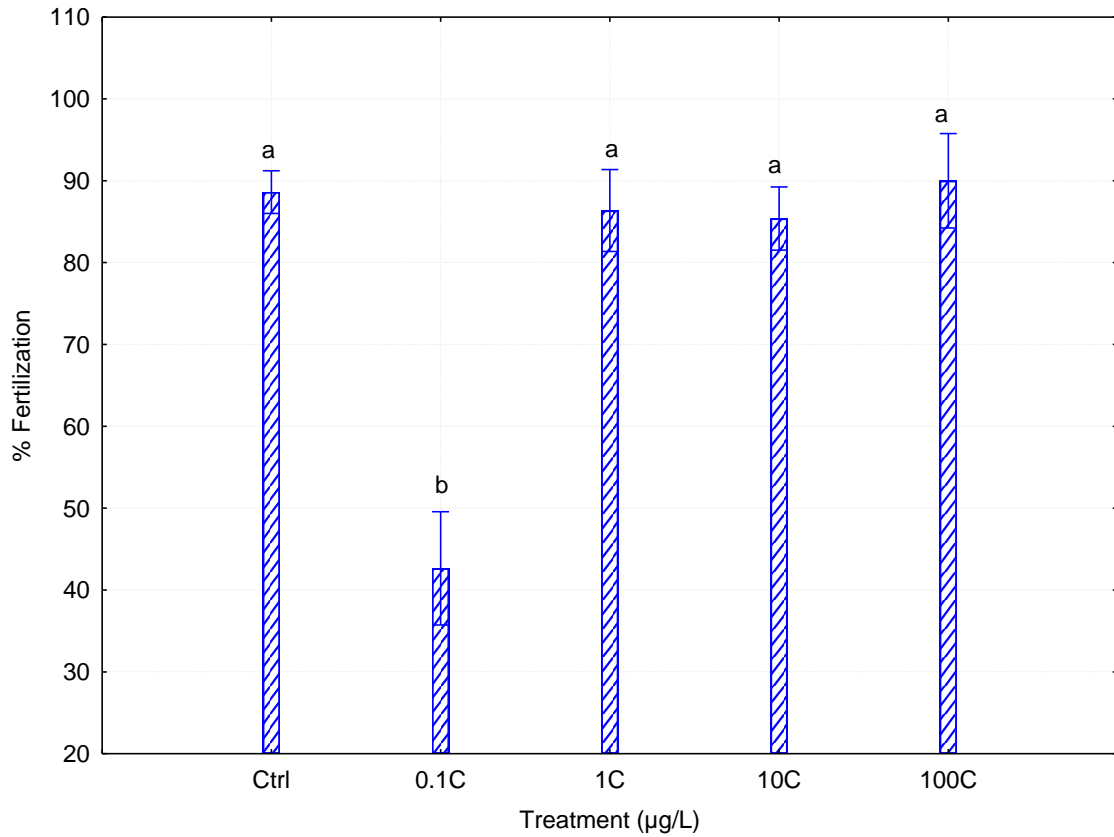


Figure 34: Impact of chronic naproxen exposure on fertilization of eggs. Error bars denote standard error. Sample sizes for control (Ctrl), 0.1, 1, 10 and 100 µg/L were 25, 13, 5, 25 and 3, respectively. Bars without a letter in common are significantly different from each other ($p \leq 0.10$).

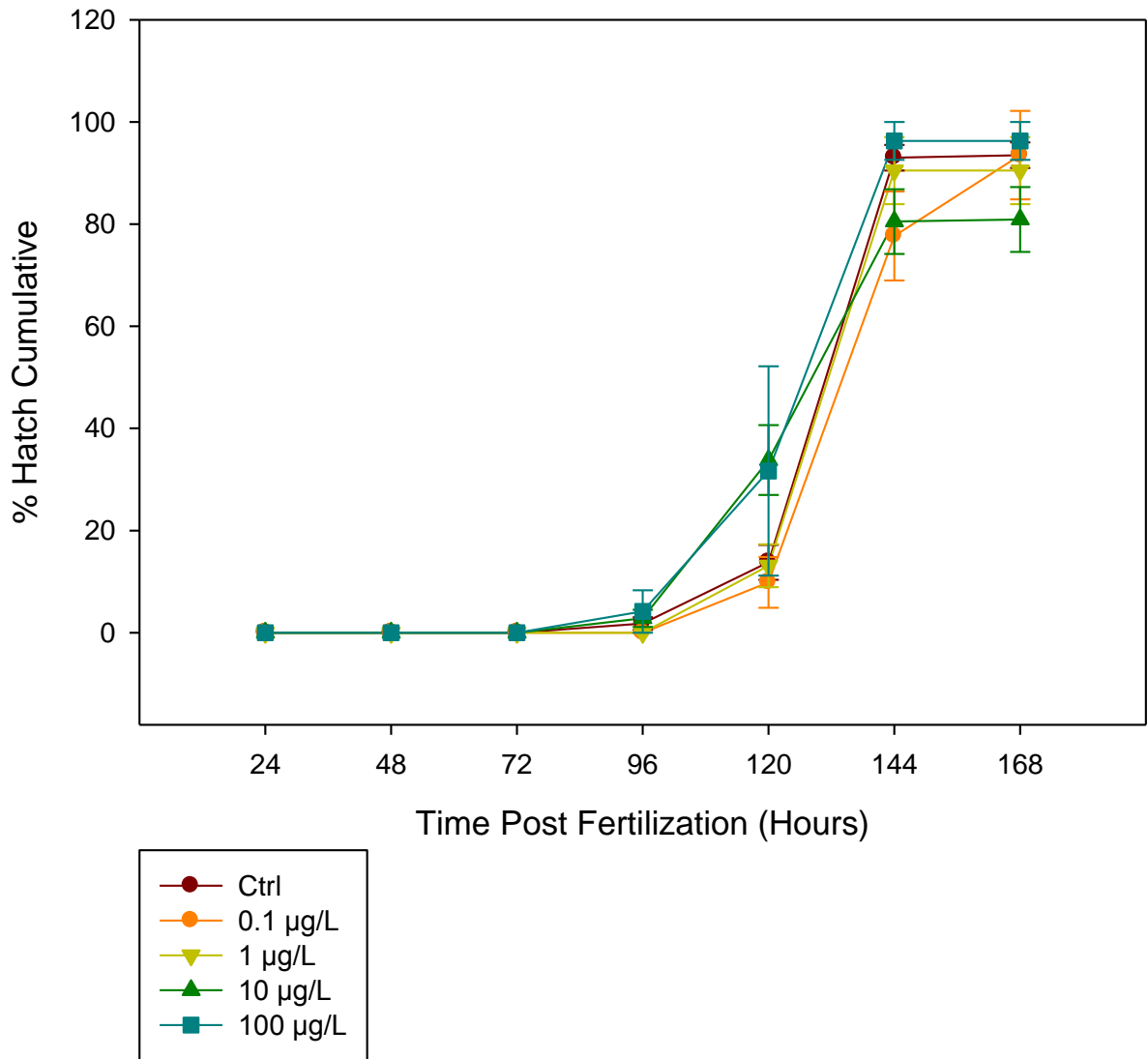


Figure 35: Effect of parental naproxen exposure on time to cumulative hatch of young. Sample sizes for control (Ctrl), 0.1, 1, 10 and 100 µg/L were 25, 13, 5, 25 and 3, respectively. Error bars denote standard error. The results of two replicates were pooled. No significant differences were detected ($p > 0.10$).

4.2.5 Mortality

No significant mortalities were found between treatments or time points throughout the experiment (Figure 36).

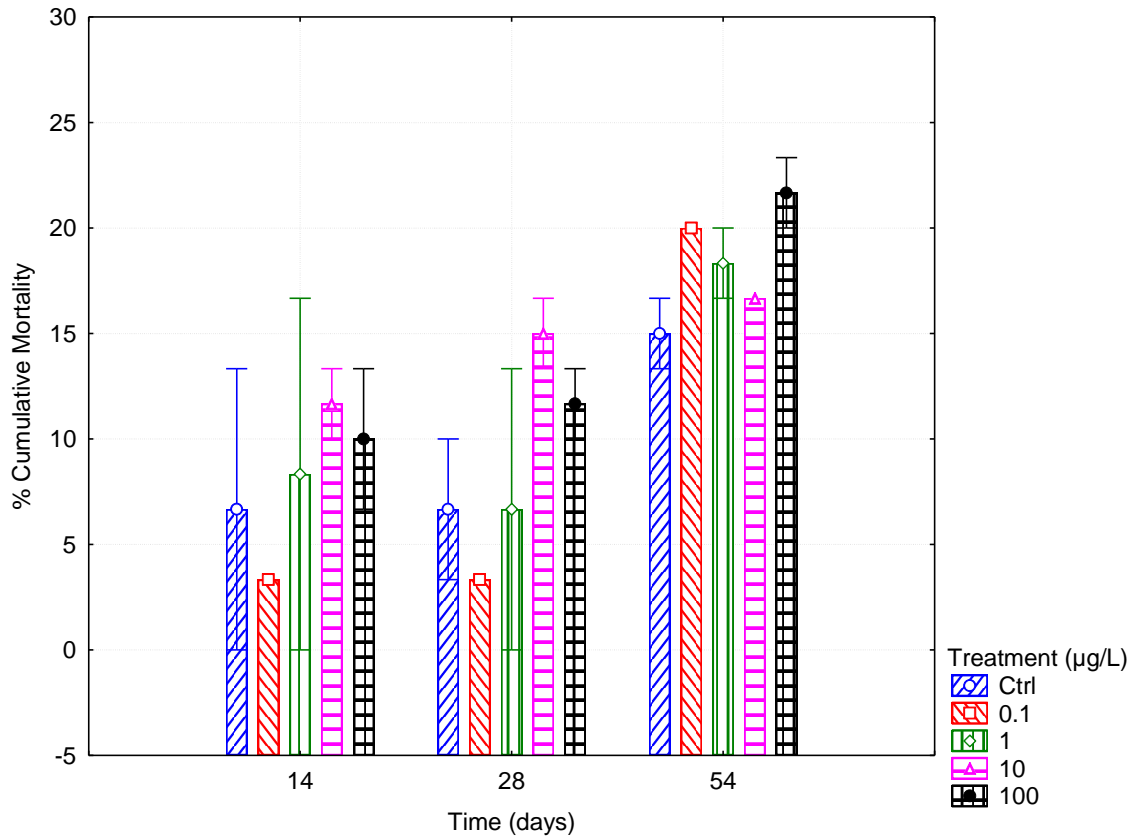


Figure 36: Cumulative mortality for 14, 28 and 54 ± 1 day-old flagfish of a chronic naproxen exposure experiment. Error bars denote standard error. Sample size for all treatments was 60. No significant differences were detected ($p > 0.10$).

4.3 Overall Effects and Comparison Between Drugs

To compare the effects between the two drugs, all treatments were taken as a fold of control with respect to all variables. In these analyses, replicates and time periods were able to be pooled. When expressing values as a fold of the control, the control values within the experiment were averaged and then treatments are expressed as a proportion of that average. This analysis was necessary to compare the ibuprofen and naproxen experiments directly. It disqualifies the differences between the experimental populations as evidenced by differences between experimental controls. Once that population based variation was removed, direct comparisons could be made.

The effects of naproxen and ibuprofen on length were assessed for the first two sampling periods, when the organisms were living in crystallization dishes. Overall during this first month, fish exposed to ibuprofen were significantly longer than those exposed to naproxen (Figure 37). *Post hoc* analysis revealed that at each treatment level, effects of ibuprofen was not significantly different from naproxen (Figure 37). Size of fish exposed to both drugs was correlated with exposure concentration ($r^2 \sim 95\%$) using a logarithmic equation (Figure 37).

Overall analyses of total length, wet weight and condition factor were made as a fold of control for the time the fish were held in 70 L aquaria, corresponding to the 54 ± 1 , 93 ± 1 and 121 ± 1 day sampling periods.

An ANOVA revealed that fish exposed to ibuprofen were globally larger than those exposed to naproxen (Figures 38, 39 and 40). However, this was not consistently revealed by the Tukey *post hoc* analysis. *Post hoc* analysis of treatments by drug

revealed that fish exposed to 100 µg/L of ibuprofen were significantly larger than those exposed to 0.1 and 1 µg/L of naproxen (Figure 38). No other significant differences were detected (Figure 38). The strong correlation between concentration and length observed in the first 28 days of the experiment for ibuprofen ($r^2 \sim 96\%$) (Figure 37) was no longer evident in the later stages of the experiment ($r^2 \sim 11\%$) (Figure 38). Despite the weaker effect size naproxen exposure had on length when compared to ibuprofen, the concentration-response relationship remained strong throughout all stages of the experiment ($r^2 \sim 94\%$) (Figure 38).

For wet weight, the strength of the concentration-response relationship was consistent with what was found for total length (Figures 38 and 39). A *post hoc* analysis did not reveal any differences between treatments: ibuprofen's stronger effect on size compared to naproxen was the only significant finding (Figure 39).

There was no difference between drugs in condition factor by treatment (Figure 40). There was also no concentration- response relationship detected with regression analysis (Figure 40).

The second generation could not be analyzed for overall effects between drugs or between generations. This was due to the second generation of each treatment lacking a cohort in one of the four treatment conditions.

Both drugs produced a drop in fertilization success following 0.1 µg/L exposure although the effect was more pronounced in the naproxen treatment (Figure 41). This was demonstrated in a greater drop in fertilization success relative to the internal experimental control (Figure 41).

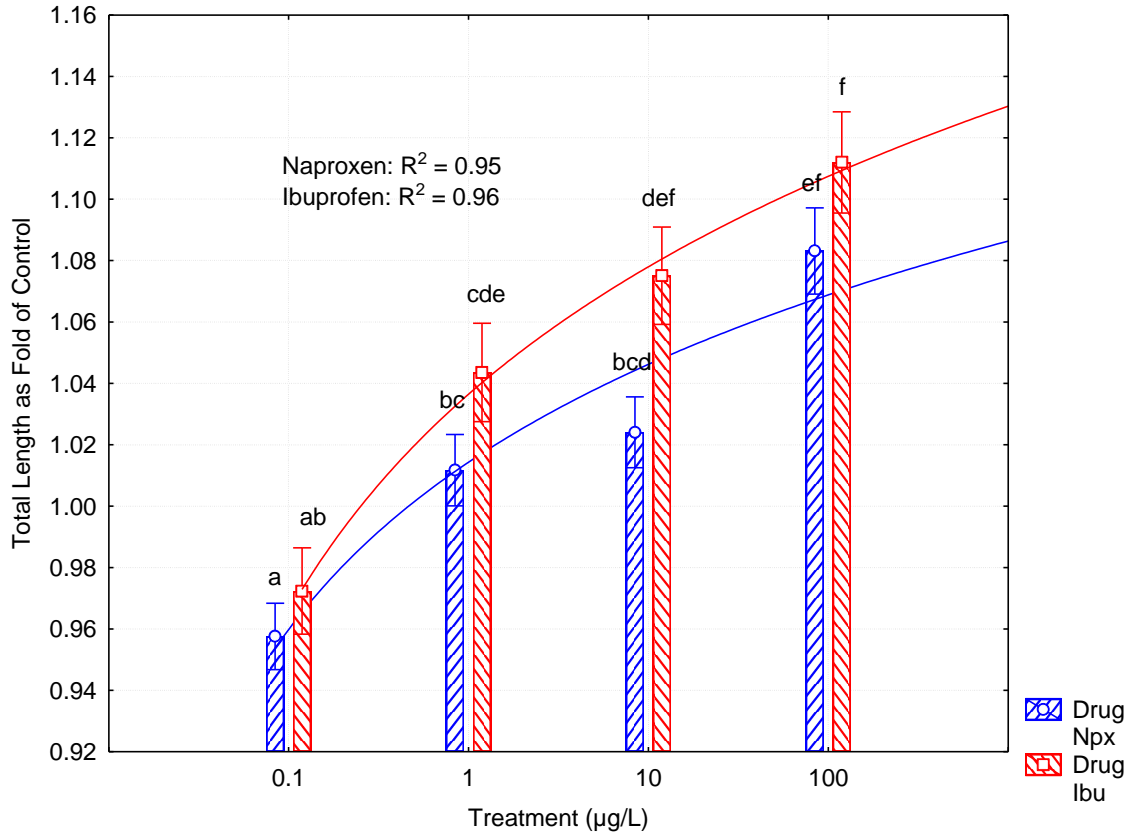


Figure 37: Impact of chronic ibuprofen and naproxen exposure on total length during the first generation dish stage of the experiment expressed as a fold of the control within each experiment at each time point used for calculation. Error bars denote standard error. Sample sizes for 0.1, 1, 10 and 100 µg/L ibuprofen treatments were 84, 88, 78 and 72, respectively. Sample sizes for 0.1, 1, 10 and 100 µg/L naproxen treatments were 100, 75, 72 and 83, respectively. ANOVA indicated that fish exposed to ibuprofen were significantly longer than fish exposed to naproxen ($p \leq 0.10$). *Post hoc* analysis revealed significant differences between treatments. Bars without a letter in common are significantly different from each other ($p \leq 0.10$).

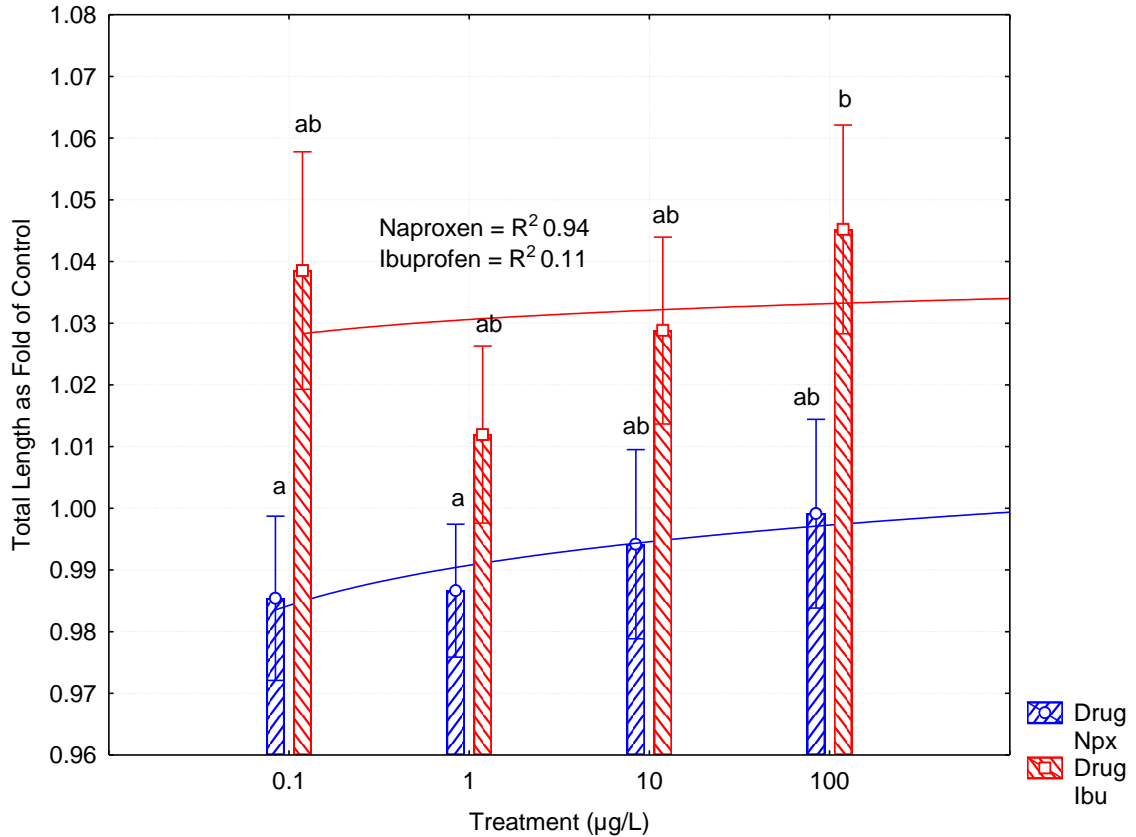


Figure 38: Impact of chronic ibuprofen and naproxen exposure on total length during the tank stage of the experiment expressed as a fold of the control within each experiment at each time point used for calculation. Error bars denote standard error. Sample sizes for 0.1, 1, 10 and 100 µg/L ibuprofen treatments were 43, 54, 45 and 43, respectively. Sample sizes for 0.1, 1, 10 and 100 µg/L naproxen treatments were 50, 52, 43 and 32, respectively. ANOVA indicated that fish exposed to ibuprofen were significantly longer than fish exposed to naproxen ($p \leq 0.10$). *Post hoc* analysis revealed significant differences between treatments. Bars without a letter in common are significantly different from each other ($p \leq 0.10$).

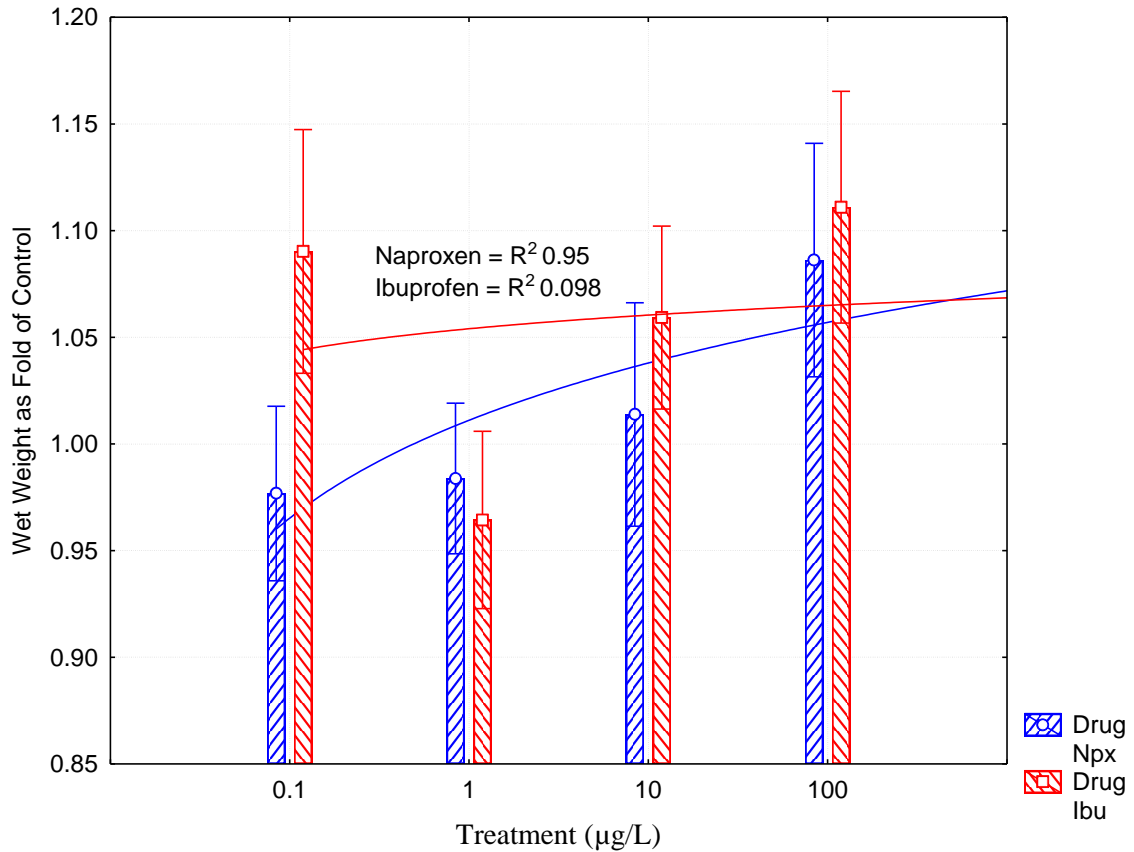


Figure 39: Impact of chronic ibuprofen and naproxen exposure on wet weight during the tank stage of the experiment expressed as a fold of the control within each experiment at each time point used for calculation. Error bars denote standard error. Sample sizes for 0.1, 1, 10 and 100 µg/L ibuprofen treatments were 43, 54, 45 and 43, respectively. Sample sizes for 0.1, 1, 10 and 100 µg/L naproxen treatments were 50, 52, 43 and 32, respectively. ANOVA indicated that fish exposed to ibuprofen were significantly heavier than fish exposed to naproxen ($p \leq 0.10$). *Post hoc* analysis did not reveal significant differences between treatments ($p > 0.10$).

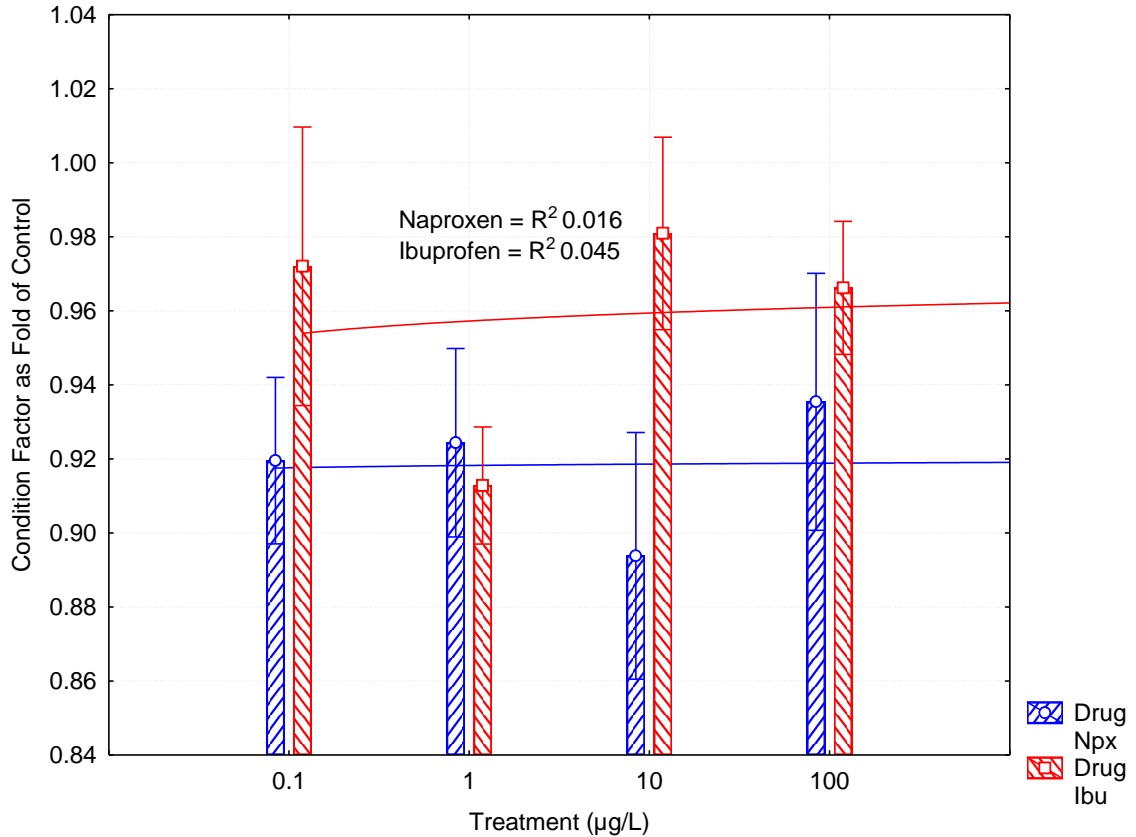


Figure 40: Impact of chronic ibuprofen and naproxen exposure on condition factor during the tank stage of the experiment expressed as a fold of the control within each experiment at each time point used for calculation. Error bars denote standard error. Sample sizes for 0.1, 1, 10 and 100 µg/L ibuprofen treatments were 43, 54, 45 and 43, respectively. Sample sizes for 0.1, 1, 10 and 100 µg/L naproxen treatments were 50, 52, 43 and 32, respectively. ANOVA indicated that fish exposed to ibuprofen had a significantly higher condition factor than fish exposed to naproxen ($p \leq 0.10$). *Post hoc* analysis did not reveal significant differences between treatments ($p > 0.10$).

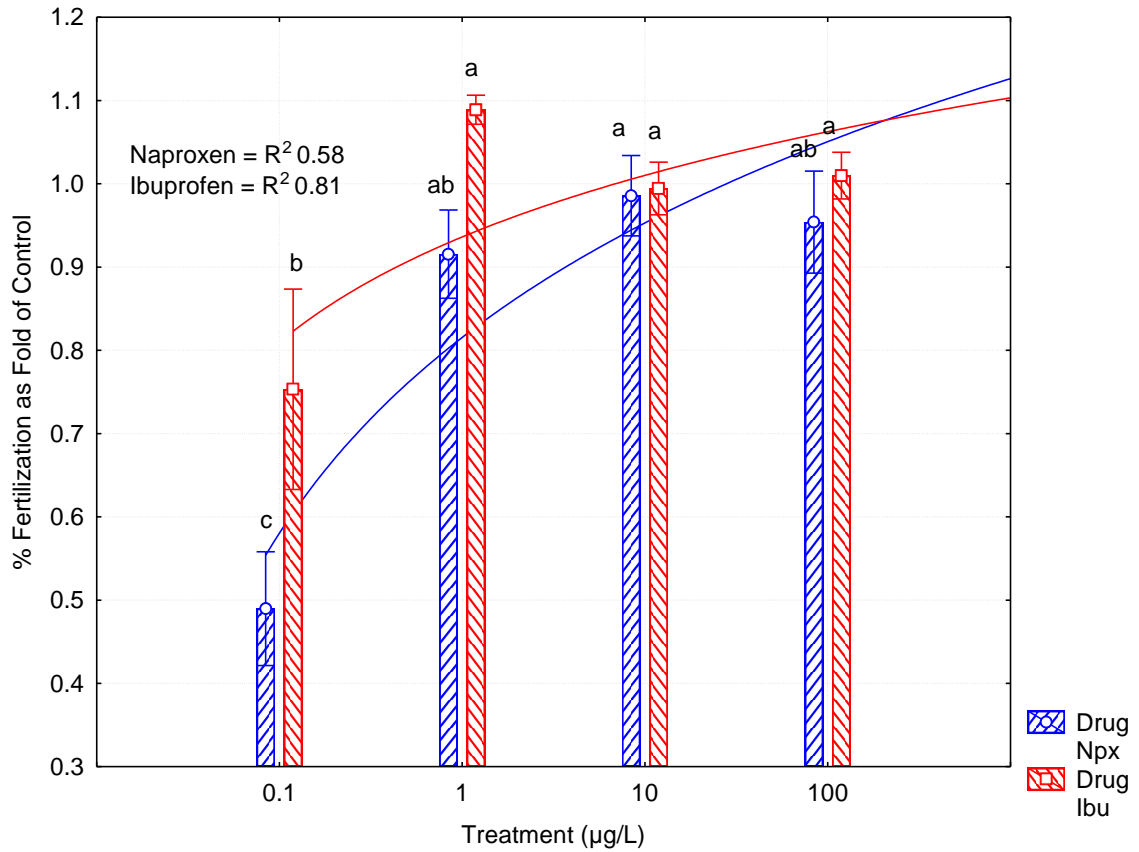


Figure 41: Impact of chronic ibuprofen and naproxen exposure on fertilization of eggs expressed as a fold of the control within each experiment. Error bars denote standard error. Sample sizes for 0.1, 1, 10 and 100 µg/L ibuprofen treatments were 8, 23, 22 and 17, respectively. Sample sizes for 0.1, 1, 10 and 100 µg/L naproxen treatments were 13, 5, 25, and 3, respectively. Bars without a letter in common are significantly different from each other ($p \leq 0.10$).

5.0 DISCUSSION

5.1 Egg Exposure Hatch Experiment

The results of this study indicate that neither ibuprofen nor naproxen have an effect on the hatchability of flagfish eggs exposed to any concentration between 0.1 and 100 µg/L (Figure 3 and 20).

David and Pancharatna (2009) exposed zebrafish to concentrations of ibuprofen similar to those used in this experiment. Their findings indicated that at concentrations of 10 µg/L and higher, the hatchability of zebrafish eggs was significantly reduced. This apparent disparity with the findings of this study are most likely due to intra-specific differences, specifically the greater sensitivity of zebrafish to toxicants compared to flagfish (Fogels and Sprague, 1977).

A chronic study of the NSAID diclofenac on Japanese medaka used similar methodology for egg exposure to that was used in this study (Lee *et al.*, 2011). Medaka eggs were exposed to either a control or to one of 1, 10, 100, 1000 or 10 000 µg/L of diclofenac. Diclofenac did not have any impact on hatchability at concentrations up to 1000 µg/L, a concentration greatly exceeding realistic environmental concentrations and well above what was used in the present study.

Diclofenac is a useful reference NSAID as it has a COX-2 / COX-1 inhibition ratio of 3/1 indicating that it would inhibit far more COX-1 than either ibuprofen or naproxen at a given concentration (Vane and Botting, 1998), indicated by low diclofenac LC₅₀ and EC₅₀ concentrations for both algae and daphnia (Cleuvers, 2004). Diclofenac has a pK_a of 4.1-4.5 which is intermediate to that of ibuprofen and naproxen (Ziylan and

Ince, 2007). Consequently, diclofenac should experience similar removal rates to ibuprofen and naproxen in WWTPs, and thus is likely present at similar concentrations in the environment.

One possible explanation for the absence of hatchability effects on eggs exposed to low concentrations of NSAIDs may be the presence of the chorion, which prevents many toxicants from reaching the developing embryo (Holdway and Dixon, 1986a; Wittbrodt *et al.*, 2001). The protective nature of the chorion was demonstrated for 3-day post fertilization flagfish eggs pulse exposed to methoxychlor (Holdway and Dixon, 1986a).

5.2 Chronic Exposure Abiotic Factors: Temperature and pH

Temperature and pH have the potential to affect flagfish growth and reproductive endpoints and as such were carefully monitored during this study (Mertz and Barlow, 1964; Foster *et al.*, 1969). Measured abiotic factors did not differ significantly from the optimal range for flagfish (Table 1 and 5) and control endpoints overlapped those in the literature.

The effect of elevated or depressed temperature and pH would be reflected in an alteration of the growth rate and reproductive indices (Foster *et al.*, 1969; St Mary *et al.*, 2004). However, the lengths and weights of control fish in this study were very similar to those found in the work of Holdway and Dixon (1986b). They conducted a life-cycle experiment on flagfish at $25.3 \pm 0.04^{\circ}\text{C}$ using a similar experimental design to that used in this study (Holdway and Dixon, 1986b). Total length and wet weight were measured on 71 and 104 days post hatch, intermediate to sampling times in this study and reflected

similar sizes (Figures 6, 7, 9, 10, 12 and 13 for Ibuprofen and Figures 23, 24, 26, 27, 29 and 30 for Naproxen).

The hatchability of control fertilized eggs, however, was lower than in this study (Holdway and Dixon, 1986b). However, another paper by the same authors reflected a high hatch rate similar to that of the controls in this study (Figure 18 and 35) (Holdway and Dixon, 1986a).

Daily egg production of the controls in this study was extremely variable, overlapping with the variability of egg production of some flagfish life-cycle literature results but not with others (Table 4 and 8). Daily egg production of the ibuprofen experiment controls (Table 4) reflected the fairly low output as found in Holdway and Sprague (1979) while the findings from the controls in the naproxen experiments (Table 8) were more similar with those of Holdway and Dixon (1986b). As these experiments were all conducted at a similar temperature and pH, it can be concluded that the variation in daily egg production seen in this study is typical of normal flagfish populations. This conclusion is somewhat supported by the findings of Craig and Baksi (1977), who reported that daily egg production of flagfish was the most sensitive endpoint to depressed pH. The average pH in the present study for both the ibuprofen and naproxen experiments was above the NOEC for flagfish egg production (Craig and Baksi, 1977).

Investigations into the time to hatch as well as hatchability indicate that temperature significantly affected both endpoints in flagfish (St Mary *et al.*, 2004). The rate of embryo development differed significantly with temperature between 27 and 33°C while hatchability did not. In the ibuprofen and naproxen experiment, the controls

hatched after 5-6 days at 27° C, the same gestation period found at that temperature by St Mary *et al.* (2004) (Figure 18 and 35). At higher temperatures, the time to hatch was further reduced to 4.5 and 4 days at 31 and 35° C, respectively, (St Mary *et al.*, 2004), though this was beyond the temperature range used in this study.

5.3 Effects of Chronic Exposure to NSAIDs Ibuprofen and Naproxen

5.3.1 F1 Growth

Growth was assessed at 5 different time points throughout the first generation of flagfish exposed to ibuprofen and naproxen. At 14 ± 1 and 28 ± 1 days post-hatch, photographs were taken to determine total length. Weight was not measured for these juvenile fish to avoid excess handling stress and the resultant excessive mortality. Wet weight was measured at 30 days post hatch, and represents one of the earliest assessments of that endpoint found in the literature (Spehar *et al.*, 1978). In this study, weight of flagfish at 30 days post hatch was almost as large as fish sampled on day 54 ± 1 for both the ibuprofen and naproxen (Figure 7 and 24).

Of the 3 growth endpoints used in the chronic exposure studies, length appeared to be the most sensitive. Wet weight and condition factor were generally insensitive to chronic ibuprofen and naproxen exposure at the concentrations selected. Of all the sampling periods, only the 54 ± 1 days post-hatch condition factor in the ≥ 10 $\mu\text{g/L}$ naproxen treatments was significantly different from controls (Figure 25). The insensitivity of these endpoints was further reflected when analyzed as a fold of the control (Figure 39 and 40). Changes in wet weight of fish exposed to naproxen showed a concentration-response relationship similar to that observed for total length during the

first 4 weeks of the experiment (Figure 39). However, neither wet weight nor length of fish exposed to either drug was significantly different from controls (Figure 38 and 39).

At 14 ± 1 days post-hatch, flagfish exposed to 1, 10 and 100 $\mu\text{g/L}$ of ibuprofen were significantly longer than the control (Figure 4). Later analysis of length did not reveal any difference between fish exposed to ibuprofen and the controls. Flagfish chronically exposed to naproxen showed a similar trend to those exposed to ibuprofen. Both at 14 ± 1 and 28 ± 1 days post hatch, flagfish exposed to 100 $\mu\text{g/L}$ of naproxen were significantly larger than the controls (Figure 21 and 22). As with the ibuprofen experiment this result was no longer visible at the later 3 sampling times. This loss of sensitivity is common in flagfish, since the natural variability in growth will mask the small effect sizes of a treatment (Beyger *et al.*, 2011).

Total length at 14 ± 1 and 28 ± 1 days revealed a strong concentration-response for both drugs (Figure 37). Although all concentrations at $\geq 1 \mu\text{g/L}$ of both drugs tended to increase the size of flagfish, concentrations of $\geq 10 \mu\text{g/L}$ of ibuprofen and $\geq 100 \mu\text{g/L}$ of naproxen increased the length of flagfish under chronic exposure conditions during the first month after hatch (Figure 37). The comparison between the two drugs also revealed that at concentrations of $\geq 1 \mu\text{g/L}$, ibuprofen produced larger fish at a given concentration than naproxen.

The cytochrome P450 pathway is a primary method of metabolising NSAIDs in an organism and may provide a mechanism to generate the growth observed in this stage of the experiment. Interestingly, cytochrome P450 also mediates an alternative pathway for AA conversion to the COX mediated production of PG (Rao and Knaus, 2008). This

alternate pathway results in the production of epoxyeicosatrienoic acid, an eicosanoid implicated in growth promotion of tumour cells (Chen *et al.*, 2011). Epoxyeicosatrienoic acid may be functioning to promote growth in young flagfish exposed to NSAIDs for two reasons. First due to the decrease in COX activity from NSAID exposure, more available AA may be converted into epoxyeicosatrienoic acid rather than PG (Heckmann *et al.*, 2007). Second, while NSAIDs do not affect the activity of cytochrome P450, they do promote a significant up regulation of the p53 (cytochrome P450 1A1 expression gene) mRNA in the liver, gills and kidneys of fish (Gravel *et al.*, 2009; Mehinto *et al.*, 2010). This up regulation was demonstrated in rainbow trout exposed to 1, 10 and 100 µg/L of diclofenac in a recent study (Mehinto *et al.*, 2010). Interestingly, this study did not find up regulation below 1 µg/L indicating that lower concentrations of NSAIDs may not be sufficient to engage this alternate pathway for AA (Mehinto *et al.*, 2010). This corroborates findings of this present study where exposure to 0.1 µg/L of ibuprofen and naproxen did not produce fish significantly larger than the control.

The only study found measuring the growth of fish exposed to NSAIDs was a recent experiment measuring growth of fathead minnows (*Pimephales promelas*) exposed to a mixture of naproxen, gemfibrozil, diclofenac, ibuprofen, triclosan, salisilic acid and acetaminophen, the total chronic exposure to NSAIDs was approximately 1-2 µg/L (Parrott and Bennie, 2009). The first growth measurement taken was at 26 days post hatch. Similarly, no difference was seen in flagfish exposed to ≤ 1 µg/L of either NSAID at 28 ± 1 days post hatch in the present study (Figures 5, 22 and 37) (Parrott and Bennie, 2009).

No studies were found in the literature which measured growth at earlier time points in fish chronically exposed to NSAIDs. However, studies exposing the invertebrate species *Daphnia magna* (Heckmann *et al.*, 2007), newly hatched turkeys (Piquer *et al.*, 1995) and cell lines (Caminada *et al.*, 2006) to ibuprofen provided further support to the findings of this study. *Daphnia* exposed to 20, 40 and 80 mg/L of ibuprofen all experienced increased somatic growth compared to the control after 14 days (Heckmann *et al.*, 2007). Despite concentrations being significantly higher than those used in this study, the findings indicate that ibuprofen has the potential to increase the growth of an organism on a fixed diet in early life stages. Newly hatched turkeys experienced a similar result when fed a diet supplemented with ibuprofen during the first 14 days of life (Piquer *et al.*, 1995). Diets including 75 mg/kg ibuprofen had significantly larger body weight than controls (Piquer *et al.*, 1995). The dose-response relationship between increasing concentrations of ibuprofen and the proliferation of the fish hepatoma cell line PLHC-1 was described as hormetic (Caminada *et al.*, 2006). In all three cases the hypothesized cause of change in growth was an increase in metabolic activity (Piquer *et al.*, 1995; Caminada *et al.*, 2006; Heckmann *et al.*, 2007). This increased metabolic activity may explain the enhanced length of flagfish exposed to ibuprofen and naproxen observed at 14 ± 1 and 28 ± 1 days post-hatch in this study.

Reproduction was also observed in the daphnia study, and analysis revealed a trade off between the size of the daphnia and reproductive capabilities (Heckmann *et al.*, 2007). While higher concentrations of ibuprofen resulted in increased size of individual organisms, it was also accompanied by a delay in reproductive maturity as well as total

reproduction (Heckmann *et al.*, 2007). As a result of this trade-off, the population growth rate declined with increasing concentrations of ibuprofen (Heckmann *et al.*, 2007).

Total length results during the 54 ± 1 , 93 ± 1 and 121 ± 1 days post-hatch sampling periods reveal a diminished affect of NSAID exposure (Figure 38). While flagfish chronically exposed to ibuprofen were still larger than those exposed to naproxen, the concentration-response relationship found in the ibuprofen experiment was no longer evident. The trend of reduced sensitivity of growth measurements to NSAID exposure has been previously reported (Lee *et al.*, 2011). Weak negative concentration-response relationships were discovered for length, weight and condition factor with r^2 values of 0.48, 0.14 and 0.03, respectively, while the trend was detected with similarly weak r^2 values at 77 days post hatch (Lee *et al.*, 2011). While some results were similar to those found in this study they also demonstrated a negative trend in growth, opposing the present experiments (Lee *et al.*, 2011). This difference could be due to NSAIDs affecting growth in a U-shaped concentration response relationship, referred to as hormesis (Calabrese and Baldwin, 2001). Hormesis indicates that while a chemical may have a negative impact on an organism at higher concentrations, the body will overcompensate for lower concentrations of the toxicant resulting in an apparent positive effect (Calabrese and Baldwin, 2001). This positive effect is usually fairly modest with only a 30-60 % increase in response being observed compared to the controls (Calabrese and Baldwin, 2001). Based on the amalgamation of several studies including this study, it is hypothesized that NSAIDs have a hormetic effect on the growth of aquatic organisms during their early life stages despite not seeing any negative impacts on growth at higher concentrations.

5.3.2 F1 Reproduction

F1 reproduction was assessed for abnormalities, egg production, fertilization and offspring hatchability. No developmental abnormalities were observed in any treatments. Egg production showed considerable variability in both the ibuprofen and naproxen chronic exposure studies (Table 4 and 8). While significant differences were detected in daily egg production of fish chronically exposed to ibuprofen, the lack of a trend in the data as well as the variability between replicates make it difficult to draw any conclusions (Table 4). Daily egg production of fish chronically exposed to naproxen showed similarly variable egg production. Interestingly, only fish exposed to 100 µg/L of naproxen showed decreased egg production compared with the controls in both replicates (Table 8).

Fertilization was significantly reduced by ibuprofen and naproxen at 0.1 µg/L but not at any other exposure concentration (Figure 17 and 34). At that concentration fertilization dropped by approximately 25 and 50 % for ibuprofen and naproxen respectively (Figure 41). As this trend was observed in both replicates of both experiments it is likely that this is a significant effect of NSAID exposure.

This observation could be attributed to a threshold response in the liver of flagfish to NSAID exposure which is not met at 0.1 µg/L. The resulting concentration which key sexual organs, sperm and eggs actually receive may be higher at 0.1 µg/L than in the ≥ 1 µg/L treatments despite the higher external exposures. A recent study examined the affect of diclofenac on both COX and cytochrome P450 1A1, the primary metabolic enzyme for NSAID degradation in the body (Mehinto *et al.*, 2010). This study found that concentrations below 1 µg/L of diclofenac significantly reduced COX-1 expression in the

liver but was not accompanied by an up regulation of cytochrome P450 to initiate the degradative metabolic response (Mehinto *et al.*, 2010). The results of the current study seem to suggest that a similar trend has occurred in the reproductive system of flagfish where a larger negative effect size was observed at 0.1 µg/L than at higher concentrations.

Despite the observed impacts on egg production and fertilization, hatchability and time to hatch were unaffected by parental NSAID exposure at any tested concentration (Figure 18 and 35).

Decreased egg production observed in the 100 µg/L of naproxen treatments may be reflective of findings in mice COX-1 and COX-2 knockout studies (Sales and Jabbour, 2003). COX-1 deficient mice show prolonged gestation periods and reduced parturition as a result. Fertilization, ovulation and implantation were unaffected in these individuals. COX-2 deficient mice exhibited multiple reproductive failures including ovulation, fertilization and implantation (Sales and Jabbour, 2003). This indicates that PG produced by COX-2 is the critical eicosanoid for ovulation.

Based on this information it might be presumed that 100 µg/L of naproxen inhibited COX-2 such that a critical threshold level of PG necessary for ovulation in flagfish was not produced. A similar finding was not observed at 100 µg/L of ibuprofen, possibly because ibuprofen inhibits less COX-2 at a given concentration than naproxen and the critical threshold of inhibition was thus not reached (Vane and Botting, 1998). However, this finding may also be evidence of the trade-off between growth and reproduction observed to occur in *Daphnia magna* exposed to high concentrations of ibuprofen (Heckmann *et al.*, 2007). Over the 14 days of the study, all ibuprofen

exposures resulted in a significantly lower rate of population growth while at the same time increasing somatic growth, both in a concentration dependent manner (Heckmann *et al.*, 2007).

Also in agreement with the knockout mouse studies were the findings reported when Japanese medaka were exposed to ibuprofen (Flippin *et al.*, 2007). These fish experienced a decrease in the total days when eggs were laid, indicative of an effect on ovulation, but demonstrated no change in total output of eggs (Flippin *et al.*, 2007).

The medaka also had no change in fertilization success at any exposure concentration of ibuprofen. Similarly, at exposure concentrations of ≤ 1 $\mu\text{g/L}$ of ibuprofen, fathead minnows also had no change in fertilization success (Parrott and Bennie, 2009). Given the consistency in fertilization for fish exposed to 1, 10 and 100 $\mu\text{g/L}$ of both ibuprofen and naproxen in this study, as well as the corroboration of findings from medaka and fathead minnows, it can be concluded that these concentrations do not have an impact on the fertilization success of flagfish. The unexpected poor fertilization detected in this study for fish exposed to 0.1 $\mu\text{g/L}$ of both ibuprofen and naproxen has not previously been reported and warrants further study.

Neither hatchability nor time to hatch of offspring from parents chronically exposed to NSAIDs in this study were significantly affected (Figure 18 and 35), consistent with other NSAID exposure studies (Parrott and Bennie, 2009; Han *et al.*, 2010)

5.3.3 F2 Success

Not all treatments were able to be carried over to the F2 generation due to the reproductive capabilities of the F1 fish. F1 fish exposed to 0.1 $\mu\text{g/L}$ of ibuprofen did not

reach steady spawning in either replicate. Eggs that were produced were poorly fertilized. Together these factors did allow a sufficient population for analysis in the F2 generation. F1 control fish and all other treatments were analyzed in the F2 generation revealing a similar trend to the F1 fish of increased growth with increasing concentration (Figure 15 and 16).

F2 fish exposed to naproxen showed a unique finding when assessed for total length at 14 ± 1 days post hatch. Here fish exposed to $0.1 \mu\text{g/L}$ of naproxen were significantly smaller than the controls (Figure 32). However, this finding may be anomalous as at the 28 ± 1 days post hatch sampling period fish exposed to $0.1 \mu\text{g/L}$ of naproxen were no different from the controls. Additionally, increased length accompanying higher NSAID concentrations was also observed at this sampling period (Figure 33).

In the naproxen experiment, parental fish exposed to $100 \mu\text{g/L}$ did not produce sufficient eggs for the F2 generation exposure. Overall, findings of the F2 generation were quite similar to those of the F1. This suggests that while the same mechanism was being affected, the result was not compounded with successive generations.

5.4 Limitations and Future Research

Exposure concentrations used in the experiments of this study were all nominal values. While efforts were made to ensure that actual concentrations if measured would be close to the nominal values, it is possible that the flagfish were exposed to lower concentrations than the reported nominal values due to adsorption to the glass of the aquaria and the plastic of the holding stock bottles (Parrott and Bennie, 2009). Future

studies using a similar methodology to the one used in this study should include measured values.

Availability of aquaria limited the experiments of the chronic exposure study from including more replicates. This did not prove to be a significant limitation in the assessment of growth, mortality or fertilization. However, the natural variability of flagfish reproduction is large enough that statistical power for analyzing egg production was not sufficient with only two replicates per treatment. Use of greater levels of replication in future studies will help determine if the low egg production observed in fish exposed to 100 µg/L of naproxen was due to the treatment or simply represents natural variability.

If the concentration-response relationship of flagfish growth to NSAID exposure is truly hormetic, future studies are needed to determine the concentration at which decreased growth would be observed. Complete life-cycle studies are also recommended to determine if there is a trade-off between increased growth observed in the early life stages of exposed flagfish and their future reproductive success.

Finally, the novel finding of this study was the decreased fertilization observed in fish exposed to 0.1 µg/L of ibuprofen or naproxen. This leaves several questions unanswered: Is this finding unique to flagfish or will similar findings be observed in other species? Also, since these detrimental effects were observed at the lowest experimental concentrations, what is the NOEC for ibuprofen or naproxen?

To answer these questions, all experiments reported here should be using other model species. Zebrafish would be an excellent candidate due to their increased

sensitivity to toxicants (Fogels and Sprague, 1977). Rainbow trout would also prove an interesting model as they have a much longer reproductive cycle, increasing the exposure duration before eggs are laid. Usage of trout would also allow enzymatic bioassays to be conducted (COX-1, COX-2, cytochrome P450 and the p53 gene) throughout the experiment to provide mechanistic information in addition to the growth, development and reproduction observations. Finally, while repeating the experiment, a wider range of NSAID concentrations should be used. This will ideally elucidate the NOEC for ibuprofen and naproxen and reveal the full hermetic to validate that model for NSAID exposure.

6.0 CONCLUSIONS

The aim of this research was to assess and compare the impacts of chronic exposure of flagfish to the NSAIDs ibuprofen and naproxen over one complete life-cycle. From the similarities of the findings in both studies, it can be concluded that these two NSAIDs impact flagfish in a similar manner.

Flagfish were most sensitive to the growth effects of chronic NSAID exposure during the first month of their life. The major finding of this study during that time period was that NSAID exposure increases the size of flagfish in a concentration-dependant manner between 0.1 and 100 µg/L. However, the highest concentrations only increased the size of flagfish between 8-10 % and the affect was no longer apparent after the first month. Since 100 µg/L is beyond the range of what is usually detected in the environment, it can be concluded that NSAID exposure will have little to no impact on the growth of individuals in the wild.

Of the reproductive endpoints measured, fertilization success proved to be the most significant finding. Both NSAIDs caused a significant reduction in hatchability of flagfish exposed to the lowest experimental test concentrations of 0.1 µg/L. This indicates that the rate of population growth might be affected in exposed wild fish populations. While NSAIDs have been detected at this level in surface waters, 1-10 µg/L concentrations are far more common.

The results of this study, in particular the experimental LOEC of 0.1 µg/L indicate a potential for the NSAIDs ibuprofen and naproxen to impact freshwater species such as *Jordanella floridae* at and below concentrations detected in the environment.

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APPENDIX

Appendix 1: Timeline of activities during a chronic exposure full lifecycle of flagfish to NSAIDs

Time	Activity	Assessment
N/A	Collect eggs (F1) from parents (F0) for use in full life cycle study	Daily
N/A	Hatching of larval flagfish (F1)	# of eggs
~Day 1	Transfer larval flagfish (F1) into crystallization dishes and begin full life cycle study	# of eggs
~Day 14	Photograph juvenile flagfish over graph paper (F1)	Total length, mortality
~Day 28	Photograph juvenile flagfish over graph paper (F1)	Total length, mortality
~Day 29	Transfer juvenile flagfish (F1) into 70 L aquaria	# of fish
~Day 54	Thinned random selection of flagfish (F1) resulting in 16 individuals remaining in each aquaria	Total length, wet weight, condition factor, mortality
~Day 75	Add breeding substrate	Guarding behaviour, presence of eggs
~Day 93	Thin adult flagfish (F1) to breeding harems; 2 males, 4 females per aquaria	Total length, wet weight, condition factor, mortality
~Day 94-114	Daily collection of eggs (F2) from breeding harems (F1)	Time to first spawn, # of eggs, Hatchability, Fertilization
~Day 107	Second generation of life cycle (F2) Begins	# of eggs
~Day 120	Photograph juvenile flagfish (F2) over graph paper on day 14 of second generation	Total length, mortality
~Day 121	Euthanize flagfish breeding harems (F1)	Total length, wet weight, condition factor, mortality
~ Day 134	Photograph juvenile flagfish (F2) over graph paper on day 28 of second generation	Total length, mortality
~Day 135	Euthanize second generation (F2) flagfish	

F0 = Parental Fish, F1 = First Generation, F2 = Second Generation

Appendix 2: AA cascade resulting in various forms of PG. Figure adapted from Simmons *et al.* 2004.

